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

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1Division of Nephrology, Department of Medicine, Vanderbilt University, Nashville, Tennessee; 2Department of Cell and Developmental Biology, Vanderbilt University, Nashville, Tennessee; 3Vanderbilt Institute of Chemical Biology, Vanderbilt University, Nashville, Tennessee; 4Department of Biochemistry and Mass Spectrometry Research Center, Vanderbilt University, Nashville, Tennessee; 5Drug Discovery Institute, University of Pittsburgh, Pittsburgh, Pennsylvania; 6Developmental Biology, University of Pittsburgh, Pittsburgh, Pennsylvania; 7Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania; 8Department of Computational and Systems Biology, University of Pittsburgh, Pittsburgh, Pennsylvania; and 9Center for Critical Care Nephrology, University of Pittsburgh, Pittsburgh, Pennsylvania

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Skrypnik NI, Sanker S, Skvarca LB, Novitskaya T, Woods C, Chiba T, Patel K, Goldberg ND, Mcdermott L, Vinson PN, Calcutt MW, Huryn DM, Vernetti LA, Vogt A, Hukriede NA, de Caestecker MP. Delayed treatment with PTBA analogs reduces postinjury renal fibrosis after kidney injury. Am J Physiol Renal Physiol 310: F705–F716, 2016. First published December 9, 2015; doi:10.1152/ajprenal.00503.2015.—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 postinjury repair in ischemia-reperfusion and aristolochic acid-induced AKI in mice. In these studies, a new panel of PTBA analogs was 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 initiation of injury.

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SEVERE ACUTE KIDNEY INJURY (AKI) affects >90,000 patients in the United States each year, while milder, non-dialysis-dependent AKI affects >1.5 million/yr (1, 15, 22). AKI is most often precipitated by major surgery, particularly cardiopulmonary bypass 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 postinjury 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, dedifferentiated, 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 postinjury 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 postinjury 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 postinjury fibrosis when administered several days after the initiating injury. We also show that UPHD186 is effective in reducing postinjury renal fibrosis after unilateral ureteric obstruction (UUO) 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 postinjury fibrosis after AKI. The discovery of UPHD186 represents an important preclinical 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.

METHODS

Zebrafish EKPC Assays

Embryos were processed for kidney expansion by in situ hybridization (ISH) or cognitive network technology (CNT) analysis.

ISH assays. Wild-type PITT-AB embryos were treated with compounds at 3.0, 1.5, 0.8, 0.4, and 0.2 μM at the 512 cell stage by serial dilution in E3 medium containing 0.5% dimethyl sulfoxide (DMSO, Sigma), as described (9). Embryos were incubated at 22°C overnight and fixed in 4% paraformaldehyde at the 10-somite stage. In situ hybridization was performed using lhx1a riboprobes, as described (14). At least 20 embryos/condition were visually scored for increased lhx1a mRNA expression. Data are presented as the percentage of embryos with expanded lhx1a.

CNT assays. CNT assays were used to quantify the half maximum effective concentrations (EC50) for compounds that increased lhx1a mRNA expression. For this we utilized transgenic zebrafish, Tg(cdh17:EGFP); as described (23). At the 512 cell stage, 10–15 embryos in 12-well plates were treated with vehicle or increasing concentrations of compounds (3.0–0.2 μM) in E3 medium containing 0.5% DMSO. Embryos were allowed to develop for 48 h, 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-h postfertilization (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, 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 and AKI Survival Assays

Maximum tolerated dose (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 postfertilization (dpf) and

![Fig. 1. Quantitation of kidney organ expansion by automated imaging.](http://ajprenal.physiology.org/)
continued for 24 h. For zebrafish larval AKI, compounds were used at one-quarter the MTD. Three-day dpf larvae were treated with 8 ng gentamicin (Sigma) diluted in 1 nl 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 postinjection (dpi), larvae showing signs of pericardial edema were transferred to E3 medium containing 1% DMSO with or without compounds. Treatment was continued for 24 h at 28°C when treatment solution was replaced with E3 medium without compounds. Larvae were assayed for survival at 4, 5, 6, and 7 dpi. The Pittsburgh University Institutional Animal Care and Use Committee approved experimental protocols in zebrafish.

Mouse IR-Induced AKI, Renal Biopsy, and UUO

Surgeries were performed on a water bath-heated platform at 38°C on 10- to 12-wk-old male BALB/c mice. To induce IR-AKI, mice underwent left renal pedicle clamping for 31 min, and a 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 1 mg/kg, 10 mg/kg or 50 mg/kg UPHD25, or 50 mg/kg PTBA, UPHD29, 36, 186, 263, or vehicle control (20% 2-hydroxypropyl-β-cyclodextrin in PBS) by daily intraperitoneal (ip) injection, starting 24 h after injury for 7 days, or 3 days after injury for 4 days, as indicated. To study the effect of UPHD186’s effect on regression of fibrosis, mice underwent left pedicle clamping for 35 min and an open-kidney biopsy on day 30 to assess fibrosis, as described (17). Mice were then randomized into vehicle or 50 mg/kg UPHD186 IP daily for 10 days, and kidneys were harvested on day 50. For 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 were performed to evaluate renal fibrosis/collagen accumulation in kidney sections, as described (7). Image capture and semiquantitative evaluation was performed by observed blinded to treatment (N. I. Skrypnyk and K. Patel). RNA isolation and quantitative RT-PCR were 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 blotting using rabbit monoclonal anti-histone H4 (clone 62) and rabbit anti-acetylated H4 lysine 5/8/12 and 16 antibodies (pan H4 acetylation, Millipore), and 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 Studies

Eight- to twelve-week male BALB/c mice were given 50 mg/ml PTBA, UPHD25, 36, 186, or 263 in 20% 2-hydroxypropyl-β-cyclodextrin in water ip, and 60 μl of blood collected in three time sets: 0.08, 0.25, and 0.5 h; 1, 2, and 4 h; and 6, 8, and 24 h postinjection (3 mice/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 (Supplemental Methods 1; all supplemental material is available on the journal web site). A noncompartmental analysis module in Phoenix WinNonlin [version (8) 6.3] was used to assess pharmacokinetic (PK) parameters. This experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) at SAI Life Sciences.

Synthetic chemistry for all of the PTBA analogs is described in supplemental data (Supplemental 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 ≥ 1 mM NADPH (Sigma-Aldrich) in 50 mM potassium phosphate buffer, pH 7.4. Time-zero samples were immediately quenched with 1.0 ml acetonitrile containing 0.1% formic acid. Test samples were incubated at 37°C for 50 min 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 (Supplemental 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’s correction for post hoc, pairwise between-group comparisons, as indicated in the figure legends. The minimal level of significance was set at P ≤ 0.05, and statistical analyses were performed using GraphPad Prism V6.

RESULTS

Screening of PTBA Analogs in EKPC Assays

To identify PTBA analogs with more favorable activities than M4PTB/UPHD25 in accelerating recovery and reducing postinjury 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 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 20 were found to be active (estimated EC50 >3 μM, Supplemental 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 Fig. 1D). Representative active analogs that contained an ester (UPHD25), a hydroxamic acid (UPHD29), two benzamides (UPHD36 and
Fig. 4. UPHD25 and 186 accelerate functional recovery and postinjury fibrosis after IR-AKI. Male BALB/c mice underwent left-sided renal pedicle clamping for 31 min 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 h after injury. A and B: functional recovery. Blood urea nitrogen (BUN; A) and serum creatinine (B) were measured at day 9, 1 day later after right nephrectomy to assess functional recovery (nos. of mice for A and 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 are 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 and 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 = 50 μm). C–G: nos. of mice: vehicle controls, n = 11; UPHD25, n = 11; UPHD29, n = 11; UPHD36, n = 10; UPHD186, n = 10. Values are means ± SE. 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–F vs. vehicle control only.
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 one-quarter their MTD (Supplemental 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 4 days. While PTBA, UPHD25, 36, and 186 significantly improved postinjury survival, UPHD29 and 263 showed no effect (Fig. 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 IR-AKI. To determine optimal dosing, we initially evaluated the effects of UPHD25 at 1, 10, and 50 mg/kg administered via ip injection 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

![Graphs and images](attachment:graphs.png)

Fig. 5. UPHD263 and PTBA do not reduce postinjury fibrosis after IR-AKI. Male BALB/c mice underwent left renal pedicle clamping for 31 min and were treated for 7 days with either vehicle or 50 mg/kg compound ip daily for 7 days starting 24 h 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. A and F: percentage of fibrosis in the outer medulla (OM). B and G: representative images of Sirius red-stained tissue section in the OM (scale bars = 50 μm). Values are means ± SE (A–D) by 2-tailed t-test. *P < 0.05, **P < 0.01 and (F–J) 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.
Col1α, α-SMA, and LoxL2 mRNAs, with maximal effects at 50 mg·kg⁻¹·day⁻¹ of UPHD25 (Fig. 3, A–C). Since we have previously shown that PTBA analogs are histone deacetylase inhibitors (HDACi) that increase levels of hyperacetylated histones in the kidney in mice (7), we compared the effects of a single 50 mg/kg ip dose of the PTBA analogs UPHD25, 29, 36, and 186 on renal histone acetylation (Fig. 3 D). All of the PTBA analogs increased renal histone H4 acetylation within an hour of treatment. Histone H4 acetylation returned to baseline 4 h after treatment with UPHD25, 29, and 186, but persisted up to 4 h with UPHD36. Based on these results, we evaluated the effects of PTBA analogs at a dose of 50 mg·kg⁻¹·day⁻¹ on days 1–7 after injury for all studies, unless otherwise indicated. We performed unilateral IR-AKI followed by a contralateral nephrectomy 8 days after injury to evaluate functional recovery and postinjury fibrosis, as described (7, 24). UPHD25- and 186-treated mice have reduced blood urea nitrogen (BUN) compared with vehicle 24 h after the contralateral nephrectomy (Fig. 4A). UPHD186 also reduced serum creatinine 24 h after contralateral nephrectomy (Fig. 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 h 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 Col1α, α-SMA, and LoxL2 mRNA in UPHD25- and 186-treated mice 28 days after injury (Fig. 4, C–E). There was also a reduction in renal fibrosis assessed by

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<th>T_max, h</th>
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Male BALB/c mice were injected with PTBA analog at 50 mg/kg ip, and plasma samples were drawn at intervals over the next 8 h. ND, not determined. Parent compound and PTBA concentrations were determined in the plasma by LC-MS/MS. C_max was determined at 0.08-h postinjection. The noncompartmental analysis module in Phoenix WinNonlin was used to derive AUC and T_1/2 for the parent and PTBA metabolite. See text for other definitions.
Liver and kidney microsome stability and activity

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Table 2. Liver and kidney microsome stability and activity

Compounds (10 μM) were incubated with liver or kidney microsomes ± NADPH, as indicated. Concentrations of the parent compound and PTBA were determined after 50-min incubation by LC-MS using standard curves. Intact UPHD25 could not be detected using electrospray ionization in positive or negative modes. Experiment was repeated 3 times with similar results. *Concentration within expected error for the calculation.

Sirius red staining in UPHD25-treated mice (Fig. 4, F and G). Consistent with effects on BUN and serum creatinine, UPHD29 had no effect on postinjury renal fibrosis, while UPHD36 reduced expression of one of three fibrosis markers but had no effect on Sirius red staining.

We next evaluated effects of UPHD263 and PTBA treatment in reducing postinjury fibrosis after IR-AKI. Treatment with UPHD263 on days 1–7 after IR-AKI had no effect on renal fibrosis 28 days after injury (Fig. 5, A and B) and increased expression of only one of three renal fibrosis markers (Fig. 5, C–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 the efficacy of PTBA with that of UPHD186 after IR-AKI in mice. Unlike UPHD186, PTBA had no effect on postinjury fibrosis and no significant effects on the expression of fibrosis markers 28 days after injury (Fig. 5, F–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 postinjury 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.

PK 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 PK study in mice after ip injection of PTBA analogs (Fig. 6, Table 1). PTBA itself was rapidly absorbed with the peak plasma level obtained within 5 min (Table 1). UPHD25 was undetectable after ip injection in mice. However, peak plasma levels of PTBA were detected 5 min after ip injection of UPHD25 (Fig. 6A, Table 1), suggesting that UPHD25 is rapidly bioconverted to PTBA. Also evident was the rapid absorption and metabolic conversion of UPHD186 to PTBA within 5 min (Fig. 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 esterases and amidases (Fig. 6, A and B; arrows indicate potential cleavage sites) (21). This is consistent with our finding that UPHD26 was only detectable in spiked mouse plasma samples if the samples were...
pretreated with an esterase inhibitor (Supplemental Methods 1). In addition, our observation that PTBA increases survival in zebrafish AKI (Fig. 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 (Fig. 5, F–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 the efficacy of UPHD25 and 186 is driven by the 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 with 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 (Fig. 6D, Table 1). Like UPHD186, UPHD36 contains an amide group that could be hydrolyzed to PTBA (Fig. 6D). However, the Cmax 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 h after injection of UPHD36 due to the absence of a hydrolyzable 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 min (Table 2). Metabolism of parent compounds and the appearance of PTBA were NADPH independent, indicating that UPHD25 and 186 metabolism is not a cytochrome P-450 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 Postinjury Fibrosis After IR-AKI

Having established the efficacy of UPHD25 and 186 in accelerating recovery and reducing postinjury fibrosis when administered 24 h 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 a 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 postinjury fibrosis, and reduced expression of all three renal fibrosis markers 28 days after injury (Fig. 7, A–D). In contrast, while UPHD25 reduced serum creatinine values 9 days after injury, delayed treatment with UPHD25 had no effect on renal fibrosis (Fig. 7, E–H).

Since delayed treatment with UPHD186 reduces postinjury fibrosis when administered late after the initiating injury, we asked whether UPHD186 could reverse established postinjury fibrosis. For this, we developed a new model of IR-AKI that

![Fig. 8. UPHD186 does not reverse established fibrosis after severe IR-AKI.](http://ajprenal.physiology.org/)

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allowed us to evaluate effects of UPHD186 on established postinjury renal fibrosis. Mice underwent prolonged unilateral renal pedicle clamping (35 min vs. 31 min used in previous studies), followed by an open renal biopsy 30 days after injury to assess baseline postinjury fibrosis. After the renal biopsy, mice were treated with 100 mg/kg UPHD186 ip for 10 days, and injured kidneys were harvested 50 days after initial injury (Fig. 8A). Kidneys harvested from mice 30 days after injury were visibly atrophic, with markedly increased expression of fibrosis markers \( \text{Col1} \) and \( \alpha\text{SMA} \) mRNAs in renal biopsies (Fig. 8, B and C). Renal \( \text{Col1} \) and \( \alpha\text{SMA} \) 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 (Fig. 8, D–G). Taken together, these data suggest that UPHD186 is only effective in reducing postinjury fibrosis after IR-AKI when administered during the active regenerative phase after IR-AKI but not once the postinjury fibrosis has been established.

**UPHD186 Reduces Renal Fibrosis After UUO in Mice**

Finally, 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 100 mg·kg\(^{-1}\)·day\(^{-1}\) starting at the time of surgery and continuing for 7 days (Fig. 9A). There was a significant reduction in two of three 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 (Fig. 9, B–E). In contrast, UPHD186-treated mice showed a significant reduction in expression of all three 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 postinjury fibrosis in IR 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 \( \text{lhx1a} \) 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 the 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 postinjury fibrosis after IR-AKI and is effective when administered several days after the initiating injury. UPHD186 also reduces postinjury 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 preclinical 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 do not depend on the nature of zinc-chelating moieties predicted to increase HDAC inhibition (13), but rather on the 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 P-450-dependent metabolism, and conversion of UPHD36 to PTBA is less efficient than for 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 the 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 postinjury 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. PK and metabolism studies suggest that the efficacy of UPHD186 in mouse AKI results from its efficient conversion to the active metabolite PTBA in the kidney.

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