**TITLE:**

**The PAR-1 antagonist Vorapaxar ameliorates kidney injury and tubulointerstitial fibrosis**

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## ABSTRACT

Protease-activated receptor (PAR)-1 has emerged as a key profibrotic player in various organs including kidney. PAR-1 activation leads to deposition of extracellular matrix (ECM) proteins in the tubulointerstitium and induction of epithelial-mesenchymal transition (EMT) during renal fibrosis. We tested the anti-fibrotic potential of vorapaxar, a clinically approved PAR-1 antagonist for cardiovascular protection, in an experimental kidney fibrosis model of unilateral ureteral obstruction (UUO) and an AKI-to-CKD transition model of unilateral ischemia-reperfusion injury (UIRI), and dissected the underlying renoprotective mechanisms using rat tubular epithelial cells. PAR-1 is activated mostly in the renal tubules in both the UUO and UIRI models of renal fibrosis. Vorapaxar significantly reduced kidney injury and ameliorated morphologic changes in both models. Amelioration of kidney fibrosis was evident from downregulation of fibronectin, collagen and α-smooth muscle actin in the injured kidney. Mechanistically, inhibition of PAR-1 inhibited MAPK ERK1/2 and TGF-β–mediated Smad signaling, and suppressed oxidative stress, overexpression of pro-inflammatory cytokines and macrophage infiltration into the kidney. These beneficial effects were recapitulated in cultured tubular epithelial cells in which vorapaxar ameliorated thrombin- and hypoxia-induced TGF-β expression and ECM accumulation. In addition, vorapaxar mitigated capillary loss and the expression of adhesion molecules on the vascular endothelium during AKI-to-CKD transition. The PAR-1 antagonist vorapaxar protects against kidney fibrosis during UUO and UIRI. Its efficacy in human CKD in addition to CV protection warrants further investigation.

## INTRODUCTION

Renal fibrosis is a common feature in most forms of chronic kidney disease (CKD), and is characterized by the accumulation of collagen, fibronectin, and other related fibrotic molecules in the kidney [1]. More importantly, tubulointerstitial fibrosis is a critical and irreversible process that heralds end-stage renal disease in diabetic [2-4] and non-diabetic kidney disease [4-6], which mandates a substantial healthcare budget as renal replacement therapy becomes necessary [7]. Currently, there is no effective treatment to halt disease progression. Transforming growth factor-beta (TGF-β) is a key mediator of renal fibrosis that activates different intracellular signaling pathways during the process of epithelial-mesenchymal transition (EMT) [8]. Excessive accumulation of extracellular matrix (ECM) components through EMT in tubular epithelial cells is thought to contribute to the pathogenesis and progression of renal fibrosis [9]. Indeed, TGF-β has emerged as a profibrotic factor involved in the cellular processes of hypertrophy, proliferation and apoptosis, resulting in phenotypic and morphological changes in tubular epithelial cells [10].

Tissue fibrosis is known to result from a dysregulated coagulation system and wound healing responses after injury, comprising various inflammatory and fibroproliferative effects on different organs [11, 12]. In pulmonary fibrosis, dysregulated epithelial-mesenchymal interaction results in aberrant tissue repair and injury [13-15]. Coagulation has long been known to be involved in different forms of kidney disease. However, the link between coagulation activation and the underlying pathological mechanisms in CKD remains unclear.

Thrombin, a serine protease that mediates cleavage of fibrinogen to fibrin, plays a key role in normal homeostasis following injury [16]. Impaired wound healing in turn results in excessive fibrin deposition, induction of pro-inflammatory cytokines, leukocyte migration and proliferation of mesenchymal cells [12]. These cellular effects of thrombin are mediated via proteolytic activation of a subfamily of transmembrane G protein-coupled receptors, the protease-activated receptors (PARs), which consists of four members (PAR-1, -2, -3 and -4)

[17]. PAR-1, also known as thrombin receptor (F2r), is the most expressed PAR in both human and rodent kidneys. Studies in mice and human suggest that coagulation signaling, via protease-activated receptor PAR-1 and PAR-2, plays a critical role in maintaining normal kidney function upon tissue injury [17-19]. However, overexpression of PAR-1 has been reported in acute and chronic progressive renal diseases including glomerulonephritis, glomerulosclerosis and diabetic nephropathy [18, 20]. In cultured human proximal tubular epithelial cells (hPTECs), fibronectin production was induced by thrombin stimulation via a TGF-β-dependent mechanism, leading to a profibrotic phenotype [21]. PAR-1 deficiency was renoprotective in mouse models of glomerulonephritis [22], type 1 diabetes [23] and chronic obstructive nephropathy [24], suggesting a pivotal role of PAR-1 in innate immunity, inflammation and renal fibrosis.

Modulation of the dysregulated coagulation system in renal fibrosis may, therefore, be a potential strategy for pharmacologic intervention against CKD. The anti-coagulants, heparin and fondaparinux, exert anti-fibrotic and anti-inflammatory effects in experimental models of renal injury, including ischemia reperfusion, ureteral obstruction and diabetic nephropathy [11]. More recently, blockade of PAR-1 with a selective PAR-1 antagonist SCH79797 attenuated inflammation from experimental ischemia-reperfusion injury via PI3K/Akt signaling [25]. However, the effect of PAR-1 inhibition on renal fibrosis, the final common pathway in CKD, remains unknown.

Here, we investigated whether administration of PAR-1 antagonist (vorapaxar) has a renoprotective effect on two robust renal fibrosis models of unilateral ureteral obstruction (UUO) and chronic kidney disease following unilateral ischemia reperfusion injury (UIRI), as well as in cultured renal rat proximal tubular epithelial cells (NRK-52E). Vorapaxar is an orally active PAR-1 antagonist, which inhibits thrombin-induced platelet activation and is undergoing clinical trials for cardiovascular diseases in reducing thrombotic cardiovascular events in patients with acute coronary or peripheral arterial disease [26, 27]. Our findings not only elucidate the underlying molecular mechanisms through which PAR-1 activation results

in renal fibrosis, but also demonstrate the renoprotective effect of vorapaxar and enhance the scope of its clinical utility.

## MATERIALS AND METHODS

### Materials

Antibodies to p-ERK1/2 (#9101), ERK1/2 (#9102), p-Smad2/3 (#9510), Smad2/3 (#3102), p-Smad3 (#9520) and Smad3 (#9523) were obtained from Cell Signaling Technology, Beverly, MA. Antibodies to fibronectin (Fn) (#F3648) and $\alpha$SMA (#A2547) were obtained from

Sigma-Aldrich, St Louis, MO. Antibodies to Collagen 1 (Col 1) (#ab34710), NADPH oxidase-4 (Nox4) (#ab133303), inducible nitric oxide synthase (iNos) (#ab3523) and CD34 (#ab8158) were obtained from Abcam, Cambridge, UK. Antibodies to PAR-1 (#sc-13503), thrombin (#sc-271449) and TGF-$\beta$ (sc-130348) were obtained from Santa Cruz

Biotechnology, Santa Cruz, CA. Antibody to $\beta$-actin (#MS-1295-P0) was obtained from

Thermo Scientific, Rochester, NY. Antibody to KIM-1 (#AF1817) was obtained from R&D Systems, Minneapolis, MN, and the F4/80 antibody (#MCA497R) was obtained from Serotec, Oxford, UK. Vorapaxar, named SCH 530348, was obtained from Axon Medchem, Groningen, the Netherlands.

### Animal Procedures and Vorapaxar Treatment

The animal experiment was approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong and was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The authorization numbers of animal experiments were CULATR 4466-17 and 4936-19. All animal works were conducted in the Centre for Comparative Medicine Research (CCMR) at University of Hong Kong. Male BALB/c and C57B6 mice weighing between 20 and 25 g at 8

weeks of age were purchased from the CCMR. Mice were anaesthetized with Midazolam/ Fentanyl/ Medetomidine (1:1:0.5; 10 ml/kg bodyweight) via intraperitoneal injection (I.P.) for the surgical procedures. Mice were euthanized with pentobarbital (150 mg/kg; I.P.) for both experiments.

Unilateral ureteral obstruction (UUO) was performed on BALB/c mice according to published methods [28]. Sham operation was performed with only abdominal cavity incision on control mice. Vorapaxar (15, 30 mg/kg), dissolved in aqueous 0.4% carboxymethylcellulose sodium (CMC) (Sigma-Aldrich) was administrated daily via oral gavage on day 1 to 7 after UUO. Control animals received CMC solution only as vehicle. All animals were sacrificed at 7 days after UUO or sham operation. Both kidneys were harvested. For each kidney, half of it was snap frozen in liquid nitrogen and stored at -80°C. The other half was fixed with 10% formalin and embedded in paraffin for histological and immunohistochemical analysis.

In AKI to CKD model, unilateral ischemia reperfusion injury was performed on the left kidney of C57B6 mice. Briefly, pedicle of the left kidney was clamped and released after 30 minutes to induce renal ischemia through the blockade of blood flow to the kidney at 37°C. Animals were administrated with vorapaxar (30 mg/kg) or vehicle on the day of surgery and daily until sacrifice at day 14. The contralateral kidney was removed one day before sacrifice. Sham operation was performed and kidneys were harvested as described in UUO model.

**Cell Culture**

Rat kidney tubular epithelial cells (NRK-52E) were cultured in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12), supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL) and 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in 5% carbon dioxide atmosphere. Serum starved NRK-52E cells were stimulated with thrombin (2 U/mL) from human plasma (Sigma-Aldrich) for 72 hours, pre-treated with vorapaxar (2 μM) dissolved in 1% DMSO or corresponding inhibitors of ERK1/2 (PD98059, 25 μM) and

Smad3 (SIS3, 5 μM) for 1 hour, and stimulated with thrombin for 0-4 hours for p-ERK1/2 and p-Smad2/3 levels.

Hypoxia experiment was performed along with pharmacological inhibition by vorapaxar on NRK-52E cells. Cells were exposed to a condition of 1% $O_2$, 5% $CO_2$ and 95% humidity in a hypoxic incubator under COY In-vitro Hypoxia system for 24 hours. Cells were pre-treated with vorapaxar (2 μM) as described prior to hypoxia treatment.

**RNA Isolation and Real Time qPCR**

Cortical total RNAs were extracted from the frozen kidney or cultured cells using NucleoSpin® RNA/Protein (Macherey-Nagel, Duren, Germany) or TRIZOL reagent (Invitrogen), respectively. The RNAs were reversely transcribed to cDNAs (High-Capacity cDNA Reverser Transcription Kit; Applied Biosystems, Carlsbad, CA). Real-time qPCR was performed using SYBR Green reagent and specific primers on StepOnePlus$^{TM}$ Real-Time PCR Systems (Applied Biosystems). Relative gene expression was obtained after normalization with β-actin, followed by comparison with respective control group using StepOne$^{TM}$ software v2.3 (Applied Biosystems). Primer sequence of target genes used in qPCR was listed in Table S1.

**Western Blot**

Total proteins were prepared from the frozen kidney or cultured cells using RIPA lysis buffer (Millipore, Bedford, MA). Protein lysate was resolved in Bolt$^{TM}$ 4-12% gel (Invitrogen) in equal amount and transferred to 0.45 μm polyvinylidene fluoride membrane (Millipore). Membranes were blotted with 5% non-fat milk and incubated with primary antibodies overnight. Peroxidase-conjugated secondary antibodies (Dako, Carpinteria, CA) were applied subsequently. Membranes were visualized with Clarity$^{TM}$ Western ECL Substrate (Bio-Rad, Hercules, CA) using ChemiDoc XRS+ system (Bio-Rad). Relative expression of target protein was normalized with β-actin expression using ImageJ analysis software (NIH, Bethesda, MD).

**Measurement of TGF-β, MCP-1, NO and Scr**

Cell culture supernatant was collected after 72 hours treatment and TGF-β and MCP-1 levels were measured by ELISA (R&D Systems, Minneapolis, MN). Serum nitric oxide level of mice was measured as total nitrite level using commercial kit (R&D Systems). Serum creatinine (Scr) level of mice was measured by enzymatic method (Stanbio Laboratory).

**Cell Proliferation Assay**

NRK-52E cells were seeded in 96-well plates with DMEM/F12 medium. After serum starvation, cells were incubated with different concentrations of vorapaxar (0.5, 1, 2, 5 and 10 μM) or vehicle for 72 hours. The number of viable cells was measured with MTT assay (Promega, Madison, MI) according to manufacturer's instruction. Experiment was performed in triplicate and data were normalized with unstimulated control sample.

**Detection of Intracellular Reactive Oxygen Species (ROS) Production**

NRK-52E cells were seeded in 8-well chamber slide (Nunc Lab-Tek II, Thermo Scientific, Waltham, MA). After stimulation with thrombin for 2 hours with or without pre-incubation with vorapaxar, cells were loaded with 10 μM fluorescence probe CM-H$_2$DCFDA (Molecular Probes, Eugene, OR) for 1 hour and fixed with 4% paraformaldehyde after washing. Slides were visualized and captured under fluorescence microscope.

**Histological Examination**

Paraffin-embedded kidney tissue was sectioned (4 μm) and deparaffinized, then rehydrated with concentration gradient of ethanol. The sections were proceeded to staining with periodic acid-Schiff (PAS) solution (Sigma-Aldrich) to evaluate tubular injury.  Tubular injury was scored from 0 to 5 according to the percentage of damaged tubules (interstitial inflammation and fibrosis, tubular dilation): 0, normal; 1, tubular lesion <10%; 2, 10-20% lesion; 3, 20-30% lesion; 4, 30-40% lesion; 5, >40% lesion. Collagen deposition on kidney sections was quantified by performing Picrosirius red staining according to manufacturer's protocol

(Polysciences, Inc., Warrington, PA). Ten images per section were acquired with light microscope (Leica Microsystems, Germany) in a blinded manner. Quantification of staining was performed using ImageJ analysis software.

**Immunohistochemical Staining (IHC)**

Paraffin-embedded kidney section was deparaffinized and rehydrated, followed by microwave-based antigen retrieval in citrate buffer (10 mM), pH 6.0 or protease K solution (20 μg/ml) in TE Buffer, pH 8.0. Sections were quenched by 3% hydrogen peroxide and blocked with 2% bovine serum albumin (BSA). Primary antibodies against PAR-1, Fn, Col 1, F4/80, Nox4, αSMA and CD34 were applied on the sections overnight, followed by incubation with peroxidase-conjugated second antibodies (Dako). Sections were developed using DAB substrate (Dako) and counterstained with haematoxylin, followed by dehydration with ethanol and clearing in xylene before mounting. Ten images per section were captured in a blinded manner. Quantification of staining was performed using ImageJ analysis software.

**Statistics**

All continuous data were expressed as mean ± SEM. Differences between experimental groups were evaluated by one-way ANOVA with two-sided *t*-test using GraphPad Prism v.6 (GraphPad Software, San Diego, CA). P<0.05 was considered statistically significant.

**RESULTS**

**PAR-1 Expression was Upregulated in the Obstructed Kidney**

Obstructed kidneys displayed progressive tubulointerstitial lesions from day 1 to day 7 after ureteral ligation (Figure 1A). Renal cortical expression of thrombin was significantly upregulated at 7 days in UUO mice compared to sham-operated controls (Figure 1B and 1C), indicating activation of the coagulation cascade in obstructive nephropathy. Similarly, PAR-1

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mRNA was significantly induced in the obstructed kidneys at 3 and 7 days after UUO (Figure 1D). PAR-1 was constitutively expressed mostly in tubular epithelial cells in sham-operated kidney, whereas its expression was selectively overexpressed in tubular epithelial cells of the obstructed kidney. (Figure 1A).

**Vorapaxar Ameliorated Kidney Fibrosis after UUO**

In a pilot study for optimal dose (5, 10, 15 and 30 mg/kg), treatment of vorapaxar at 15 and 30 mg/kg significantly inhibited PAR-1 gene induction in UUO mice (Figure S1A). Administration of vorapaxar at 15 and 30 mg/kg not only significantly inhibited induction of PAR-1 expression at mRNA (Figure 2A), but also reduced its protein expression (Figure 2B and 2C) after 7 days in the obstructed kidneys. Serum aspartate transaminase (AST) level was not elevated in UUO mice given with 15 and 30 mg/kg vorapaxar (Figure S1B). Gene expression of other PAR isoforms remained unaffected with vorapaxar treatment (Figure S1C).

UUO kidneys displayed severe morphological lesions with tubular dilation and interstitial macrophage infiltration. UUO mice treated with vorapaxar exhibited less morphological abnormalities and had significant improvement in tubular injury on the obstructed kidneys (Figure 2C). Sirius red staining showed a significant increase in collagen deposition in the UUO kidney (8.7%), compared to that from sham-operated mice (3.7%). In 15 and 30 mg/kg vorapaxar treated UUO kidneys, the positively stained area was reduced to 7.1% and 4.1%, respectively (Figure 2C).

Renal cortical mRNA levels of Fn, Col 1 and αSMA were significantly induced in UUO mice compared to sham-operated mice. Vorapaxar at 15 mg/kg significantly attenuated overexpression of Fn and Col 1 (Figure 2D), though suppression of αSMA overexpression required a higher dose (30 mg/kg, Figure 2E).

Immunohistochemical staining for Fn and Col 1 was barely detected in sham-operated kidneys, whereas in UUO kidneys their expression were significantly increased to 1% and 2%

of the total kidney area, respectively (Figure S1D). Fn staining was significantly decreased to 0.3% with 30 mg/kg vorapaxar, and Col 1 staining was significantly reduced to 1.1% with 15 mg/kg vorapaxar in the UUO group (Figure S1D).

## Vorapaxar Ameliorated Thrombin-Induced EMT and Fibrotic Responses in Rat Tubular Epithelial Cells

To validate the effect of vorapaxar *in vitro*, cultured rat tubular epithelial (NRK-52E) cells was pretreated with vorapaxar, subsequent to thrombin stimulation. Thrombin upregulated PAR-1 protein expression after 48 hours (Figure 3A and 3B) and mRNA expression through 24 to 72 hours in NRK-52E cells (Figure 3C). NRK-52E cell viability decreased with increasing concentrations of vorapaxar after 72 hours of incubation (Figure S2A). The maximal inhibitory concentration on PAR-1 expression was 2 μM (Figure S2B), which was used for all subsequent experiments.

The gene expression of Fn and Col 1 were significantly induced in thrombin-stimulated NRK-52E cells, whereas pretreatment with vorapaxar significantly inhibited their induction (Figure 3D). In addition, thrombin also induced EMT as evidenced by a loss in the epithelial marker E-cadherin and a gain in mesenchymal markers including α-smooth muscle actin (αSMA), Snai1 and vimentin. Treatment with vorapaxar inhibited thrombin-induced αSMA, Snai1 and vimentin expression, and prevented the loss of E-cadherin (Figure 3D). Likewise, thrombin-induced protein expression of Fn, αSMA and Col 1 was suppressed by antagonizing PAR-1 with vorapaxar (Figure 3E and 3F) but did not reach statistical significance. Comparable reduction of Fn and Col 1 expression by vorapaxar was also observed in cells stimulated with a PAR-1 activating peptide (TFLLR-NH$_2$) (Figure S2C).

## Vorapaxar Suppressed ERK1/2 and TGF-β/Smad3 Activation in UUO

To dissect the PAR-1-induced mechanistic pathways in the obstructed kidney, upregulation of phospho-p42 and phospho-p44 MAPK protein levels were significantly inhibited by 30 mg/kg vorapaxar (Figure 4A). In addition, renal cortical mRNA levels of TGF-β were

markedly induced in UUO mice compared to sham-operated animals, and this was significantly downregulated with 15 mg/kg of vorapaxar treatment (Figure 4B). The reduced trend in latent TGF-β1 production after PAR-1 inhibition in UUO mice was further confirmed by Western blot analysis (Figure 4A and 4B). Canonical TGF-β1 signaling via Smad3 phosphorylation was enhanced in the obstructed kidneys, which was significantly suppressed by vorapaxar (Figure 4A).

Our results demonstrated that inhibition of PAR-1 by vorapaxar could blunt the activation of ERK1/2 MAPK pathway, and suppress the production of TGF-β and downstream Smad3 signaling.

**Vorapaxar Inhibited Thrombin-induced MAPK and TGF-β/Smad Signaling in Tubular Cells**

In cultured tubular epithelial cells, thrombin-induced PAR-1 mRNA expression was significantly suppressed by (PD98059). Other thrombin-induced fibrosis-associated molecules including Fn, Col 1, Snai1 and TGF-β1 mRNA were also reduced by ERK1/2 inhibitor (Figure 4C). Pretreatment with vorapaxar inhibited thrombin-induced phospho-ERK1/2 levels in cultured tubular cells (Figure 4D). Upregulation of ERK1/2 MAPK phosphorylation was associated with inflammatory responses upon acute thrombin stimulation as the increase in MCP-1 production in thrombin-stimulated cells was abolished by pretreatment with ERK1/2 inhibitor. Likewise, vorapaxar also attenuated MCP-1 induction to the same extent as ERK1/2 inhibitor (Figure 4E).

TGF-β1 mRNA was significantly upregulated upon thrombin stimulation, but suppressed by vorapaxar pretreatment in NRK-52E cells (Figure 4F). Similarly, the production of TGF-β1 in cell culture supernatant was markedly increased by thrombin and was significantly attenuated with vorapaxar pretreatment (Figure 4G). Vorapaxar significantly inhibited the downstream phosphorylated Smad2/3 level of TGF-β canonical signaling pathway (Figure 4D), which

regulated the production of thrombin-activated expression of profibrotic factors αSMA and Col 1 in cells pretreated with small molecule inhibitor of Smad3 (SIS3) (Figure 4H).

The above findings indicate the important role of ERK1/2 signaling pathway in the inhibitory mechanism of vorapaxar and the subsequent TGF-β/Smad signaling pathway in thrombin-induced fibrotic changes in tubular epithelial cells.

**Vorapaxar Attenuated UUO-induced Macrophage Infiltration and Oxidative Stress**

F4/80 staining was hardly detected in control kidneys while the positive staining of macrophage infiltration was significantly increased to 7.3% of total kidney section in the obstructed kidneys. Vorapaxar treatment ameliorated macrophage infiltration with a reduction to 4.4% of F4/80 positive staining (Figure 5A). Consistent with F4/80 staining, the renal cortical mRNA level of MCP-1 and TNF-α was significantly upregulated in UUO mice and the induction was significantly abrogated in animals treated with 15 mg/kg and 30 mg/kg vorapaxar respectively (Figure 5B).

To determine the effect of vorapaxar on oxidative stress in UUO mice, upregulated protein expression of Nox4 in renal tissue was determined by Western blotting and showed significant inhibition with vorapaxar treatment (Figure 5C). These results were also confirmed with immunohistochemical staining, showing upregulation of Nox4 positive stained area (11.7%) in tubular cells in the UUO kidney compared to the sham-operated kidney. Nox4-positive area decreased to 7.4% and 9.3% in 15 and 30 mg/kg vorapaxar treated UUO kidney respectively (Figure 5D). Total serum nitrite level was significantly increased in UUO mice compared to control, which was significantly reduced in vorapaxar treated mice (Figure 5E).

**PAR-1 Mediated ROS Formation and Oxidative Stress in Tubular Cells**

Among the NADPH oxidase (Nox) family, Nox4 is highly expressed in renal tubular epithelial cell [29]. As shown in Figure 5F, thrombin induced protein expression of Nox4 and

inducible nitric oxide synthase (iNos) and these effects were suppressed by vorapaxar. Thrombin-induced mRNA level of Nox4 is ERK1/2 dependent, as evidence by its significant suppression with ERK1/2 inhibition (Figure 5G). In NRK-52E cells, thrombin significantly induced endogenous reactive oxygen species (ROS) formation as demonstrated by an increase in green fluorescence signal of oxidized $H_2DCFDA$, whereas pre-incubation with vorapaxar markedly decreased this phenomenon (Figure 5H).

**Vorapaxar Reduced Kidney Fibrosis in UIRI**

We next evaluated whether vorapaxar could ameliorate kidney fibrosis in a model of acute to chronic kidney disease transition following unilateral renal ischemia reperfusion injury in mice [30]. PAS staining revealed prominent kidney damage including tubular dilation, cast formation and inflammatory cell infiltration 14 days after UIRI, whereas mice treated with 30 mg/kg vorapaxar developed less morphological lesion with significant improvement in tubular injury after UIRI (Figure 6A). Upregulation of the AKI marker KIM-1 in the renal cortex was also ameliorated at protein and mRNA levels with vorapaxar treatment (Figure 6B and Figure S3A). Renal dysfunction in term of serum creatinine level induced by UIRI was vaguely improved by vorapaxar (Figure 6C).

With vorapaxar, induction of PAR-1 protein expression, predominantly in tubular cells, was significantly inhibited (Figure 6B) while the increased mRNA level was considerably less after UIRI (Figure S3A). Thrombin, the endogenous ligand of PAR-1, was significantly suppressed by vorapaxar in kidney cortical tissue with UIRI (Figure 6B). Similar to UUO model, gene expression of other PAR isoforms remained unaffected with vorapaxar treatment (Figure S3B).

UIRI significantly upregulated Fn, αSMA and Col 1 expression at both mRNA and protein levels. Vorapaxar significantly reduced Fn and showed a trend toward decreasing αSMA and Col 1 levels (Figure S3C and S3D). There was heavy immunohistochemical staining for Fn, Col 1 and αSMA with total positive staining areas of 4.5%, 1.5% and 2.9% respectively in the

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tubulointerstitium of UIRI kidneys. Staining for Fn, Col 1 and αSMA was decreased to 4%, 0.3% and 2.3% by vorapaxar (Figure 6D).

Activation of TGF-β/Smad3 signaling was evident in the UIRI kidneys compared to sham-operated kidneys. Vorapaxar reduced TGF-β1 protein expression and significantly suppressed phospho-Smad3 levels (Figure 6E).

**Vorapaxar Decelerated IR-induced Interstitial Inflammation and Endothelial Dysfunction**

IR-induced CKD also features interstitial inflammation and endothelial dysfunction [31]. UIRI increased the area of F4/80 +ve staining to 0.8%, which was suppressed to 0.3% by vorapaxar (Figure 7A). Macrophage infiltration associated with the induction of MCP-1 and TNF-α mRNA levels in UIRI kidneys was also significantly inhibited by vorapaxar (Figure 7B).

Endothelial dysfunction that was determined by immunohistochemical staining of CD34. UIRI reduced the area of CD34 +ve staining, which reflects peritubular capillary density, from 0.9% in sham-operated kidneys to 0.4% after UIRI, and was restored to 0.8% with vorapaxar treatment (Figure 7A). Likewise, overexpression of leukocyte adhesion molecule VCAM-1 observed after UIRI was significantly blunted by vorapaxar (Figure 7C).

In hypoxic NRK-52E cells incubated in a low $O_2$ (1%) milieu, PAR-1 expression was significantly induced after 24 h, which was prevented by vorapaxar pretreatment. The marked overexpression of the ECM proteins Fn and Col 1 under this hypoxic condition was reduced by vorapaxar, which also significantly suppressed the induction of TGF-β1 (Figure 7D).

**DISCUSSION**

Thromboembolic events triggered by the robust generation of thrombin in patients with CKD [32, 33] contribute to the increased risk of cardiovascular disease. However, other evidence reveals that the hypercoagulable state in CKD is correlated with the degree of renal dysfunction and inflammation, rather than endothelial function, suggesting a therapeutic potential of anticoagulation strategies in slowing the progression of kidney fibrosis in CKD. Among the components of the coagulation system, PAR-1 is a key mediator for thrombin-activated inflammatory and fibrotic responses. Increasing studies have demonstrated the pivotal role of PAR-1 in lung [15, 34-36], liver [35-38] and pulmonary fibrosis [37, 38]. Until recently, PAR-1 deficient mice exhibited diminished UUO-induced kidney fibrosis through the suppression of profibrotic MCP-1 and TGF-β production by tubular epithelial cells rather than EMT [24]. Besides, PAR-1 deficiency was found be protective from tubular injury and neutrophil infiltration in acute ischemia reperfusion injury, and PAR-1 inhibitor SCH 79797 exerted an anti-inflammatory effect in 24h IR rat model [25, 39]. In line with these observations, our findings further demonstrated a mechanistic pathway through which PAR-1 mediates thrombin-induced ERK1/2 MAPK and TGF-β/Smad3 signaling in cultured rat tubular epithelial cells that contributes to renal inflammation and the progression of tubulointerstitial fibrosis. Here, we showed that PAR-1 activation mediates thrombin-induced ERK1/2 MAPK signaling under hypercoagulable state, that modulates its downstream effector TGF-β on ECM protein production. Activation of ERK1/2 MAPK signaling further induces PAR-1 transcript expression, resulting in PAR-1 overexpression in disease state. Likewise, vorapaxar inhibits the expression of TGF-β1 and in turn revokes the canonical Smad3 signaling pathway. Suppression of PAR-1-mediated TGF-β signaling could reduce ECM accumulation, possibly via attenuation of EMT as vorapaxar inhibited the induction of EMT markers in cultured tubular epithelial cells. Meanwhile, vorapaxar attenuated the upregulation of PAR-1 expression and the activation subsequently. This further enhanced the

inhibitory effect of vorapaxar. However, the definitive efficacy of vorapaxar in ameliorating EMT *in vivo* has yet to be confirmed as different studies have shown conflicting data [40].

Chronic inflammation plays a critical role in the development of renal fibrosis [41]. Injured tubular epithelial cells modulate the immune responses through activation of various pro-inflammatory cytokines [42]. Here, vorapaxar significantly inhibited the production of MCP-1 via ERK1/2 MAPK signaling in rat tubular cells after thrombin stimulation. ERK1/2 MAPK signaling has been extensively studied and is associated with proinflammatory responses in experimental obstructive nephropathy [43, 44]. Our results showed that pharmacological inhibition of PAR-1 by vorapaxar attenuated ERK1/2 phosphorylation resulting in diminished production of ECM protein and inflammatory cytokines in UUO mice. More importantly, the recruitment of macrophage in association with induction of MCP-1 and TNF-α was endured by antagonizing PAR-1 in both UUO and UIRI-induced CKD models. TNF-α is a major cytokine associated with the progression of AKI to CKD [45]. The pro-inflammatory TNF-α aggravates M1-subtype cell polarization, leading to persistent tubular injury and macrophage infiltration in progressive renal fibrosis [42, 46].

Oxidative stress also contributes to the pathogenesis of UUO and numerous markers of oxidative stress including heme oxygenase-1, 8-hydroxy-2'-deoxyguanosine and nitrotyrosine are increased in mouse kidneys after UUO [47, 48]. Thrombin binds to PAR-1 and induces the production of intracellular ROS via Nox activation in extravillous trophoblasts [49]. On the other hand, antagonizing PAR-1 prevented upregulation of the receptor for oxidized LDL, a prooxidative marker, in response to factor X (FXa) activation in human atrial tissue [50]. In tubular epithelial cells, we demonstrated the production of ROS via a thrombin/PAR-1/ERK/Nox4 activated pathway. Expression of Nox4 is upregulated in UUO-induced fibrotic kidney and is induced by TGF-β in tubular epithelial cells [51]. Thus, vorapaxar protects against tubulointerstitial fibrosis in UUO partly via suppression of TGF-β and the subsequent Nox4-dependent ROS production. In addition, vorapaxar reduced NO production in UUO mice. Under normal conditions, NO regulates vasoreactivity by promoting vasodilation to

increase renal blood flow through a PAR-1/eNOS interaction [52, 53]. However, the bioactivity of NO is reduced by reacting with excessive superoxide $O_2^-$ under pathological conditions, leading to the formation of peroxynitrite ($ONOO^-$), a powerful oxidant and nitrosating agent that accelerates oxidative stress [53]. Another study also supports the detrimental role of NO in mediating hypoxia/reperfusion injury in renal proximal tubules [54]. Therefore, an increase of NO production together with oxidative stress in UUO aggravates kidney injury, and our findings suggest that targeting PAR-1 activation by vorapaxar can reduce the production of NO and the ensuing oxidative stress signals in tubular epithelial cells. Consistent with findings on protection against oxidative stress, PAR-1 activation was detected in hypoxic tubular epithelial cells while its inhibition by vorapaxar inhibited TGF-β-mediated ECM proteins accumulation in tubular epithelial cells, indicating the important role of PAR-1 activation in hypoxia-induced oxidative stress during both AKI and CKD.

Apart from maladaptive tubular repair, capillary rarefaction after AKI further potentiates the development of fibrosis, and subsequently CKD. During AKI, hypoxia-induced endothelial damage and capillary loss confine the microenvironment around injured tubules, exacerbating renal hypoxia, ECM proteins accumulation and inflammatory cell infiltration [55]. PAR-1 is constitutively expressed in vascular endothelial cells, which enhances capillary permeability and leukocyte adhesion [56]. In our study, vorapaxar prevented the loss of capillary density as evidenced by unaffected CD34 staining in the ischemic kidneys. Elevated VCAM-1 level was also inhibited by vorapaxar to restrict the recruitment of leukocytes into the interstitium and retain the development from AKI to CKD. Although vorapaxar did not significantly improve renal functional loss in the presence of continuous tubular injury at 14 days after UIRI. Serum creatinine and BUN levels were preserved with vorapaxar treatment in the early stage of UIRI-induced CKD model (data not shown). The markedly reduced expression of KIM-1, a biomarker for tubular injury in both acute and chronic kidney diseases, after vorapaxar

treatment suggests that inhibition of PAR-1 activation protects against tubular injury during the process of AKI to CKD.

Vorapaxar is an FDA-approved drug for the reduction of thrombotic cardiovascular events in patients with a history of myocardial infarction or peripheral artery disease [26, 57]. It is well tolerated in human, and is rapidly absorbed and distributed in the body to reach a peak plasma level within 60-90 min with an effective half-life of 3-4 days [27]. The major side effect of concern is an elevated bleeding risk in Phase III clinical trials after long-term administration of up to 1080 days [58, 59]. In patients with impaired renal function, vorapaxar has been reported in a recent study to confer similar CV benefits [60]. In our study, there is no bleeding observed in both mouse models as PAR-1 is absent in mouse platelets [61], which requires a different PAR isoform, PAR-3 for aggregation [62]. Although high dosages of vorapaxar (15 and 30 mg/kg) were used in our UUO and UIRI model, which were far higher than the human equivalent dose, the drug displayed high specificity without off-target effect as evidenced by the significant inhibition of PAR-1 expression, but not in other PAR family members, at 30 mg/kg of vorapaxar. Another study also showed that vorapaxar treatment of mice (30 mg/kg) improved the heart function of Dox-induced cardiotoxicity to a level seen in Dox treated PAR-1 deficient mice, suggesting the absence of off-target effects of vorapaxar treatment [63].

In light of increased thrombin generation in renal disease, vorapaxar has been tested in preclinical studies and found to improve diabetic complications including reduction of albuminuria, mesangial expansion and glomerular fibronectin deposition in STZ-induced mice [23]. However, in a type 2 diabetic model using BTBR *ob/ob* mice, vorapaxar only lowered blood glucose levels without any beneficial effects on renal function [64]. Our findings of the renoprotective effect of vorapaxar are in line with the observation that absence of PAR-1 protected kidneys from UUO-induced injury [24] and IR-induced injury [39]. Beneficial effects of vorapaxar not only apply to the maladaptive repair process in tubular cells, but also to the endothelial compartment during the development and progression of

CKD. A recent study also showed a beneficial effect of direct FXa inhibition with edoxaban on fibrotic changes in UUO model [65]. Edoxaban is mainly eliminated via the kidneys and is contraindicated in advanced CKD [66], whereas vorapaxar is mainly eliminated through feces (58%), with only 25% renal excretion [67]. Hence, vorapaxar may have a therapeutic edge over edoxaban in patients with moderate to severe CKD.

In conclusion, pharmacological inhibition of PAR-1 using vorapaxar may provide a novel renoprotective strategy in patients with CKD by virtue of its anti-oxidative, anti-inflammatory and anti-fibrotic effects, mediated partly via inhibition of ERK1/2 MAPK and Smad-dependent TGF-β signaling (Figure 8).

**CLINICAL PERSPECTIVES**

- Increased thrombin generation and abnormal activation of the coagulation system are common in patients with chronic kidney disease (CKD), indicating a role of protease-activated receptor (PAR)-1 (or the thrombin receptor) in thrombin-induced kidney fibrosis.
- Our results demonstrated that PAR-1 mediates thrombin-induced oxidative stress and overproduction of inflammatory and fibrotic molecules in tubular epithelial cells, via MAPK and TGF-β signaling.
- By targeting PAR-1, vorapaxar improves kidney injury by reducing intrarenal oxidative stress and ECM accumulation in UUO and UIRI mice. These novel findings suggest a potential PAR-1 targeted therapeutic strategy by vorapaxar in CKD.

**COMPETING INTERESTS**

All the authors declare no competing interests.

## DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and in its online supplementary material.

## FUNDING

## AUTHOR CONTRIBUTIONS

SWY Lok, WH Yiu designed and performed experiments, analysed data and wrote the manuscript. H Li, R Xue, Y Zou, B Li and KW Chan performed experiments and data collection. LYY Chan and JCK Leung helped with experimental design and interpretation of data. KN Lai and SCW Tang conceived and supervised the study.

## ACKNOWLEDGEMENTS

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**FIGURE LEGENDS**

**Figure 1. Thrombin induces PAR-1 expression in UUO kidneys.** (A) Representative periodic acid-Schiff (PAS) and PAR-1 staining of kidney sections from UUO (1, 3 and 7 days after surgery) and sham-operated (control) kidneys (scale bar, 100μm). (B) The generation of thrombin in control and UUO kidney by Western blotting. Band intensity of thrombin was normalized against β-actin for quantification in (C). (D) Progressive induction of PAR-1 mRNA expression from day 1 to day 7 UUO kidneys compared to sham-operated kidneys. For all experiments, mRNA expression was quantified by qPCR; *P<0.05 versus Sham, **P<0.01 versus Sham.

**Figure 2. Vorapaxar inhibited PAR-1 induction, accumulation of ECM proteins, and improved kidney morphology in UUO kidneys.** (A and B) PAR-1 mRNA and protein expression in sham-operated, vorapaxar treated and untreated UUO kidneys by Western blotting and qPCR, respectively. Band intensity of PAR-1 was normalized against β-actin. (C) PAR-1 expression, representative periodic acid-Schiff (PAS) and picrosirius red staining of kidney sections from sham-operated (control), UUO (7 days after UUO surgery) and vorapaxar-treated UUO kidneys (scale bar, 100μm). (D) Effect of vorapaxar on Fn, Col 1 and αSMA mRNA expression in UUO kidneys by qPCR. (E) Protein expression of Fn, Col 1 and αSMA in sham-operated, UUO and vorapaxar-treated UUO kidney by Western blot. Band intensity of Fn, Col 1 and αSMA was normalized against β-actin for quantification. All results

were expressed as mean ± SEM of data from sham-operated mice (n=6), UUO mice (n=8) and UUO mice treated with vorapaxar at different dosages (n=5-6); #P<0.05 versus Sham, ##P<0.01 versus Sham, ###P<0.001 versus Sham, *P<0.05 versus UUO, **P<0.01 versus UUO, ***P<0.001 versus UUO.

**Figure 3. Vorapaxar reduced thrombin-induced fibrotic and EMT markers in NRK-52E cells.** (A) The activation of PAR-1 after 48 hours of thrombin stimulation in NRK-52E cells by Western blotting. Band intensity of PAR-1 was normalized against β-actin for quantification in (B). (C) Thrombin-induced PAR-1 mRNA expression in NRK-52E cells at different time points. (D) The effect of vorapaxar on thrombin-induced mRNA expression of Fn and Col 1, E-cadherin, αSMA, Snai1 and Vimentin by qPCR. (E) Effect of vorapaxar on thrombin-induced protein expression of Fn, Col 1 and αSMA by Western blot. Band intensity of Fn, Col 1 and αSMA were normalized against β-actin for quantification in (F). For all graphs, results were expressed as mean ± SEM of data from 3-6 experiments; #P<0.05 versus Ctl, ###P<0.001 versus Ctl, *P<0.05 versus Thrombin, **P<0.01 versus Thrombin, ***P<0.001 versus Thrombin, ****P<0.0001 versus Thrombin.

**Figure 4. Vorapaxar ameliorated UUO-induced ERK1/2 and Smad3 signaling, and thrombin-induced TGF-β/MAPK signaling in NRK-52E cells.** . (A) Effect of vorapaxar on phosphorylated p42/44 and Smad3 protein and total latent TGF-β1 level by Western blotting. Band intensity of phosphorylated p42, p44 and Smad3 was normalized against their respective total protein. (B) Quantification of latent TGF-β1 band intensity against β-actin and mRNA level of TGF-β1 in UUO kidneys by qPCR. (C) Thrombin-induced mRNA level of PAR-1, Fn, Col 1, Snai1 and TGF-β1 pre-incubated with ERK1/2 inhibitor PD98059 (25 μM) after 72 hours of thrombin stimulation in NRK-52E cells. (D) Effect of vorapaxar on phosphorylation of p42/44 MAPK and Smad2/3 in thrombin-stimulated cells by Western blotting. Band intensity of phosphorylated p42/44, Smad3 were normalized against total Smad3 and p42/44, respectively. (E) Effect of vorapaxar and ERK1/2 inhibitor PD98059 on MCP-1 levels from thrombin-induced cell culture media. (F) Effect of vorapaxar on

thrombin-induced TGF-β1 mRNA expression was quantified by qPCR. (G) Effect of vorapaxar on active TGF-β1 levels from thrombin-induced cell culture media. (H) mRNA expression of αSMA and Col 1 in cells pre-incubated with Smad3 inhibitor SIS3 (5 μM) after 72 hours of thrombin stimulation. Results were expressed as mean ± SEM of data from sham-operated mice (n=6), UUO mice (n=8) and UUO mice treated with vorapaxar in different dosages (n=5-6); #P<0.05 versus Sham or Ctl, ##P<0.01 versus Sham or Ctl, ###P<0.001 versus Sham or Ctl, *P<0.05 versus UUO or Thrombin, **P<0.01 versus UUO or Thrombin, ***P<0.001 versus UUO or Thrombin.

**Figure 5. Blockade of PAR-1 inhibited renal inflammation, oxidative stress in UUO mice, and ROS production in thrombin-induced NRK-52E cells.** (A) Representative immunohistochemical staining of F4/80 on kidney section (scale bar, 100μm). Percentage of positively stained area for 10 random fields of the renal cortex was counted. (B) Effect of vorapaxar on MCP-1 and TNF-α mRNA expression in UUO kidneys by qPCR. (C) Protein expression of Nox4 from sham-operated, UUO and vorapaxar-treated UUO kidneys by Western blotting. Band intensity was normalized against β-actin. (D) Representative immunohistochemical staining of Nox4 on kidney section (scale bar, 100μm). (E) Total serum nitric oxide level from sham-operated, UUO and vorapaxar-treated UUO mice. (F) Effect of vorapaxar on thrombin-induced protein expression of Nox4 and iNos by Western blotting in NRK-52E cells. Bands intensity was normalized against β-actin. (G) Nox4 mRNA expression in cells pre-incubated with ERK1/2 inhibitor PD98059 (25 μM) after 72 hours of thrombin stimulation. (H) Detection of reactive oxygen species (ROS) by green fluorescence signal of oxidized H$_2$DCFDA (scale bar, 100μm) in thrombin-stimulated cells and quantified as ROS positive staining per field. Results were expressed as mean ± SEM of data from each group (n≥4); #P<0.05 versus Sham or Ctl ##P<0.01 versus Sham or Ctl, ###P<0.001 versus Sham, ####P<0.0001, *P<0.05 versus UUO or Thrombin, **P<0.01 versus UUO or Thrombin.

**Figure 6. Vorapaxar alleviated tubular injury and kidney fibrosis in UIRI mice.** (A) Representative periodic acid-Schiff (PAS) of kidney sections by immunohistochemistry from sham-operated (control), UIRI (14 days after UIRI surgery) and vorapaxar-treated UIRI kidneys (Scale bar, 100μm). PAS was quantified with tubular injury score. (B) Protein expression of thrombin, KIM-1 and PAR-1 expression in sham-operated, vorapaxar treated and untreated UIRI kidneys by Western blotting. Band intensity was normalized against respective β-actin. (C) Serum creatinine (Scr) level of sham-operated, vorapaxar treated and untreated UIRI mice. (D) Representative immunohistochemical staining of Fn, Col 1 and αSMA on sham-operated, vorapaxar treated and untreated UIRI kidney sections (scale bar, 100μm) and percentage of positively stained area for 10 random fields of the renal cortex. (E) Effect of vorapaxar on TGF- β1 and phosphorylated Smad3 level in UIRI kidneys by Western blotting. Band intensity was normalized against β-actin and respective total protein. All results were expressed as mean ± SEM of data from each group (n≥5); #$P<0.05$ versus Sham, ##$P<0.01$ versus Sham, ###$P<0.001$ versus Sham, ####$P<0.0001$, *$P<0.05$ versus UIRI, **$P<0.01$ versus UIRI, ****$P<0.0001$ versus UIRI.

**Figure 7. Effect of vorapaxar on IR-induced macrophage infiltration, endothelial dysfunction, and hypoxia-induced fibrosis in NRK-52E cells.** (A) Representative immunohistochemical staining of F4/80 and CD34 on sham-operated, vorapaxar treated and untreated UIRI kidney sections (scale bar, 100μm) and percentage of positively stained area for 10 random fields of the renal cortex. (B) mRNA expression of MCP-1 and TNF-α in UIRI kidneys by qPCR (C) Effect of vorapaxar on VCAM-1 mRNA level in UIRI kidneys by qPCR. (D) Effect of vorapaxar on PAR-1, Fn, Col 1 and TGF- β1 mRNA expression in hypoxia-induced NRK-52E cells by qPCR. All results were expressed as mean ± SEM of data from each group (n≥5); #$P<0.05$ versus Sham or Ctl, ##$P<0.01$ versus Sham or Ctl, ####$P<0.0001$, *$P<0.05$ versus UIRI or Hypoxia, ***$P<0.001$ versus UIRI or Hypoxia.

**Figure 8. Schema of PAR-1 signaling in tubular cells.** Seven-transmembrane G protein-coupled receptor PAR-1 signaling is mediated through cleavage of the extracellular N-

terminal domains by the endogenous serine protease, thrombin, which causes a conformational change of the receptor to activate the ERK1/2 MAPK pathway. PAR-1 transcript expression is further induced via ERK1/2 signaling, resulting in the activation of the TGF-β/Smad pathway, which mediates ECM protein accumulation. ERK1/2 signaling also increases the production of pro-inflammatory cytokines MCP-1 and TNF-α, which stimulates macrophage recruitment. In addition, oxidative stress is mediated through PAR-1/ERK/Nox4/ROS production. In summary, thrombin-induced PAR-1 signaling leads to tubular injury by mediating fibrosis, inflammation and oxidative stress. Administration of vorapaxar, a PAR-1 antagonist, abrogates these detrimental effects.