Inhibition of Histone Deacetylase Expands the Renal Progenitor Cell Population


Departments of *Microbiology and Molecular Genetics, †Pharmaceutical Sciences, ‡Pharmacology and Chemical Biology, and §Chemistry and ¶Drug Discovery Institute, University of Pittsburgh, Pittsburgh, Pennsylvania

ABSTRACT
One of the first hallmarks of kidney regeneration is the reactivation of genes normally required during organogenesis. Identification of chemicals with the potential to enhance this reactivation could therapeutically promote kidney regeneration. Here, we found that 4-(phenylthio)butanoic acid (PTBA) expanded the expression domains of molecular markers of kidney organogenesis in zebrafish. PTBA exhibits structural and functional similarity to the histone deacetylase (HDAC) inhibitors 4-phenylbutanoic acid and trichostatin A; treatment with these HDAC inhibitors also expanded the renal progenitor cell population. Analyses in vitro and in vivo confirmed that PTBA functions as an inhibitor of HDAC activity. Furthermore, PTBA-mediated renal progenitor cell expansion required retinoic acid signaling. In summary, these results support a mechanistic link among renal progenitor cells, HDAC, and the retinoid pathway. Whether PTBA holds promise as a therapeutic agent to promote renal regeneration requires further study.


The zebrafish embryo is a viable model organism for use in chemical library screens.12 Such screens have provided insight into developmental events and have yielded lead compounds for combating human disease.3–5 In zebrafish, the pronephric kidney serves as the functional larval kidney. The pronephros consists of a pair of nephrons connected at the dorsal midline to a compound glomerulus.6 Despite this simplicity, the pronephros contains cell types typical of more complex kidneys.7 Pronephric development depends on the renal progenitor cells that populate the intermediate mesoderm.8 These cells express several genes that define the size of the kidney field, including lhx1a (formerly lim1), pax2a, and pax8.8–10

Regenerating proximal tubule kidney cells express genetic markers normally associated with embryonic renal progenitor cells, including Lhx1, Pax2, Wnt4, Bmp7, and the Notch signaling pathway.11–16 These markers appear within the first 24 hours after injury.12 Expression of these developmental markers is thought to be required for the differentiation of regenerating tubule cells. The mechanism regulating gene reactivation after kidney damage is unknown; however, one study demonstrated that regeneration after renal ischemia is associated with a reduction in histone deacetylase (HDAC) activity.16

A majority of chemicals that function as HDAC inhibitors (HDACis) demonstrate equal efficacy against multiple HDAC classes.17 This functional conservation across diverse structures suggests that the effect of an individual HDACi in vivo could re-

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Correspondence: Dr. Neil A. Hukriede, Department of Microbiology and Molecular Genetics, University of Pittsburgh, 3501 5th Avenue, 5061 BST3, Pittsburgh, PA 15213. Phone: 412-648-9918; Fax: 412-383-5918; E-mail: hukriede@pitt.edu

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reveal a general mechanistic effect. 

One functional consequence of HDAC activity is to regulate retinoic acid (RA) signaling. RA activates transcription by binding to RA receptor (RAR) dimers located on RA response elements in target promoters. The absence of RA results in the enlistment of co-repressors by RARs to silence gene activity by engaging HDACs. The RA pathway plays an important role in formation of the kidney field. Ectopic activation of RA signaling increases the size of the kidney field, whereas blocking the pathway impairs development. It has been proposed that HDACs can attenuate RA–RAR complexes, lowering the threshold of RA necessary to activate transcription.

Evidence suggests that the mechanisms of kidney repair parallel those of organogenesis; therefore, compounds capable of stimulating renal progenitor cell proliferation could improve the regeneration response. Toward this end, we designed a simple small molecule screen using zebrafish embryos. We paired a straightforward indicator of potential kidney effects with a small but diverse chemical library and identified 4-(phenylthio)butanoic acid (PTBA). We demonstrate that PTBA treatment induces an expansion of the renal progenitor cells through a mechanism that involves HDAC inhibition.

RESULTS

PTBA Treatment Expands the Kidney Field

We performed a kidney screen in zebrafish embryos using a library of small molecules with diverse structures (see the Concise Methods section). From this screen, we identified a compound, PTBA (Figure 1A), that caused pericardial edema and axis curvature at 72 hours postfertilization (hpf; Figure 1, B and C). We re-synthesized PTBA and confirmed its structure (see the Supplemental Methods section).

To determine whether PTBA affected the kidney field, we performed pax2a in situ hybridization. Embryos treated with 1 to 5 μM PTBA exhibited a concentration-dependent expansion of pax2a expression that coincided with developmental delay (Supplemental Figure 1). To maximize the PTBA effect on expansion of the kidney field while minimizing developmental delay, we chose 3 μM PTBA as our working concentration. Treatment with 3 μM PTBA caused 92% (n = 88) of the embryos to develop edema by 72 hpf without eliciting significant toxicity (as assayed by death; Supplemental Figure 2).

The effects of PTBA on the kidney field prompted us to examine whether the compound influenced renal progenitor cells. To address this, we determined the expression patterns and relative abundance of lhx1α, pax2a, and pax8 at the 10-somite stage. Lhx1α expression was expanded in embryos treated with PTBA, representing a three-fold increase in relative transcript quantity as determined by quantitative PCR (qPCR; Figure 1, D and E). Increased lhx1α expression appeared in the bilateral stripes of intermediate mesoderm that give rise to the pronephros (Figure 1E, arrowheads), as well as

Figure 1. PTBA treatment expands the pool of renal progenitor cells. (A) Structure of PTBA. (B and C) Zebrafish larvae at 72 hpf treated with 0.5% DMSO (B) or 3 μM PTBA (C). Arrow indicates pericardial edema. (D through I) in situ hybridization for lhx1α (D and E), pax2a (F and G), pax8 (H and I), in 10-somite embryos treated with 0.5% DMSO (D, F, and H) or 3 μM PTBA (E, G, and I). As compared with controls (n = 60 [D], n = 60 [F], and n = 59 [H]), observed expansion in response to PTBA treatment is 95% for lhx1α (n = 60 [E]), 97% for pax2a (n = 60 [G]), and 95% for pax8 (n = 59 [I]). Arrowheads indicate renal progenitor cells, asterisk indicates notochord. Relative qPCR for lhx1α, pax2a, and pax8 in the trunk region of 10-somite embryos (n = 4, 240 embryos) is displayed under corresponding in situ image. Data are mean expression plus 95% confidence interval. Expression is normalized to β-actin and SDHA transcript levels. (J through O) Confocal projections of 10-somite Tg(lhx1α:EGFP) embryos treated with 0.5% DMSO (n = 18 [J and L through N]) or 3 μM PTBA (n = 21 [K and O through Q]). Boxed areas are regions that were counted for GFP- and DAPI-positive nuclei (J and K) and are shown in L and O (GFP), M and P (DAPI), and N and Q (merge). Cell counts are mean number of positive cells plus 95% confidence interval for each condition (J and K).
in the axial mesoderm (notochord; Figure 1E, asterisk). Expression of pax2a and pax8 was also expanded, with qPCR detecting an approximately two-fold increase for pax2a and a 50% increase for pax8 (Figure 1, F through I).

Although these studies demonstrated that PTBA treatment resulted in increased gene expression, they did not indicate whether there are more renal progenitor cells or simply higher expression levels per cell. To differentiate between these two possibilities, we treated the Tg(lhx1a:EGFP)Wt1a reporter line with PTBA and counted the number of renal progenitor cells. As compared with control embryos, PTBA-treated embryos showed a 2.4-fold increase in the number of renal progenitor cells (Figure 1, J through Q).

We next performed a temporal assay to determine the timing of PTBA efficacy. PTBA treatments from 2 hpf through 10 somites (14 hpf) resulted in an increase in pax2a expression as assayed at 24 hpf, but treatments at 15 somites (16.5 hpf) resulted in no kidney field expansion (Supplemental Figure 3). In addition, treatment at 15 somites or later did not affect the functional kidney as assayed by a lack of edema in larvae at 72 hpf (data not shown). The effective temporal treatment window coincides with the period when renal progenitor cells are present.6,8

To determine whether PTBA treatment resulted in a transient or persistent expansion of the kidney field, we examined the kidney at 48 hpf using markers of glomerulus and tubule.29–31 As compared with controls, PTBA-treated embryos displayed an expansion of the pan-tubule markers cdh17 and NaK-ATPase at 48 hpf, which indicated a persistent expansion of the kidney field size could result from the fate transformation of nonrenal cells and/or proliferation of renal progenitor cells. To assess the first possibility, we examined the effects of PTBA on markers of two mesodermal tissues juxtaposed to renal progenitor cells: myod1 (somites) and fli1a (vasculature). By in situ hybridization, we observed that myod1 expression in the somites showed a slight decrease after PTBA treatment (Figure 3, A and B); however, subsequent qPCR analysis did not confirm the significance of this observed decrease (Figure 3, A and B). Expression of fli1a in the vasculature remained unchanged by both in situ hybridization and qPCR (Figure 3, C and D). In addition to an increased lhx1a expression in renal progenitor cells, we observed increased lhx1a expression in the notochord (Figure 1E, asterisk). To determine whether this expansion reflected an effect on notochord size or a general increase in lhx1a expression, we assayed the notochord-specific marker ntla. Ntla displayed an expansion that resulted in an approximately 80% increase by qPCR analysis after PTBA treatment (Figure 3, E and F). These results suggest that PTBA treatment cannot be definitively linked to a fate transformation event.

To examine the alternative possibility that PTBA-mediated renal progenitor cell expansion depended on cell proliferation, we tested the efficacy of PTBA in the presence of hydroxyurea and aphidicolin (HUA). HUA treatment is known to block cell division without affecting tissue specification.32 As compared with controls, PTBA-treated embryos exhibited an expansion of lhx1a expression at 10 somites (Figure 3, G and H). HUA treatment alone elicited little effect on lhx1a expression (Figure 3I); however, treatment with a combination of HUA and PTBA diminished the PTBA-mediated expansion of lhx1a expression in renal progenitor cells (Figure 3J). These results suggest that cell proliferation is required for PTBA efficacy.

PTBA Structure-Activity Studies

We performed structure-activity analyses using a series of seven analogs (Figure 4). In situ hybridization for lhx1a was performed on 10 somite embryos treated with each analog at 3 μM. The results were compared with control (Figure 4A) and PTBA-treated (Figure 4B) embryos. We observed that replacement of the phenylthio ether with a phenylsulfonyl linkage stripped the compound of its effects on renal progenitor cells (Figure 4C); therefore, the oxidation state of the sulfur atom is a critical activity determinant. However, 4-(naphthalen-2-yl)thio)butanoic acid, an analog carrying a naphthalene ring in place of the phenyl moiety of PTBA, still expands lhx1a expression (Figure 4D). Thus, modifications of the ring structure are tolerated and suggest a site for future analog synthesis. Two analogs containing substitutions of the butanoic acid backbone 2-amino-PTBA and 3-(phenylthio)benzoic acid had no effect on lhx1a expression (Figure 4, E and F), suggesting a requirement for a flexible hydrocarbon backbone for biological activity. We also examined 4-phenoxybutanoic acid and 5-phenylpentanoic acid, which contain oxygen and carbon substitutions for the sulfur atom, respectively. 4-Phenoxybutanoic acid exhibited reduced efficacy compared with the parent compound (Figure 4G), whereas 5-phenylpentanoic acid was inactive (Figure 4H). These results suggest that an atom with a nonbonding electron pair(s) must occupy this position to provide activity. Finally, we determined whether esterification of PTBA elicited any effect on its function. The analog methyl-4-(phenylthio)butanoate demonstrated higher potency than the parent compound (Figure 4I).

HDAC Is Mimic the Effects of PTBA

The structure-activity analyses suggested that PTBA functional domains are analogous to 4-phenylbutanoic acid (PBA; Figure 5A), a known HDACi. Furthermore, both compounds resem-
related inhibitors should also expand renal progenitor cells. Concentration-response experiments were performed with PBA and TSA to determine the concentrations necessary to elicit lhx1a expansion (Supplemental Figures 4 and 5). We determined that treatment with 25 μM PBA or 200 nM TSA produced an expansion of renal progenitor cells consistent with that elicited by 3 μM PTBA (Figure 5, C through E, versus Figure 1E); therefore, at least two families of HDACis mimic the ability of PTBA, supporting the idea that PTBA may act through HDAC inhibition.

We tested how trunk mesoderm juxtaposed to the kidney field is affected by PBA and TSA treatments. TSA is a broad-spectrum HDACi and has been documented to cause disruption of multiple tissues in zebrafish. In addition, TSA and a second member of the hydroxamic acid family, SAHA, have demonstrated renal toxicity. As previously demonstrated, PTBA treatment did not result in drastic structural changes to the somites (myod1), vasculature (fli1a), or notochord (ntl); Figure 3). We treated embryos with 25 μM PBA or 200 nM TSA and compared expression with that of control embryos (Figure 5, F through N). PBA treatment resulted in decreased expression of myod1 and fli1a and increased expression of ntl (Figure 5, G, J, and M). TSA treatment caused severe defects in all three tissues assayed. The somites were almost completely absent, the vasculature was reduced, and the notochord was malformed (Figure 5, H, K, and N).

To determine the toxicity of PBA and TSA, we performed phenotypic concentration-response experiments. Embryos were treated from 2 to 72 hpf over the same concentration range used to test for expansion of the kidney field. When observed at the respective concentrations required to expand the kidney field, PBA treatment resulted in minimal but significant death (7%; n = 90; Supplemental Figure 6), whereas TSA treatment resulted in a high percentage of lethality (61%; n = 90; Supplemental Figure 7).

**Figure 2.** PTBA treatment results in a persistent expansion of the kidney field. (A through D) In situ hybridization for cdh17 expression in embryos at 48 hpf treated with 0.5% DMSO (A [higher magnification in B]) or 3 μM PTBA (C [higher magnification in D]). As compared with controls (n = 54 [A and B]), 89% of PTBA-treated embryos exhibit expansion of cdh17 expression at 48 hpf (n = 56 [C and D]). (E and F) Whole-mount antibody staining for NaK-ATPase in embryos at 48 hpf treated with 0.5% DMSO (E) or 3 μM PTBA (F). As compared with controls (n = 10 [E]), 100% of PTBA-treated embryos exhibit expansion of NaK-ATPase expression at 48 hpf (n = 10 [F]). (G and H) Proximal tubule cross-sections (5 μm) taken from cdh17 in situ hybridization of embryos at 48 hpf treated with 0.5% DMSO (G) or 3 μM PTBA (H). Black arrowheads indicate cdh17 expression. Cross-sections are taken from the location indicated in B and D by yellow lines. (I and J) Distal tubule cross-sections (5 μm) taken from NaK-ATPase antibody-stained embryos at 48 hpf treated with 0.5% DMSO (I) or 3 μM PTBA (J). White arrowheads indicate NaK-ATPase protein expression. Cross-sections are taken from the locations indicated in E and F by yellow lines. (K through P) In situ hybridization for wt1a (K and L), slc4a4 (M and N), and slc12a1 (O and P) in embryos at 48 hpf treated with 0.5% DMSO (K, M, and O) or 3 μM PTBA (L, N, and P). As compared with controls (n = 57 [K], n = 58 [M], and n = 56 [O]), observed expansion in response to PTBA treatment is 74% for wt1a (n = 50 [L]), 92% for slc4a4 (n = 60 [N]), and 64% for slc12a1 (n = 59 [P]). Brackets in K and L indicate the expression domain of wt1a.

ble the HDACi TSA (Figure 5B). This reflects the general structure-activity relationship of this class of compounds; therefore, we hypothesized that PTBA is an HDACi and, if so, inhibit HDACs. We measured the deacetylation of a fluorescent peptide substrate in the presence of HDACs. HDAC activity increased in direct proportion to the amount of HeLa cell
PTBA Treatment Affects RA Signaling

HDACis have been posited to lower the threshold of RA necessary to activate transcription. If PTBA treatment hyperactivates the RA pathway, then expression of genes responsive to RA signaling should change. We focused on two genes for this assay, cyp26a1, which is directly activated by RA signaling, and the cardiac gene cmlc2, because the heart field is reduced by RA treatments. To visualize changes to both genes at the same stage, we collected the treated embryos at the 18-somite stage. Expression of cyp26a1 was increased in PTBA-treated embryos (Figure 7, A and B), whereas cmlc2 expression was reduced (Figure 7, C and D).

To provide a stronger link between PTBA treatment and RA signaling, a dominant-negative RARα construct (DN-RARα), which is known to block RA signaling, was injected before nuclear extract added to the assay (Figure 6A, black triangle). Addition of TSA completely blocked HDAC activity at all input levels of nuclear extract added (Figure 6A, gray triangle). Previous work showed that PBA decreased HDAC activity in DS19 mouse erythroleukemia cells to 19% of the control value at 5 mM. To elucidate whether PTBA had similar HDAC inhibition characteristics as PBA, we examined the extent of inhibition for both compounds at 5 mM in HeLa cell nuclear extracts. The two compounds showed similar potency, reducing the HDAC activity elicited by 10 μg HeLa extract to 30% of the control value (Figure 6A, blue circle, red diamond). The PTBA analog 4-(naphthalen-2-yl thio)butanoic acid (PSOBA), showed no apparent ability to expand renal progenitor cells in structure-activity studies (Figure 3C); therefore, we hypothesized that it would function poorly in vitro as an HDACi. At 5 mM, PSOBA reduced the HDAC activity elicited by 10 μg HeLa extract to approximately 70% of the control value (Figure 6A, green square).

We next sought to determine whether PTBA HDACi function was measurable in vivo. We treated 24 hpf embryos with PTBA, PBA, or TSA at their effective concentrations. In addition, to ascertain whether the HDACi activity is concentration-dependent, we also treated at five-fold higher concentrations. After a 6-hour treatment, protein extracts were prepared and immunoblotted with an anti-hyperacetylated histone H4 antibody. A concentration-dependent histone H4 hyperacetylation was observed with all three HDACis (Figure 6B). TSA elicited the strongest hyperacetylation, followed by PTBA then PBA. The in vitro and in vivo results confirm that PTBA functions as an HDACi.
PTBA treatment. As compared with controls, we observed an expansion of *lhx1a* expression in 90% of the mock-injected PTBA-treated embryos (Figure 7, E and F). Injection of 200 pg of DN-RARα mRNA did not grossly affect *lhx1a* expression (Figure 7G). Less than 20% of the embryos injected with 200 pg of DN-RARα mRNA and subsequently treated with 3 μM PTBA showed expanded *lhx1a* expression (Figure 7H); therefore, these data suggest that PTBA-mediated expansion of renal progenitor cells is dependent on the retinoid pathway.

**DISCUSSION**

The zebrafish is a viable model organism for kidney organogenesis-based small molecule screens. By screening in live embryos, toxic events, such as those elicited by TSA, can be readily identified. From a small molecule screen of 1990 compounds, we identified PTBA, which had not been reported as a positive hit in previously published screens using the National Cancer Institute (NCI) Diversity Set. This compound was found to expand renal progenitor cell populations and was determined to be a new member of the carboxylic acid HDACi family.

PTBA was originally identified for its ability to cause pericardial edema in treated larvae. The edema-associated phenotype suggests that expansion of the renal progenitor cell population disrupts kidney organogenesis and function. In PTBA-treated embryos, *wnt1a* expression domains are expanded, and these domains fail to coalesce and migrate to the midline. This suggests that properly organized glomeruli are absent in treated larvae.29

In addition to PTBA, we demonstrated that PBA and TSA treatments expand the renal progenitor cell population. The ability of several HDACis to expand renal progenitor cell populations fits well with the known functional characteristics of HDACis. Most HDACis share a general structure that facilitates docking in the catalytic pocket of HDACs. What makes PTBA unique is a thioether moiety in the position of the connecting unit, a site that is typically occupied by an amide bond.33 The connecting unit has recently garnered interest as a target for new drug design efforts.
PTBA (B and D). As compared with controls (n = 58 [A]), cyp26a1 expression is increased in 100% of PTBA-treated embryos (n = 57 [B]). Arrowheads highlight expression domains in cyp26a1 embryos. As compared with controls (n = 58 [C]), cmlc2 expression is decreased in 100% of PTBA-treated embryos (n = 57 [D]). (E through H) In situ hybridization for lhx1a in 10-somite embryos mock-injected with 1% fluorescein dextran (E and F) or injected with 200 pg of DN-RARα mRNA and 1% fluorescein dextran (G and H). At 5 hpf, embryos were treated with 0.5% DMSO (E and G) or 3 µM PTBA (F and H). As compared with controls (n = 80 [E]), lhx1a expression increased in 93% of the mock-injected PTBA-treated embryos (n = 78 [F]) and 19% of DN-RARα-injected PTBA-treated embryos (n = 125 [H]). Normal lhx1a expression is apparent in 92% of the control embryos injected with the DN-RARα construct (n = 130 [G]).

Although all three HDACis expanded the renal progenitor cell population, they exhibited varying effects on other tissues in the embryo. Unlike PTBA, both PBA and TSA caused severe disruption of both somitic and vascular tissue at their effective dosages. This may be indicative of toxicity. Survival studies demonstrated that both PBA and TSA treatment caused significant larval death at their effective concentrations. Interestingly, PTBA and TSA treatment also elicited an expansion of the notochord. This expansion is likely not proliferation based, because PTBA-treated embryos still displayed an expanded notochord in HUA studies. Further investigation is warranted to determine how HDACis of the carboxylic class drive higher gene expression in the notochord.

Mechanistically, PTBA treatment is likely inhibiting HDACs that are part of the RA transcriptional repression complex. This hypothesis is supported by the hyperactivation of a direct target of RA, cyp26a1, in PTBA-treated embryos; therefore, we posit that in the presence of an HDACi, endogenous RA levels can drive the hyperplastic expansion of renal progenitor cells. Interestingly, retinoids have been proposed to be direct target of RA, thereby driving the hyperplastic expansion of renal progenitor cells. Although all three HDACis expanded the renal progenitor cell population, they exhibited varying effects on other tissues in the embryo. Unlike PTBA, both PBA and TSA caused severe disruption of both somitic and vascular tissue at their effective dosages. This may be indicative of toxicity. Survival studies demonstrated that both PBA and TSA treatment caused significant larval death at their effective concentrations. Interestingly, PTBA and TSA treatment also elicited an expansion of the notochord. This expansion is likely not proliferation based, because PTBA-treated embryos still displayed an expanded notochord in HUA studies. Further investigation is warranted to determine how HDACis of the carboxylic class drive higher gene expression in the notochord.

TSA treatment has been shown to attenuate renal injury in mice. One study also demonstrated that renal ischemia-mediated regeneration is associated with a reduction in HDAC5 activity, increased histone acetylation, and reactivation of bone morphogenetic protein 7 (BMP7); therefore, inhibitor-mediated attenuation of HDAC activity may increase the expression of reactivated embryonic genes in regenerating renal tubular cells. The ability of HDACis to abate renal damage and increase the number of renal progenitor cells points to a promising therapeutic role for this class of small molecules in kidney regeneration.

**CONCISE METHODS**

**Zebrafish Husbandry**

Zebrafish were maintained under standard conditions and staged as described previously. Embryos were collected from group matings of wild-type AB adults. All animal husbandry adheres to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Small Molecule Screening**

The screen was performed in zebrafish embryos using the NCI’s Developmental Therapeutics Program (NCI/DTP) Diversity Set. This library contains 1990 compounds selected by pharmacophore modeling to represent more than 140,000 small molecules maintained in the NCI/DTP Open Repository. Compounds from the NCI/DTP Diversity Set were diluted to 10 µM in E3 embryo medium (5 mM NaCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, and 0.17 mM KCl) in a final DMSO concentration of 0.5% and arrayed in 96-well plates. Beginning at approximately 2 hpf, embryos were transferred to each well in groups of five using a glass pipette. The plates were incubated at 28.5°C for 70 hours. Individual wells were then scored for a dominant phenotype, representative of at least four of the five embryos. The objective was to identify compounds that caused edema in treated embryos at 72 hpf. Edema can indicate a compromised pronephros, potentially reflecting changes in kidney structure and/or cell number. Small molecules generating edema were retested once for verification, before obtaining additional compound from the NCI/DTP Open Repository.

**Compound Sources and Treatments**

PTBA and methyl-4-(phenylthio)butanoate were synthesized as described in the Supplemental Methods. 4-(Naphthalen-2-yl thio)butanoic acid (NSC2733), 3-(phenylthio)benzoic acid (NSC113994), and 2-amino-PTBA (NSC140113) were obtained from the NCI/DTP Open Repository. PBA, TSA, 4-phenoxybutanoic acid, 5-phenylpen- tanoic acid, hydroxyurea, and aphidicolin were obtained from Sigma-Aldrich. PSOBa was obtained from Matrix Scientific.

Groups of 20 to 30 chorionated 2-hpf embryos were arrayed in individual wells of 12-well plates. E3 medium was removed with a glass pipette and replaced with 1.5-ml treatment solutions containing 0.5% DMSO in E3 with or without compound at the reported concentrations. Treatments for all studies were initiated at 2 hpf, except for the temporal studies (treated as described in the Results section and Supplemental Information), and the HUA studies. HUA studies were performed as described previously, with the following modifications. HUA in 0.5% DMSO was added at early gastrulation (5 hpf).
and PTBA was subsequently added at late gastrulation (8 hpf) to allow for penetration of the proliferation inhibitors. All embryos were incubated at 28.5°C until the required developmental stage.

In Situ Hybridization and Immunocytochemistry

In situ hybridization was performed as described previously with some modifications. 46 Hybridization temperature was 65°C. Embryos were blocked in 2% blocking reagent (Roche) with 5% sheep serum in MAB (100 mM maleic acid and 150 mM NaCl [pH 7.5]). Whole-mount immunocytochemistry with 1:25 mouse anti-a6F antibody (Developmental Studies Hybridoma Bank) and 1:100 Cy3 secondary antibody (Jackson ImmunoResearch) was performed as described previously. 29 Embryos were embedded in JB-4 for sectioning per the manufacturer’s instructions (Polysciences), sectioned at 5 μm, and mounted with Cytoseal 60 (Richard-Allan Scientific).

Relative qPCR

Samples for trunk RNA extraction were prepared by cutting embryos just above the first somite with microscissors and discarding the anterior portion. The trunk portions were homogenized with a plastic microcentrifuge pestle in 500 μl of TRI reagent (Ambion), and RNA was isolated using an RNeasy Micro Kit (Qiagen) per the manufacturer’s instructions.

Relative qPCR was performed as described previously47 with minor modifications. Details of these modifications, reaction conditions, and information regarding primer sets, primer selection criteria, and qPCR data analysis can be found in the Supplemental Methods section. Mean expression levels (normalized to the control group) and the corrected expression SD were used to generate 95% confidence intervals for each data set.

Transgene Design and Cell Counting

An 8.8-kb genomic fragment of the lhx1a locus, containing green fluorescent protein (GFP) inserted in-frame with the first nine nucleotides of exon 1, was cloned into the pSceI vector and injected into one-cell-stage zebrafish embryos to establish lines. Three independent transgenic lines were isolated, and Tg(lhx1a:EGFP)pt303 was used for subsequent analysis.

After treatment with PTBA, Tg(lhx1a:EGFP)pt303 embryos were fixed in 4% paraformaldehyde in PBS for 8 hours at 4°C. Embryos were washed in PBS containing 0.1% TWEEN 20 (PBT) and incubated in 1 μg/ml DAPI in PBT for 30 minutes at room temperature. Embryos were flat-mounted on glass slides with Cytoseal 60 and imaged with either a Leica M205 FA epifluorescent scope or an Olympus Fluoview 1000 confocal microscope. Confocal projections contained stacks of six 3-μm images.

For cell counting, a predefined box was positioned at the most posterior region of the notochord and encompassed the most lateral GFP-positive cell in a kidney field. The cells that were positive for both GFP and DAPI within this box were counted using Image J. Variances of the control and PTBA-treated groups were compared by F test and found to be unequal; therefore, a two-tailed t test with unequal variance was used to determine significance (α = 0.05).

Histone Hyperacetylation Assays

SDS-PAGE and Western blotting were performed as described previously38 with some modifications. Proteins were separated on 18% SDS-PAGE gels. Membranes were incubated at 4°C overnight with 1:1000 anti-hyperacetylated histone H4 antibody (06-946; Millipore) or 1:1000 anti-α-tubulin antibody (Sigma-Aldrich) in PBT containing 5% nonfat milk.

Fluorescence HDAC Assays

In vitro HDAC activity assays were performed using a fluorescence HDAC assay kit (Active Motif) according to the manufacturer’s instructions. For maintaining compound solubility at 5 mM, the final DMSO concentration in all assay wells was increased to 5%. Fluorescence was detected using an M5 Plate Reader (Molecular Dynamics).

mRNA Synthesis and Microinjections

Synthetic mRNA was generated from the XRARα1405/pCD61 construct49 (NotI digested) using a T7 mMessage mMachine kit (Ambion). Zebrafish embryos were injected at the one-cell stage either with 200 pg of synthetic mRNA and 1% fluorescein dextran (Sigma-Aldrich) or with 1% fluorescein dextran alone (mock) and allowed to develop in E3 culture medium at 28.5°C. At the 256-cell stage, only fluorescein dextran–positive embryos were selected for PTBA treatment, which occurred at 5 hpf.

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DISCLOSURES

None.

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See related editorial, “From Proteus to Prometheus: Learning from Fish to Modulate Regeneration,” on pages 726–728.

Supplemental information for this article is available online at http://www.jasn.org/.
**Supplemental Figure 1.** PTBA causes larval edema commensurate with embryonic expansion of the kidney field. Representative examples of 72 hpf larvae treated from 2 hpf with: 0.5% DMSO (A, n = 90), 1 µM PTBA (B, n = 90), 2 µM PTBA (C, n = 90), 3 µM PTBA (D, n = 88), 4 µM PTBA (E, n = 83), 5 µM PTBA (F, n = 67). Corresponding in situ hybridization for pax2a in 24 hpf embryos treated from 2 hpf with: 0.5% DMSO (G, n = 54), 1 µM PTBA (H, n = 48), 2 µM PTBA (I, n = 52), 3 µM PTBA (J, n = 49), 4 µM PTBA (K, n = 44), 5 µM PTBA (L, n = 43). Insets contain enlargements of pax2a in the lower kidney field.
Supplemental Figure 2. PTBA elicits concentration-dependent effects on larval edema and survival. Embryos were treated with 0 to 10 µM PTBA from 2 hpf, and larvae were scored at 72 hpf using a phenotype-based classification system. Wild-type (WT): no visible edema or developmental delay (A). Edemic 1 (E1): pericardial edema evident, may exhibit slight developmental delay, little or no axis curvature, axis length normal (B). Edemic 2 (E2): pericardial edema evident, slight to moderate developmental delay, axis curvature, axis length normal or slightly reduced (C). Edemic 3 (E3): pericardial edema evident, moderate to severe developmental delay, gross axis curvature frequently accompanied by tail kink, axis noticeably shortened (D). (E) Graph of observed phenotypes after treatment with 0 to 10 µM PTBA (n = 90 per concentration). Asterisk denotes the concentration where PTBA begins to exhibit a significant effect (p < 0.05) on survival as determined by two-tailed Fisher’s exact test in comparison with the 0 µM PTBA treatment group.
Supplemental Figure 3. Effects of temporal PTBA treatments on the kidney field. *In situ* hybridization for *pax2a* in 24 hpf embryos treated with 0.5% DMSO (A, \(n = 132\)) from 2 hpf or 3 \(\mu\)M PTBA from: 2 hpf (B, \(n = 67\)), shield (C, 6 hpf, \(n = 100\)), 2 somites (D, 10.7 hpf, \(n = 71\)), 5 somites (E, 11.7 hpf, \(n = 87\)), 10 somites (F, 14 hpf, \(n = 89\)), 15 somites (G, 16.5 hpf, \(n = 72\)). Insets contain enlargements of *pax2a* in the lower kidney field.
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Supplementary Figure 5. Treatment with TSA, a hydroxamic acid HDACi, expands renal progenitor cells. In situ hybridization for lhx1a in 10 somite embryos treated from 2 hpf with: 0.5% DMSO (A, n = 60), 100 nM TSA (B, n = 57), 150 nM TSA (C, n = 57), 200 nM TSA (D, n = 52), 250 nM TSA (E, n = 42), 300 nM TSA (F, n = 40).
Supplemental Figure 6. PBA elicits concentration-dependent effects on larval edema and survival. Embryos were treated with 0-30 µM PBA from 2 hpf, and phenotypes were scored at 72 hpf using the classification system described in Supplemental Figure 1. Examples of treated larvae classified as WT (A), E1 (B), E2 (C). No E3 larvae were observed in response to PBA treatment at the listed concentrations. (D) Graph of observed phenotypes after treatment with 0-30 µM PBA (n = 90 per concentration). Asterisk denotes the concentration where PBA begins to exhibit a significant effect (p < 0.05) on survival as determined by two-tailed Fisher’s exact test in comparison with the 0 µM PBA treatment group.
Supplemental Figure 7. TSA elicits concentration-dependent effects on larval edema and survival. Embryos were treated with 0-300 nM TSA from 2 hpf, and phenotypes were scored at 72 hpf using the classification system described in Supplemental Figure 1. Examples of treated larvae classified as WT (A), E1 (B), E2 (C), E3 (D). (E) Graph of observed phenotypes after treatment with 0-300 nM TSA ($n = 90$ per concentration). Asterisk denotes the concentration where TSA begins to exhibit a significant effect ($p < 0.05$) on survival as determined by two-tailed Fisher’s exact test in comparison with the 0 nM TSA treatment group.
SUPPLEMENTAL METHODS

Synthesis of 4-(phenylthio)butanoic acid and methyl 4-(phenylthio)butanoate.

Methyl 4-(phenylthio)butanoate (1) was prepared from thiophenol, potassium carbonate, and methyl 4-bromobutyrate in refluxing acetone as described in the literature.\(^1\) 4-(phenylthio)butanoic acid (2) was prepared either in quantitative yield by saponification of 1 with aqueous KOH in MeOH overnight at room temperature followed by acidification with aqueous HCl, or in 97% yield from reaction of the sodium salt of thiophenol and γ-butyrolactone in refluxing EtOH and subsequent acidification with aqueous HCl as described in the literature.\(^2\) All compounds gave \(^1\)H and \(^{13}\)C NMR (400/100 MHz and/or 600/150 MHz), mass spectra (GC-EI-MS, LC-ESI-MS and high resolution MALDI-TOF-MS) and melting points consistent with the literature and their structures. All spectral and melting point data suggested >99% purity.\(^1\)\(^-\)\(^3\)

Relative qPCR

**Primer design.** Primer sets were designed using NetPrimer and Beacon Designer (ver. 7.51) primer analysis software (PREMIER Biosoft). In each set, one primer was designed to span an exon boundary. In addition, at least one primer was confirmed to exhibit no significant cross-
homology when compared against the NCBI zebrafish RefSeq mRNA library by BLAST search. Primer melting temperatures were maintained between 60 and 64 °C as determined by NetPrimer. Each primer set was observed to generate a single amplicon of expected length following qPCR.

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**cDNA synthesis.** 1 \(\mu\)g RNA was heated to 75 °C for 5 min and then placed on ice. The following reagents were then added to a final volume of 29 \(\mu\)l: 1X Expand High-Fidelity PCR buffer without MgCl\(_2\) (Roche), 3 mM MgCl\(_2\), 500 \(\mu\)M dNTPs, 3.3 \(\mu\)M random hexamers, and 30 U Protector RNase Inhibitor (Roche). The mixture was preincubated to 42 °C for 5 min. 1 \(\mu\)l of 200 U/\(\mu\)l SuperScript II Reverse Transcriptase (RT) or RNase-free water was added for +RT or –RT reactions, respectively. Reactions were incubated at 42 °C for 1 h and then stopped by heating to 95 °C for 5 min. Reaction products were stored at -20 °C.
qPCR conditions. 25 µl reactions were prepared containing the following reagents: 12.5 µl 2X iQ SYBR Green Supermix (Bio-Rad), 5 µl 1 µM primer mix (1 µM each of forward and reverse primer), 5.5 µl RNase-free water, and 2 µl 1:10-diluted template (+RT or –RT product) or 2 µl RNase-free water (no template control). Each assay was performed in triplicate wells using an iQ5 Real-Time PCR Detection System (Bio-Rad). Thermal cycling was performed for 40 cycles, each consisting of 94 °C for 15 s, then 59 °C for 1 min. Following amplification, melt curve analysis was performed to assess non-specific amplification. Each primer set yielded a single peak, indicative of specific amplification. Reactions performed using –RT product or no template controls were observed to exhibit little or no amplification in comparison with their +RT counterparts.

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Data analysis. Relative gene expression was calculated using iQ5 software (ver. 2.0, Bio-Rad) to determine normalized expression levels (ΔΔCt method). For comparison of fold-differences,
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