Delayed treatment with PTBA analogs reduces post injury renal fibrosis after kidney injury


Department of Medicine, Division of Nephrology, Department of Cell and Developmental Biology, Vanderbilt Institute of Chemical Biology, Department of Pharmacology, Department of Biochemistry and Mass Spectrometry Research Center, Vanderbilt University; Drug Discovery Institute, Developmental Biology, Department of Pharmaceutical Sciences, Department of Computational and Systems Biology and the Center for Critical Care Nephrology, University of Pittsburgh

* These authors contributed equally to the paper (co-first authors)

11 These authors contributed equally to the paper (co-senior authors)

# Corresponding author

Mark de Caestecker M.B., B.S., Ph.D., Nephrology Division, Vanderbilt University, S3223, Medical Center North, 1161 21st Avenue South, Nashville, TN 37232, USA. Telephone: 615-343-2844. Email: mark.de.caestecker@vanderbilt.edu: Neil Hukriede Ph.D., Department of Developmental Biology, University of Pittsburgh, 3501 5th Ave. 5061 BST3, Pittsburgh, PA 15213. Telephone: 412-648-9918. Email: hukriede@pitt.edu

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Abstract

No therapies have been shown to accelerate recovery or prevent fibrosis after acute kidney injury (AKI). In part this is because most therapeutic candidates have to be given at the time of injury and the diagnosis of AKI is usually made too late for drugs to be efficacious. Strategies to enhance post-AKI repair represent an attractive approach to address this. Using a phenotypic screen in zebrafish we identified 4-(phenylthio)butanoic acid (PTBA), which promotes proliferation of embryonic kidney progenitor cells (EKPCs), and the PTBA methyl ester, UPHD25, which also increases post-injury repair in ischemia reperfusion and aristolochic acid induced AKI in mice. In these studies a new panel of PTBA analogs were evaluated. Initial screening was performed in zebrafish EKPC assays followed by survival assays in a gentamicin-induced AKI larvae zebrafish model. Using this approach we identified UPHD186, which in contrast to UPHD25, accelerates recovery and reduces fibrosis when administered several days after ischemia reperfusion AKI, and reduces fibrosis after unilateral ureteric obstruction in mice. UPHD25 and 186 are efficiently metabolized to the active analog, PTBA, in liver and kidney microsome assays, indicating both compounds may act as PTBA prodrugs in vivo. UPHD186 persists longer in the circulation than UPHD25, suggesting that sustained levels of UPHD186 may increase efficacy by acting as a reservoir for renal metabolism to PTBA. These findings validate use of zebrafish EKPC and AKI assays as a drug discovery strategy for molecules that reduce fibrosis in multiple AKI models and can be administered days after initiating injury.
Introduction

Severe acute kidney injury (AKI) affects more than 90,000 patients in the US each year, while milder, non-dialysis dependent AKI affects more than 1.5 million per year(1, 15, 22). AKI is most often precipitated by major surgery-particularly cardiopulmonary by-pass surgery, prolonged hypotension from any cause, sepsis, drug and systemic toxicities, and urinary obstruction(1, 22). AKI is a precursor to chronic kidney disease (CKD), and there is increased risk of end stage renal disease in patients with severe AKI(2-4, 8, 28, 29). Despite this, to date, no therapeutic interventions have been shown to either prevent injury, accelerate recovery, reduce post-injury fibrosis, or prevent progressive CKD in patients after an episode of AKI (19). In part, this is because drug candidates usually have to be administered within hours of the initiating injury and the diagnosis of AKI is usually made late, often days after the injury has occurred(1).

Therapeutic strategies designed to enhance post-AKI repair mechanisms represent an attractive approach, as repair occurs largely by proliferation of surviving, de-differentiated, tubular epithelial cells a number of days after the initiating injury(12). To address this, we developed a high content phenotypic screen using zebrafish embryos to identify compounds that promote proliferation of embryonic kidney progenitor cells (EKPCs)(9, 23). Since proliferating tubular epithelial cells express markers of kidney progenitor cells after injury(5), we reasoned that compounds promoting expansion of zebrafish EKPCs might also increase proliferation of surviving epithelial cells after AKI. Using this approach we identified the small molecule, 4-(phenylthio)butanoic acid (PTBA), a histone deacetylase inhibitor (HDACi) that expands EKPCs in zebrafish embryos(9, 23), and the methyl ester of PTBA, methyl-4-(phenylthio)butanoate (M4PTB/UPHD25), which increases tubular cell proliferation, accelerates recovery and reduces renal fibrosis when administered after injury in two different mouse models of AKI, ischemia reperfusion-induced AKI (IR-AKI) and aristolochic acid induced AKI (AA-AKI)(7, 20).

In these studies, a new series of PTBA analogs were evaluated for efficacy in accelerating recovery and reducing post-injury fibrosis after AKI. Primary screening was performed using two complementary
zebrafish EKPC assays. This was followed by secondary and tertiary screens in larval zebrafish and mouse models of AKI, respectively, to evaluate the most promising PTBA analogs for their ability to
enhance post-injury repair. Using this functional phenotypic screening approach we found that activity in zebrafish and mice is dependent on exposure to PTBA itself, and developed the PTBA prodrug, UPHD186, which accelerates recovery and reduces post-injury fibrosis when administered several days after the initiating injury. We also show that UPHD186 is effective in reducing post-injury renal fibrosis after unilateral ureteric obstruction in mice, extending the spectrum of PTBA analog activity from ischemia and toxin-induced injury to a model of post-renal AKI. These findings establish the sequential use of zebrafish EKPC and AKI models as a drug discovery strategy to identify molecules that accelerate recovery and reduce post-injury fibrosis after AKI. The discovery of UPHD186 represents an important pre-clinical advance since no established treatments have been shown to accelerate recovery or reduce progressive CKD when administered late after the diagnosis of AKI has been made.
Results

Screening of PTBA analogs in embryonic kidney progenitor cell assays. To identify PTBA analogs with more favorable activities than M4PTB/UPHD25 in accelerating recovery and reducing post-injury fibrosis after AKI, we synthesized and tested a new series of PTBA analogs in zebrafish EKPC assays. Based on our finding that PTBA and the PTBA methyl ester, UPHD25, expand zebrafish EKPCs by in situ hybridization (ISH)(9, 23), we screened a panel of PTBA analogs for effects on expansion of the lhx1a mRNA positive embryonic kidney fields(9). Sixty-four PTBA analogs were screened and twenty were found to be active (estimated EC₅₀ >3μM, S. Table). These compounds contained structural modifications in the benzene ring (R¹, predicted to enhance stability), and modifications to the acid functionality with different zinc-chelating moieties (R², predicted to increase HDAC inhibition(13)) (see Figure 1D). Representative active analogs that contained an ester (UPHD25), a hydroxamic acid (UPHD29), two benzamides (UPHD36 and 186), as well as an inactive ketone lacking a zinc-chelating moiety as a negative control (UPHD263), were further evaluated further using the Cognition Network Technology (CNT) EKPC assay. The CNT assay uses the transgenic zebrafish line Tg(cdh17:EGFP)pt305, and complements the ISH assay by providing an independent, quantitative measure of changes to the size of the kidney field in larvae exposed to compounds for 48 hours post fertilization (hpf) (Figure 1A/B) (23). Using this assay we measured the EC₅₀ for expansion of the kidney field for each compound (Figure 1C/D). In addition to PTBA (EC₅₀=1.48 uM), we evaluated: 1) UPHD25 (EC₅₀=0.65 uM), the PTBA methyl ester (9); 2) UPHD29 (EC₅₀=1.44 uM) a fluoro-substituted hydroxamic acid analog of PTBA; 3) UPHD36 (EC₅₀ – 1.41 uM) an analog in which the acid was replaced with a 2-aminoanilide moiety; and 4) UPHD186 (EC₅₀=0.77 uM) an analog in which the acid was replaced with a 2-hydroxyanilide moiety (S. Table, Figure 1C, D). In addition, as anticipated based on the ISH lhx1a assay, the inactive ketone, UPHD263, had no effect on expansion of the cdh17:EGFP domain in the CNT assay.

PTBA analog efficacy in zebrafish AKI assays. Previous studies using our zebrafish ISH assay indicate
that it predicts efficacy in mouse models of AKI(7, 20). However it was impractical to screen the large
number of positive compounds identified in the EKPC assay in mice. To address this we utilized a model
of gentamicin-induced AKI in zebrafish larvae and scored for larval survival. Since gentamicin toxicity is
specific to renal epithelial cells and the inner ear due to selective uptake in these organs(16), and edema
is a sign of renal dysfunction(10), we selected edematous larvae for treatment and scored for organism
survival as an initial indicator of renal injury and repair following compound treatment, as previously
described (6, 7). We evaluated PTBA, UPHD25, 29, 36, 186, and 263 for efficacy in improving survival of
zebrafish larvae after gentamicin-induced AKI at ¼ their maximum tolerated doses (MTD) (S. Figure)(7).
In this model we treated zebrafish larvae with a single dose of compound 2 days after gentamicin
injection and assayed for survival over the next four days. While PTBA, UPHD25, 36 and 186 significantly
improved post-injury survival, UPHD29 and 263 showed no effect (Figure 2).

**PTBA analog efficacy in accelerating recovery and reducing renal fibrosis after AKI in mice.** To
determine whether results from the larval AKI screen were predictive of efficacy in mice, we first
evaluated the efficacy of UPHD25, 36 and 186, which showed activity in the zebrafish EKPC and AKI
assays, and of UPHD29, which showed activity in zebrafish EKPC but not the zebrafish AKI assays, in a
mouse model of ischemia reperfusion induced AKI (IR-AKI). To determine optimal dosing, we initially
evaluated the effects of UPHD25 at 1, 10 and 50mg/kg administered via intra-peritoneal injection (IP) on
days 1-7 on expression of fibrosis markers in the injured kidney 28 days after unilateral IR-AKI. Dose
ranges were based on previous experience treating mice with UPHD25 (7, 20). There was a dose
dependent reduction in expression of the fibrosis markers *Col1α1*, α-SMA and *LoxL2* mRNAs, with
maximal effects at 50mg/kg/day of UPHD25 (Figure 3A-C). Since we have previously shown that PTBA
analogs are histone deacetylase inhibitors (HDACi) that increase levels of hyper-acetylated histones in
the kidney in mice(7), we compared the effects of a single 50mg/kg IP dose of the PTBA analogs UPHD25,
29, 36 and 186, on renal histone acetylation (Figure 3D). All of the PTBA analogs increased renal histone
H4 acetylation within an hour of treatment. Histone H4 acetylation returned to baseline 4 hours after
treatment with UPHD25, 29 and 186, but persisted up to 4 hours with UPHD36. Based on these results we evaluated the effects of PTBA analogs at a dose of 50mg/kg/day from days 1-7 after injury for all studies, unless otherwise indicated. We performed unilateral IR-AKI followed by contralateral nephrectomy 8 days after injury to evaluate functional recovery and post-injury fibrosis, as described(7, 24). UPHD25 and 186 treated mice have reduced blood urea nitrogen (BUN) compared with vehicle 24 hours after the contralateral nephrectomy (Figure 4A). UPHD186 also reduced serum creatinine 24 hours after contralateral nephrectomy (Figure 4B). The relatively high BUN levels that we observed in some of the mice relative to changes in serum creatinine may reflect a degree of dehydration that occurs at the time these assays were performed, 24 hours after contralateral nephrectomy surgery. It is well recognized that dehydration preferentially increases BUN vs. serum creatinine values after AKI(25). In contrast, UPHD36 had no effect on BUN or serum creatinine after AKI. Renal fibrosis markers showed a reduction in Col1α1, α-SMA and LoxL2 mRNA in UPHD25 and 186 treated mice 28 days after injury (Figure 4C-E). There was also a reduction in renal fibrosis assessed by Sirius red staining in UPHD25 treated mice (Figure 4F/G). Consistent with effects on BUN and serum creatinine, UPHD29 had no effect on post-injury renal fibrosis, while UPHD36 reduced expression of 1/3-fibrosis markers but had no effect on Sirius red staining.

We next evaluated effects of UPHD263 and PTBA treatment in reducing post-injury fibrosis after IR-AKI. Treatment with UPHD263 from day 1-7 after IR-AKI had no effect on renal fibrosis 28 days after injury (Figure 5A/B), and increased expression of only 1 out of 3 renal fibrosis markers (Figure 5C-E). Since UPHD263 was inactive in zebrafish EKPC assays, this suggests that lack of activity in the EKPC assay is predictive of a lack of efficacy in mouse AKI. Since PTBA was active in both the EKPC and larval AKI models, we also compared efficacy of PTBA with that of UPHD186 after IR-AKI in mice. Unlike UPHD186, PTBA had no effect on post-injury fibrosis and no significant effects on the expression of fibrosis markers 28 days after injury (Figure 5F-J). Taken together, these data indicate that lack of activity in zebrafish EKPC and/or larval AKI assays can be used to eliminate PTBA analogs that are ineffective in reducing
post-injury fibrosis after mouse IR-AKI. However, these data also indicate that not all of the compounds that show activity in both of the zebrafish assays are effective in reducing renal fibrosis in mouse IR-AKI.

**Pharmacokinetics of PTBA analogs in mice.** Since biological activity in mice is dependent on absorption, distribution, metabolism and excretion (ADME) characteristics, we performed a comparative plasma pharmacokinetic (PK) study in mice after IP injection of PTBA analogs (Figure 6, Table 1). PTBA itself was rapidly absorbed with the peak plasma level obtained within 5 minutes (Table 1). UPHD25 was undetectable after IP injection in mice. However, peak plasma levels of PTBA were detected 5 minutes after IP injection of UPHD25 (Figure 6A, Table 1), suggesting that UPHD25 is rapidly bio-converted to PTBA. Also evident was the rapid absorption and metabolic conversion of UPHD186 to PTBA within 5 minutes (Figure 6B; Table 1). This suggests UPHD25 and 186 may be acting as PTBA prodrugs, most likely hydrolyzed to the acid, PTBA, by ubiquitous and promiscuous tissue and serum esterase and amidases (Figure 6A/B, arrows indicate potential cleavage sites)(21). This is consistent with our finding that UPHD25 was only detectable in spiked mouse plasma samples if the samples were pretreated with an esterase inhibitor (S. Methods 1). In addition, our observation that PTBA increases survival in zebrafish AKI (Figure 2A) is consistent with the hypothesis that PTBA is the active metabolite of UPHD25 and 186. However, PTBA itself was ineffective in reducing post-AKI fibrosis in mice (Figure 5F-J). It is notable that the area under the curve (AUC) for PTBA was markedly reduced compared with PTBA levels after injecting UPHD25 and 186 (Table 1). Since the peak plasma levels of PTBA after IP injection of UPHD25, 186 and PTBA were similar (Cmax, Table 1), this suggests that efficacy of UPHD25 and 186 is driven by AUC, while decreased efficacy of PTBA in mice may result from decreased tissue exposure to PTBA associated with a reduced AUC. Since zebrafish are soaked continuously in PTBA, it is likely that efficacy in zebrafish models reflects greater tissue exposure in this model compared to mice.

UPHD36 was active in zebrafish EKPC and AKI assays but had only minor effects on fibrosis in mice after AKI. Similar Cmax and AUC levels of PTBA were detected after IP dosing of UPHD36, 25 and 186 (Figure
Like UPHD186, UPHD36 contains an amide group that could be hydrolyzed to PTBA (Figure 6D). However, the $C_{\text{max}}$ for the parent UPHD36 was higher than that for UPHD186 (Table 1). This suggests that hydrolysis of UPHD36 to PTBA is less efficient than UPHD186. As expected, we were unable to detect PTBA in the plasma up to 8 hours after injection of UPHD263 due to absence of a hydrolysable amide (Table 1). Since UPHD263 is inactive in mouse and zebrafish AKI models, these findings suggest that conversion to PTBA is necessary but not sufficient for biological activity in zebrafish and mouse AKI.

**Metabolic stability and activity of PTBA analogs in vitro.** To explore the metabolism of PTBA analogs further, we performed *in vitro* stability and activity (appearance of PTBA) assays using mouse liver and kidney microsome preparations. PTBA was stable in liver and kidney microsome preparations, while UPHD25 and 186 were efficiently metabolized and converted to PTBA when incubated with both liver and kidney microsomes for 50 minutes (Table 2). Metabolism of parent compounds and the appearance of PTBA were NADPH independent, indicating that UPHD25 and 186 metabolism is not a cytochrome P450 reaction (30). This contrasts with UPHD36 metabolism as this is NADPH-dependent and occurs at a slower rate than UPHD186, particularly in kidney microsomes (Table 2). These findings lend further support to our hypothesis that UPHD25, 36 and 186 are prodrugs for the active species, PTBA, and differences in efficacy in mice are likely to result from differences in the rates of metabolism and release of PTBA at the site of injury.

**Delayed treatment with UPHD186 reduces post-injury fibrosis after IR-AKI.** Having established the efficacy of UPHD25 and 186 in accelerating recovery and reducing post-injury fibrosis when administered 24 hours after the initiating injury, we evaluated the effects of delayed treatment on IR-AKI outcomes in mice. For these studies we again performed unilateral IR-AKI followed by delayed contralateral nephrectomy 8 days after the initial injury, but now PTBA analog treatment was delayed until 3 days after injury and continued for only 4 days. Treatment with UPHD186 3 days after injury reduced serum creatinine, decreased post-injury fibrosis and reduced expression of all 3 renal fibrosis
markers 28 days after injury (Figure 7A-D). In contrast, while UPHD25 reduced serum creatinine values 9 days after injury, delayed treatment with UPHD25 had no effect on renal fibrosis (Figure 7E-H).

Since delayed treatment with UPHD186 reduces post-injury fibrosis when administered late after the initiating injury, we asked whether UPHD186 could reverse established post-injury fibrosis. For this we developed a new model of IR-AKI that allowed us to evaluate effects of UPHD186 on established post-injury renal fibrosis. Mice underwent prolonged unilateral renal pedicle clamping (35 minutes vs. 31 minutes used in previous studies), followed by an open renal biopsy 30 days after injury to assess baseline post-injury fibrosis. After the renal biopsy, mice were treated with 100mg/kg UPHD186 IP for 10 days, and injured kidneys harvested 50 days after initial injury (Figure 8A). Kidneys harvested from mice 30 days after injury were visibly atrophic, with markedly increased expression of the fibrosis markers, \(Col1\alpha1\) and \(\alphaSMA\) mRNAs in renal biopsies (Figure 8B/C). Renal \(Col1\alpha1\) and \(\alphaSMA\) mRNAs were still increased 50 days after injury, but there was no change in expression of either marker in UPHD186 treated mice, and the extent of renal fibrosis as determined by Sirius red staining was no different in UPHD186 and vehicle treated mice (Figure 8D-G). Taken together these data suggest that UPHD186 is only effective in reducing post-injury fibrosis after IR-AKI when administered during the active regenerative phase after IR-AKI but not once the post-injury fibrosis has been established.

**UPHD186 reduces renal fibrosis after unilateral ureteric obstruction (UUO) in mice.** Finally, in order to determine whether UPHD186 is also effective in other models of AKI, we compared the efficacy of UPHD25 and 186 in reducing renal fibrosis after UUO. For these studies UPHD25 and 186 were administered at a dose of 100mg/kg/day starting at the time of surgery and continued for 7 days (Figure 9A). There was a significant reduction in 2/3 fibrosis mRNA markers in mice treated with UPHD25 8 days after surgery compared with vehicle control, but no significant reduction in renal fibrosis assessed by Sirius red staining for renal collagen deposition (Figure 9B-E). In contrast, UPHD186 treated mice showed a significant reduction in expression of all 3 fibrosis markers and there was less renal fibrosis by
Sirius red staining in UPHD186 compared with UPHD25 treated mice. These data indicate that UPHD186 is more effective than UPHD25 in reducing renal fibrosis after UUO.


**Discussion**

Prior studies from our laboratories used phenotypic screens in zebrafish embryos to identify the PTBA analog, UPHD25, which accelerates recovery from AKI in zebrafish and mice and reduces post-injury fibrosis in ischemia-reperfusion and aristolochic acid-induced models of AKI in mice (7, 20). In the current study we have refined this approach to characterize a phenotypic drug discovery paradigm in which screening was performed using ISH for expression of the EKPC marker, *lhx1a*, and an automated fluorescence assay to quantify expansion of kidney structures in zebrafish embryos. This was followed by zebrafish larval AKI survival assays to evaluate efficacy in reducing injury and accelerating renal repair after AKI. Both models are largely unaffected by drug delivery and absorption problems that often occur in early drug discovery in mice. Using this approach we identified the PTBA prodrug, UPHD186, which accelerates recovery and reduces post-injury fibrosis after IR-AKI and is effective when administered several days after the initiating injury. UPHD186 also reduces post-injury fibrosis after UUO. In contrast, UPHD25 has no effect on renal fibrosis when administered late after injury in IR-AKI, and has limited effects on fibrosis after UUO. These findings establish the sequential use of zebrafish EKPC and larval AKI models as an effective drug discovery platform to identify molecules that reduce renal fibrosis after AKI in mice. The ability of UPHD186 to reduce fibrosis when administered late after injury and its efficacy in an obstruction model of AKI represent important new pre-clinical advances. Agents that can be administered late after the initiation of renal injury are more likely to be effective in human AKI as patients often present late in the course of their illness (1). In addition, agents that are active in multiple models of AKI are more likely to be effective in human AKI in which pathogenesis is often complex and multifactorial.

A retrospective analysis of FDA approved drugs over the last decade showed that the majority of first-in-class agents were discovered in phenotypic, not target based, screens (27). The discovery of UPHD186 validates use of our phenotypic screen in zebrafish to identify compounds that are effective in accelerating recovery and reducing fibrosis when administered several days after the initiating injury.
The discovery of UPHD186 was only possible through screening in a living organism since the molecular events mediating recovery are poorly understood. These studies also provide insight into efficacy, since they suggest that differences in activity of PTBA analogs in mice does not depend on the nature of zinc chelating moieties predicted to increase HDAC inhibition (13), but rather on efficiency with which they are metabolized to PTBA.

Our studies show that PTBA is active in zebrafish AKI assays and is present as the dominant metabolite in mice treated with UPHD25 and 186. This suggests that PTBA is the active species for the prodrugs UPHD25 and 186. However, when delivered IP PTBA is not effective in preventing fibrosis in mice with AKI. Since plasma PTBA decreases rapidly after IP injection, it is likely its lack of efficacy results from rapid elimination from the circulation, and that UPHD25 and 186 are active because there is increased total exposure to PTBA. However, UPHD36 is also metabolized to PTBA in mice. Unlike UPHD186, UPHD36 undergoes cytochrome P450-dependent metabolism, and conversion of UPHD36 to PTBA is less efficient than UPHD25 and 186. This suggests that while UPHD36 is effective in zebrafish AKI assays because metabolite production is not limited by decreasing levels of the parent compound in these assays, UPHD36 is ineffective in mice because there is inefficient conversion to PTBA. On this basis, we propose that differences in efficacy of UPHD25, 36 and 186 in mouse AKI result from differences in the rate of metabolism and release of PTBA. Since UPHD186 is efficiently converted to PTBA in kidney microsomes, it is likely that increased efficacy of delayed treatment with UPHD186 compared with UPHD25 results from prolonged local release of PTBA in the kidney from UPHD186.

In summary, our studies establish a novel, sequential phenotypic screening approach using zebrafish to identify molecules that accelerate recovery and reduce post-injury fibrosis in mice. Using this approach we have identified a novel prodrug, UPHD186, which is particularly effective when administered days after the initiating injury in mouse AKI. Pharmacokinetic and metabolism studies suggest that efficacy of UPHD186 in mouse AKI results from its efficient conversion to the active metabolite PTBA in the kidney.
Methods

Zebrafish Embryonic Kidney progenitor cell (EKPC) assays. Embryos were processed for kidney expansion by *in situ* hybridization or Cognitive Network technology (CNT) analysis: 1) *In situ* hybridization assays. Wild type PITT-AB embryos were treated with compounds at 3.0, 1.5, 0.8, 0.4 and 0.2µM at 512 cell stage by serial dilution in E3 medium containing 0.5% dimethyl sulfoxide (DMSO, Sigma), as described(9). Embryos were incubated at 22ºC overnight and fixed in 4% paraformaldehyde at 10-somite stage. *In situ* hybridization was performed using *lhx1a* riboprobes, as described(14). At least 20 embryos per condition were visually scored for increased *lhx1a* mRNA expression. Data are presented as the percentage of embryos with expanded *lhx1a*. 2) Cognition Network Technology (CNT) assays. CNT assays were used to quantify the half maximum effective concentrations (EC50) for compounds that increased *Lhx1* mRNA expression. For this we utilized transgenic zebrafish, Tg(cdh17:EGFP)pt305, as described(23). At 512 cell stage 10 to 15 embryos in 12-well plates were treated with vehicle or increasing concentrations of compounds (3.0 to 0.2 µM) in E3 medium containing 0.5% DMSO. Embryos were allowed to develop for 48 hours, dechorionated, arrayed in flat-bottom 96-well plates and anesthetized with 40 µg/mL MS222 (tricaine methanesulfonate; Sigma) to restrict movement during imaging. Images were acquired at a single wavelength (GFP). Because the elongated morphology of 48 hour post fertilization (hpf) embryos necessitated capture of the entire well, a MetaXpress journal was generated that acquired two sites that were stitched together into a single montage. Archived fluorescence micrographs were uploaded into Developer (Definiens AG, Munich, Germany) using the Cellenger module. CNT rule sets were used that quantified Cdh17-EGFP expression within the developing embryo, as described(23).

Zebrafish larval maximum tolerated dose (MTD) and AKI survival assays. MTD assays were performed with minor modifications, as described(7). Cohorts of 15 zebrafish larvae were arrayed in 6
well plates and treated with 1% DMSO vehicle, or increasing concentrations of compounds in E3 medium plus 1% DMSO. Treatment was initiated at 5 days post fertilization (dpf) and continued for 24 hours. For zebrafish larval AKI, compounds were used at one-quarter the MTD. 3dpf larvae were treated with 8ng gentamicin (Sigma) diluted in 1nL normal saline injected into the common cardinal vein. Lucifer yellow dextran (10 kDa; 1 mg/ml; Invitrogen) was included with the gentamicin to verify successful microinjection, as described(6). After injection, larvae were incubated at 28°C for 2 days. At 2 days post injection (dpi), larvae showing signs of pericardial edema were transferred to E3 medium containing 1% DMSO with or without compounds. Treatment was continued for 24 hours at 28°C when treatment solution was replaced with E3 medium without compounds. Larvae were assayed for survival at 4, 5, 6 and 7dpi. Pittsburgh University Institutional Animal Care and Use Committee approved experimental protocols in zebrafish.

**Mouse ischemia reperfusion-induced AKI (IR-AKI), renal biopsy and unilateral ureteric obstruction (UUO).** Surgeries were performed on a water bath-heated platform at 38°C on 10- to 12-week-old male BALB/c mice. To induce IR-AKI, mice underwent left renal pedicle clamping for 31 minutes, and delayed contralateral nephrectomy was performed after 8 days, as described(24). None of the mice died in either the vehicle or treatment groups over the course of these studies. Serum creatinine was evaluated using enzymatic cascade assays (Pointe Scientific, Canton MI). Mice were dosed with 1mg/kg, 10mg/kg or 50 mg/kg UPHD25, or 50mg/kg PTBA, UPHD29, 36 186, 263 or vehicle control (20% 2-hydroxypropyl-β-cyclodextrin in phosphate buffered saline (PBS) by daily IP injection, starting 24 hours after injury for 7 days, or 3 days after injury for 4 days, as indicated. To study the effect of UPHD186 effect on regression of fibrosis, mice underwent left pedicle clamping for 35 min and open kidney biopsy on day 30 to assess fibrosis, as described(17). Mice were then randomized into vehicle or 50mg/kg UPHD186 IP daily for 10 days and kidneys harvested on day 50. For unilateral ureteric obstruction (UUO) surgery, male Balb/c mice underwent left sided ureteral ligation, and treated with
either vehicle or 50 mg/kg UPHD25 or 186 IP daily for 7 days starting at the time of surgery. Obstructed kidneys were harvested 8 days after surgery for analysis of renal fibrosis. Experimental protocols were approved by the Vanderbilt Institutional Animal Care and Use Committee.

**Analysis of renal fibrosis and histone acetylation.** To assess fibrosis, kidneys were harvested, fixed and mounted in paraffin, and Sirius red staining and quantification performed to evaluate renal fibrosis/collagen accumulation in kidney sections, as described(7). Image capture and semi-quantitative evaluation was performed by observed blinded to treatment (N.I.S. and K.P.). RNA isolation and quantitative RT-PCR was performed to evaluate expression of renal fibrosis markers in snap frozen kidneys using primer sequences and RNA extraction, as previously described(7). Histone acetylation was determined in snap frozen kidneys by Western blot using rabbit monoclonal anti-histone H4 (Clone 62) and rabbit anti-acetylated H4 Lysine 5/8/12 and 16 antibodies (Millipore, pan H4 acetylation), infrared 680LT conjugated donkey anti-rabbit IgG secondary antibodies (LI-COR), and fluorescence images were captured using the Odyssey Laser Fluorescence Detection System, as previously described(7).

**Pharmacokinetic (PK) studies.** 8-12 week male BALB/c mice were given 50mg/ml PTBA, UPHD25, 36, 186 or 263 in 20% 2-hydroxypropyl-β-cyclodextrin in water IP, and 60μL of blood collected in 3 time sets: 0.08, 0.25 and 0.5 hours; 1, 2 and 4 hours; and 6, 8 and 24 hours post injection (3 mice per compound in 3 time sets). Plasma samples were separated, stored below -70°C, and processed for analysis after precipitation using acetonitrile followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) to identify compounds and PTBA (S. Methods 1). Non-compartmental analysis module in Phoenix WinNonlin® (Version (8)6.3) was used to assess PK parameters. This experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) at SAI Life Sciences Ltd,

**Synthetic chemistry for all of the PTBA analogs** is described in supplemental data (S. Methods 2).
In vitro stability and activity assays. We used a solution containing 1 mg/mL mouse liver and kidney microsomes (CD-1 male, Xenotech), 10 µM test compound +/- 1mM Nicotinamide adenine dinucleotide phosphate (NADPH, Sigma-Aldrich) in 50mM potassium phosphate buffer, pH 7.4. Time zero samples were immediately quenched with 1.0mL acetonitrile containing 0.1% formic acid. Test samples were incubated at 37°C for 50 minutes before quenching. Standard curves were generated by spiking time zero microsomal preparations with compounds. Supernatants were dried under nitrogen and reconstituted in water for analysis by liquid chromatography and mass spectrometry (LC-MS) in the Vanderbilt Mass Spectrometry Core Laboratory, as described in the supplemental data (S. Methods 3).

Statistical analyses. Statistical analyses performed included by Student’s two-tailed t test for paired group comparisons, one-way ANOVA for multiple between-group comparisons using Tukey correction for post-hoc, pair-wise between group comparisons, as indicated in the figure legends. The minimal level of significance was set at P≤0.05 and statistical analyses performed using GraphPad Prism V6.
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Disclosures: Mark de Caestecker performed consultancy work for NephroGenex.
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Figure and Table Legends

**Figure 1.** Quantitation of kidney organ expansion by automated imaging. *Tg(chd17:EGFP)* transgenic zebrafish (A) were treated for 48 hours at the 256-512 cell stage with vehicle (0.5% DMSO) or specified compounds, followed by washout. At 72hpf embryos were dechorionated and imaged in 96 well plates (8 embryos per condition). Images were acquired in the GFP channel and analyzed by a Definiens Cognition Network Technology (CNT) rule set. Starting from the original image (A), the rule-set successively detected the entire embryo and the fluorescently labeled kidney (B, orange). Further sub-segmentation identified the cloaca based on brightness and proximity to the zebrafish edge (B, blue), enabling final measurements to be made in a defined length segment relative to the cloaca (B, red). (C) Quantitation of kidney expansion by UPHD20, 25, 29, 36, 186, and 263. Images were analyzed by CNT and total transgene expression in the tubular segment calculated. Each data point represents the averages +/-SEM of three independent experiments. (D) Structure of PTBA analogs with sites of modification (R¹ and R²) and EC₅₀ for compounds UPHD20 (PTBA), 25, 29, 36, 186 and 263.

**Figure 2.** Augmented survival after treatment with PTBA analogs in zebrafish larval AKI. (A) Survival after gentamicin-induced AKI. Gentamicin-injected larvae were treated with either 1% DMSO vehicle (V, white triangle) or at ¼ MTD for compounds (black triangles): (A) PTBA (1 μM), (B) UPHD25 (2 μM), (C) UPHD29 (2 μM), (D) UPHD36 (8 μM), (E) UPHD186 (4 μM), and (F) UPHD263 (4 μM) at 2 days post gentamicin injection (dpi). Legend indicates PTBA and UPHD identification numbers. The survival rate was scored at 4, 5, 6 and 7 dpi. Data pooled from 3 survival assays are shown expressed as mean+/−SEM. 2-tailed T-test on day 7 post injection: *p <0.05, **p<0.01, ***p<0.005, #<0.0001.

**Figure 3.** Dosing with PTBA analogs in mice. (A-C) Dose-dependent effects of UPHD25 on post-injury fibrosis after IR-AKI. Male Balb/c mice underwent left sided renal pedicle clamping for 31 minutes to
induce IR-AKI, were treated for 7 days with vehicle, 1mg/kg, 10mg/kg or 50 mg/kg UPHD25 IP daily for 7
days starting 24 hours after injury, as indicated. Injured kidneys were harvested 28 days after injury and
evaluated for expression of renal fibrosis markers, Col1α1, α-SMA and LoxL2 mRNA relative to Gapdh
mRNA control. Results expressed as means +/- SEM (injured vehicle controls, n=17; 1mg/kg, n=7;
10mg/kg, n=8; 50mg/kg, n=11 mice). 1-way ANOVA (p<0.05) with Dunnett’s correction for multiple
between-group testing: *p<0.05, **p<0.01 vs. injured vehicle control, as indicated. (D) Kinetics of renal
histone hyper-acetylation after IP injection of PTBA analogs in mice. CD1 mice were injected once with
50mg/kg of PTBA analogs UPHD25, 29, 36 and 186 IP, as indicated. Kidneys were isolated at different
time point for histone extraction and analysis of histone H4 acetylation. Fluorescence immunoblots for
total renal histone H4 and acetylated histone H4 K5/8/11/16 (Acetyl H4) captured using Odyssey Infrared
Imaging System. Replicates for each of the time points obtained from different mice.

**Figure 4. UPHD25 and 186 accelerate functional recovery and post-injury fibrosis after IR-AKI.**

Male Balb/c mice underwent left sided renal pedicle clamping for 31 minutes to induce IR-AKI, and were
treated with vehicle or 50 mg/kg UPHD25, 29, 36 or 186 IP daily for 7 days starting 24 hours after injury.

(A/B) Functional recovery. Blood urea nitrogen (BUN, A), serum creatinine (B) was measured at day 9,
one day later after right nephrectomy to assess functional recovery (mouse numbers for A/B: uninjured
mice, n=12/15, day 9 after injury, vehicle controls, n=20/15; UPHD25, n=10/11; UPHD29, n=11 each;
UPHD36, n=12/10; UPHD186, n=9 each). Individual data points and means for each group shown. (C-G)
Renal fibrosis 28 days after IR-AKI. (C-E) Expression of renal fibrosis markers. Col1α1, α-SMA and LoxL2
mRNA relative to Gapdh mRNA control. (F/G) Sirius red staining for interstitial collagen. (F)
Quantification of Sirius red staining (% total area). (G) Representative images for Sirius red stained
tissues (outer medulla; scale bars, 50µm). (C-G) Mouse numbers: vehicle controls, n=11; UPHD25, n=11;
UPHD29, n=11; UPHD36, n=10; UPHD186, n=10). Results expressed as means +/-SEM. We performed 1-
way ANOVA (p<0.05) with Dunnett’s correction for multiple between-group testing: *p<0.05, **p<0.01 vs. uninjured control or vs. injured vehicle control, as indicated. (C-G) vs. vehicle control only.

**Figure 5.** UPHD263 and PTBA do not reduce post-injury fibrosis after IR-AKI. Male Balb/c mice underwent left renal pedicle clamping for 31 minutes and were treated for 7 days with either vehicle or 50 mg/kg compound IP daily for 7 days starting 24 hours after injury. Injured kidneys were harvested 28 days after injury and evaluated for markers of renal fibrosis. (A-E) UPHD263 (injured vehicle controls, n=12; UPHD263, n=10). (F-J) UPHD186 and PTBA (injured vehicle controls, n=7; UPHD186, n=9; PTBA, n=9). (A-C, F-H) Expression of renal fibrosis markers. Col1α1, α-SMA and LoxL2 mRNA relative to Gapdh mRNA control. (D/I) Percent of fibrosis in outer medulla (OM). (E/J) Representative images of Sirius red stained tissue section in the OM (scale bars, 50µm). All results expressed as means +/- SEM. (A-C, D) 2-tailed T-Test, *p<0.05, **p<0.01; (F-H, I) 1-way ANOVA (p<0.05) with Dunnett’s correction for multiple between-group testing: *p<0.05, **p<0.01 vs. injured vehicle control, as indicated.

**Figure 6.** Pharmacokinetics of PTBA analogs in mice. Male Balb/c mice were injected with a single dose of PTBA analog at 50mg/kg IP, and plasma samples drawn at the indicated intervals over the next 8 hours. Parent compound (▲) and PTBA metabolite (Δ) concentrations determined in ng/ml plasma by LC-MS/MS. (A) UPHD25. No detectable parent compound was detected at any time point up to 8 hours after IP injection of UPHD25. Data are shown for the UPHD25 metabolite, PTBA, only. (B) UPHD186. (C) PTBA. (D) UPHD36. Chemical structures are shown. Potential esterase (UPHD25) and amidase (UPHD36 and 186) cleavage sites are indicated with arrows. Three mice per compound and time points. Results are expressed graphically as the mean of 3 measurements per time point (3 mice) +/- SEM.
Figure 7. Delayed treatment with UPHD186 accelerates recovery and reduces post–injury fibrosis after IR-AKI. Male Balb/c mice underwent left sided renal pedicle clamping for 31 minutes to induce IR-AKI, were treated with either vehicle or 50 mg/kg UPHD25 or 186 IP daily for 4 days starting 3 days after injury. Mice underwent right nephrectomy on day 8, and injured kidneys harvested 28 days after injury and evaluated for markers of renal fibrosis. (A-D) UPHD186 (injured vehicle controls, n=13; UPHD186 treatment, n=12). (E-H) UPHD25 (injured vehicle controls, n=7; UPHD25 treatment, n=8). (A/E) Serum creatinine values 9 days after injury, one day after right nephrectomy to assess functional recovery. Individual data points and means for each group shown. (B/F) Expression of renal fibrosis markers. Col1α1, α-SMA and LoxL2 mRNA relative to Gapdh mRNA control. (C/D, G/H) Sirius red staining for interstitial collagen. (C/G) Quantification of Sirius red stained (% total area). (D/H) Representative images for Sirius red stained tissues (outer medulla; scale bars, 50µm). Results expressed as means +/- SEM. 2-tailed T-test vs. vehicle controls: *p<0.05, **p<0.01, ***p<0.001.

Figure 8. UPHD186 does not reverse established fibrosis after severe IR-AKI. (A) Male Balb/c mice underwent left sided renal pedicle clamping for 36 minutes to induce severe IR-AKI, and were treated with vehicle or 50 mg/kg UPHD186 IP daily for 10 days starting 30 days after injury, as indicated in the model. Prior to starting UPHD186, mice underwent an open renal biopsy to assess fibrosis, and kidneys were harvested 50 days after the initial injury to evaluate the effect of delayed treatment with UPHD186 on post-injury fibrosis. (B/C) Expression of renal fibrosis markers α-SMA and Col1α1 mRNA relative to Gapdh mRNA control in uninjured mouse kidneys (UI), and injured mouse kidneys 30 days after injury before treating with UPHD186 (uninjured, n=5; IR-AKI, n=14). (D-G) Renal fibrosis 50 days after IR-AKI in mice treated with UPHD186 (injured vehicle controls, n=7; UPHD186 treatment, n=7). (D/E) Expression of renal fibrosis markers α-SMA and Col1α1 mRNA relative to Gapdh mRNA control. (F) Quantification of Sirius red staining. (G) Representative images for Sirius red stained tissues (outer medulla; scale bars, 50µm). Results expressed as means +/- SEM. 2-tailed T-test vs. controls: ***p<0.001.
**Figure 9. UPHD186 prevents renal fibrosis after unilateral ureteric obstruction (UOO).** (A) Male Balb/c mice underwent left sided ureteric ligation and were treated with vehicle, 50 mg/kg UPHD25 or 186 IP daily for 7 days starting at the time of surgery. Obstructed kidneys were harvested 8 days after surgery and evaluated for markers of fibrosis. (B-D) Expression of renal fibrosis markers. 

Col1α1, α-SMA and LoxL2 mRNA relative to Gapdh mRNA control. (vehicle controls, n=6; UPHD25, n=5-6; UPHD186, n=5). (D) Quantification of Sirius red staining (injured controls, n=5, UPHD25, n=5; UPHD186, n=4). Results expressed as means +/- SEM. 1-way ANOVA (p<0.05) with Dunnett’s correction for multiple testing: *p<0.05, **p<0.01, #p<0.0001 vs. injured vehicle, or vs. UPHD25, as indicated by brackets.

**Table 1. Pharmacokinetics of plasma PTBA, UPHD25, 36, 186 and 263 after IP injection in mice.** Male BALB/c mice were injected with PTBA analog at 50mg/kg IP, and plasma samples drawn at intervals over the next 8 hours. Parent compound and PTBA concentrations were determined in the plasma by LC-MS/MS. $C_{\text{max}}$ was determined at 0.08 hours post injection. The non-compartmental analysis module in Phoenix WinNonlin® was used to derive $AUC$ and $T_{1/2}$ for the parent and PTBA metabolite.

**Table 2. Liver and kidney microsome stability and activity.** 10μM compounds were incubated with liver or kidney microsomes +/- NADPH, as indicated. Concentrations of the parent compound and PTBA were determined after 50 minutes incubation by LC-MS using standard curves. Intact UPHD25 could not be detected using electrospray ionization in positive or negative modes. * Concentration within expected error for the calculation. Experiment repeated three times with similar results.
**Figure 1**

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Figure 2

A

% Survival

Days after gentamicin injection

V

PTBA

B

% Survival

Days after gentamicin injection

V

25

C

% Survival

Days after gentamicin injection

V

29

D

% Survival

Days after gentamicin injection

V

36

E

% Survival

Days after gentamicin injection

V

186

F

% Survival

Days after gentamicin injection

V

263
Figure 3

A

Col1α1/Gapdh mRNA

B

dSMA/GAPDH mRNA

C

Lox12/Gapdh mRNA

D

Hours after IP injection at 50mg/kg

KDa

0 1 2 4

Ac-H4

Total H4

UPHD25

UPHD29

UPHD36

UPHD186
Figure 6

A

B

C

D

Plasma concentration (ng/ml)

Hours post-injection
Figure 7

A

B

C

D

E

F

G

H

Creatinine, mg/dL

0.0 1.0 2.0

V 186

Collα1/Gapdh mRNA

0 100 200 300 400

V 186

α-SMA/Gapdh mRNA

0 100 200 300 400

V 186

Lox1/Gapdh mRNA

0 10 20 30 40

V 186

Percent fibrosis

0 2 4 6 8

V 186

Vehicle

Vehicle

Creatinine, mg/dL

0.0 1.0 2.0

V 25

Collα1/Gapdh mRNA

0 100 200 300 400

V 25

α-SMA/Gapdh mRNA

0 100 200 300 400

V 25

Lox1/Gapdh mRNA

0 20 40 60 80

V 25

Percent fibrosis

0 2 4 6 8

V 25

Vehicle

Vehicle
Figure 8

A

L-IR 36min → L-biopsy → Drug Treatment → Fibrosis

0 30 40 50 Days

B

\[ \frac{\alpha \text{SMA}}{\text{Gapdh mRNA}} \]

\[ \text{Col1}\alpha1/\text{Gapdh mRNA} \]

UI IR-AKI

**

C

\[ \text{Col1}\alpha1/\text{Gapdh mRNA} \]

UI IR-AKI

***

D

E

F

G

\[ \text{Percent fibrosis (SR staining)} \]

Vehicle 186

\[ \alpha \text{SMA/Gapdh mRNA} \]

\[ \text{Col1}\alpha1/\text{Gapdh mRNA} \]

\[ \text{Percent fibrosis (SR staining)} \]

\[ \text{Vehicle} \]

\[ 186 \]
Figure 9

A. Schematic diagram of the experimental timeline:
- UUO
- PTBA analogue (100 mg/kg/day)
- Harvest

B. Bar graph showing Col1α1/Gapdh mRNA levels:
- V, 25, 186

C. Bar graph showing αSMA/Gapdh mRNA levels:
- V, 25, 186

D. Bar graph showing LoxL2/Gapdh mRNA levels:
- V, 25, 186

E. Bar graph showing Sirius red (% area):
- V, 25, 186
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