The antihelmenthic phosphate niclosamide impedes renal fibrosis by inhibiting homeodomain-interacting protein kinase 2 expression

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Renal fibrosis is the final common pathway of all varieties of progressive chronic kidney disease. However, there are no effective therapies to prevent or slow the progression of renal fibrosis. Niclosamide is a US Food and Drug Administration–approved oral antihelminthic drug used for treating most tapeworm infections. Here, we demonstrated that phosphate niclosamide, the water-soluble form of niclosamide, significantly reduced proteinuria, glomerulosclrotic lesions, and interstitial fibrosis in a murine model of adriamycin nephropathy. In addition, phosphate niclosamide significantly ameliorated established renal interstitial fibrosis a murine model of unilateral ureteral obstruction. Mechanistically, phosphate niclosamide directly inhibited TGF-β-induced expression of homeodomain-interacting protein kinase 2 (HIPK2) by interfering with the binding of Smad3 to the promoter of the HIPK2 gene, and subsequently mitigated the activation of its downstream signaling pathways including Smad, Notch, NF-κB and Wnt/β-catenin pathway both *in vitro* and *in vivo*. Thus, phosphate niclosamide mitigates renal fibrosis at least partially by inhibiting HIPK2 expression. Hence, phosphate niclosamide might be a potential therapeutic agent for renal fibrosis.

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The prevalence of chronic kidney disease in the general population is high and is increasing worldwide.[1,2] Renal fibrosis is the final common pathway of all kinds of progressive chronic kidney disease leading to end-stage renal disease.[3–6] Although great efforts have been made in recent years, there are no effective therapies to prevent or slow the progression of renal fibrosis,[5] and the treatment options for patients with end-stage renal disease are limited to dialysis and renal transplantation. Therefore, it is imperative to develop satisfactory therapeutic drugs for renal fibrosis.

One promising approach to slow the progression of fibrosis is to target important fibrogenic pathways. Accumulating studies have shown that multiple signaling pathways such as transforming growth factor (TGF)-β/Smads, Wnt/β-catenin, mTOR (mechanistic target of rapamycin), nuclear factor κB (NF- κB), and Notch are critical in the pathogenesis of renal fibrosis.[3,5,7] Recently, homeodomain-interacting protein kinase 2 (HIPK2), a member of an evolutionary conserved family of serine/threonine kinases, has been identified as a key regulator in kidney fibrosis and idiopathic pulmonary fibrosis that acts upstream of several major profibrotic and proinflammatory pathways including TGF-β/Smad, Wnt/β-catenin, Notch pathway, and NF-κB pathway,[8–11] indicating that HIPK2 might be a potential target for antifibrosis therapy.[5,9,12]

Niclosamide (NICLO), whose systematic name is (5-chloro-N-2-chloro-4-nitrophenyl)-2-hydroxybenzamide, is a US Food and Drug Administration–approved oral antihelminthic drug used for treating most tapeworm infections.[13] It is also used as a molluscicide for water treatment in schistosomiasis control programs.[13,14] The activity of NICLO against these parasites is believed to be mediated by inhibiting mitochondrial oxidative phosphorylation and anaerobic adenosine triphosphate production.[13,15] High-throughput screening campaigns have identified NICLO as a potential anticancer agent.[14,16–18] However, NICLO has poor water solubility.[13,19] To improve its water solubility, a phosphate of NICLO (P-NICLO) is synthesized and has been proven to be able to induce apoptosis of acute myelogenous leukemia cells.[13,20]

It has been shown that P-NICLO exerts antitumor function by targeting multiple signaling pathways including NF-κB, Wnt/β-catenin, and the Notch pathway.[13,14] Given the critical role of these pathways in the pathogenesis of renal fibrosis, the aim of this study was to explore the potential therapeutic effect of P-NICLO on renal fibrosis. We found that administration of P-NICLO significantly inhibited the progression of renal fibrosis in adriamycin (ADR) nephropathy and unilateral ureteral obstruction (UUO) models. Mechanistically, the antifibrotic effect of P-NICLO was at least partially mediated by inhibiting TGF-β1–induced HIPK2 expression and the activation of its downstream multiple profibrotic pathways.

RESULTS

P-NICLO attenuates glomerular injury and interstitial fibrosis induced by ADR

We first investigated the effects of P-NICLO on ADR nephropathy, a model characterized by initial podocyte injury and albuminuria and subsequent renal fibrosis. BALB/c mice were injected with ADR, and 2 weeks later, mice were given P-NICLO (30 mg/kg per day) by i.p. injection. As shown in Figure 1a, at 5 weeks after ADR injection, albuminuria was markedly elevated in the ADR group compared with the control group, P-NICLO administration significantly attenuated ADR-induced albuminuria. In addition, periodic acid–Schiff staining showed attenuated glomerulosclerosis, as evidenced by decreased mesangial expansion and less accumulation of extracellular matrix in the mesangium of P-NICLO–treated mice (Figure 1b). A semiquantitative glomerulosclerotic index of kidney sections confirmed that P-NICLO administration led to a marked reduction in the index (Figure 1c), suggesting that P-NICLO attenuates ADR nephropathy.

We further examined the effects of P-NICLO on interstitial fibrosis. As shown in Figure 1d–f, Western blot and immunohistochemistry staining revealed marked upregulation of α-smooth muscle actin (α-SMA), fibronectin, and collagen I at 5 weeks after ADR injection. Administration of P-NICLO significantly attenuated their induction together with reduced interstitial collagen deposition (Figure 1g and h). These data indicate that P-NICLO attenuates ADR-induced interstitial fibrosis.

P-NICLO inhibits multiple profibrotic signaling pathways *in vitro* and *in vivo*

Previous studies show that niclosamide could inhibit the activity of Wnt/β-catenin and Notch signaling, which are implicated in renal fibrosis.[13,21–24] Therefore, we evaluated whether P-NICLO could affect their activity. NRK52E cells were incubated with 10 ng/ml of TGF-β1 with or without P-NICLO for 24 hours, and cell lysate was harvested. As shown in Figure 2c and d, P-NICLO significantly inhibited TGF-β1–induced expression of *PAI-1*, *MMP-7*, and *Snail*, which are the target genes of Wnt/β-catenin, and expression of *Hes1*, *Hey1*, and *Hey2*, which are the target genes of Notch signaling. In addition, P-NICLO attenuated TGF-β1–induced Smad3 phosphorylation in a dose-dependent manner (Figure 2a and b). Consistently, the levels of p-Smad3, PAI-1, MMP-7, Snail, Hes1, Hey1, and Hey2 in the ADR model were significantly inhibited by p-NICLO (Figure 3). Collectively, these data suggest that P-NICLO inhibits the activation of multiple profibrotic pathways.

P-NICLO inhibits the expression of profibrosis markers through targeting HIPK2

Because HIPK2, a key regulator in kidney fibrosis, acts upstream of multiple antifibrotic pathways,[8–10,12] we hypothesized that HIPK2 might be the target of P-NICLO. To test this hypothesis *in vitro*, we first examined the cytotoxicity of P-NICLO by MTT assay. Rat proximal tubular cells (NRK52E) were incubated with the indicated amount of P-NICLO for 24 hours and then harvested for analysis. No obvious cell mortality was observed when cells were incubated with as much as 4 μM of P-NICLO (Supplementary Figure S1). Therefore, 0 to 2 μM of P-NICLO was used for the following experiments.

First, we examined whether P-NICLO could modulate the expression of HIPK2. NRK52E cells were incubated with 10 ng/ml of TGF-β1 with or without P-NICLO for 24 hours. Consistent with a previous study,[8] TGF-β1 significantly upregulated HIPK2 expression at both mRNA and protein levels measured by Western blot and real-time polymerase chain reaction (PCR). P-NICLO significantly attenuated TGF-β1–augmented HIPK2 expression in a dose-dependent manner (Figure 4a–c). Similarly, Western blot and real-time PCR showed that the expression of renal HIPK2 in the ADR model was inhibited by P-NICLO treatment (Figure 4d–f). Bioinformatic analysis revealed a putative Smad3 binding motif on the promoter region of both human and murine *HIPK2* genes (Figure 4g). Chromatin immunoprecipitation assay demonstrated the binding of Smad3 on the promoter region of the human *HIPK2* gene in human proximal tubular (HK-2) cells. The amount of Smad3 on the promoter of HIPK2 was significantly attenuated by P-NICLO (Figure 4h). These data indicate that P-NICLO inhibits HIPK2 transcription by interfering with the binding of Smad3 to the promoter region of the *HIPK2* gene.

To further confirm that the antifibrotic effect of P-NICLO is mediated by HIPK2, HIPK2 was overexpressed in HK-2 cells by transient transfection of the expression construct WT-HIPK2. As shown in Figure 5a and b, P-NICLO treatment inhibited TGF-β–increased phosphorylation of Smad3. However, this inhibitory effect of P-NICLO was significantly diminished in cells overexpressing HIPK2. Moreover, real-time PCR showed that HIPK2 overexpression partially attenuated the inhibitory effect of P-NICLO on TGF-β1–augmented transcription of profibrotic markers such as vimentin and fibronectin (Figure 5c).

P-NICLO attenuates NF-κB activation and renal inflammation *in vitro* and *in vivo*

NF-κB is downstream of HIPK2.[8,12] We examined the effects of P-NICLO on TGF-β1–induced NF-κB activation. NRK52E cells were incubated with 10 ng/ml of TGF-β1 with or