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Inhibition of Glycolate Oxidase With Dicer-substrate siRNA Reduces Calcium Oxalate Deposition in a Mouse Model of Primary Hyperoxaluria Type 1

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Primary hyperoxaluria type 1 (PH1) is an autosomal recessive, metabolic disorder caused by mutations of alanine-glyoxylate aminotransferase (AGT), a key hepatic enzyme in the detoxification of glyoxylate arising from multiple normal metabolic pathways to glycine. Accumulation of glyoxylate, a precursor of oxalate, leads to the overproduction of oxalate in the liver, which accumulates to high levels in kidneys and urine. Crystalization of calcium oxalate (CaOx) in the kidney ultimately results in renal failure. Currently, the only treatment effective in reduction of oxalate production in patients who do not respond to high-dose vitamin B_6 therapy is a combined liver/kidney transplant. We explored an alternative approach to prevent glyoxylate production using Dicer-substrate small interfering RNAs (DsiRNAs) targeting *hydroxyacid oxidase 1* (*HAO1*) mRNA which encodes glycolate oxidase (GO), to reduce the hepatic conversion of glycolate to glyoxylate. This approach efficiently reduces GO mRNA and protein in the livers of mice and nonhuman primates. Reduction of hepatic GO leads to normalization of urine oxalate levels and reduces CaOx deposition in a preclinical mouse model of PH1. Our results support the use of DsiRNA to reduce liver GO levels as a potential therapeutic approach to treat PH1.

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INTRODUCTION

The primary hyperoxalurias (PH) are a group of autosomal recessive disorders caused by the overproduction of oxalate.[1–4] Oxalate is an end product of glyoxylate metabolism that is produced in the liver and excreted by the kidney. Overproduction of oxalate causes precipitation of highly insoluble calcium oxalate (CaOx) crystals in the kidney, resulting in urolithiasis, nephrocalcinosis, and eventually renal failure. As PH patients progress to end-stage renal disease (ESRD), systemic oxalosis (oxalate deposited in all tissues) often occurs as the combination of overproduction and limited excretion of oxalate.[5,6] There are three forms of PH in which the underlying defects have been identified, designated as PH types 1 (PH1), 2 (PH2), and 3 (PH3). PH1, the most common (around 80% of all PH patients) and severe subtype, is caused by mutations in the *AGXT* gene leading to impaired activity of the enzyme AGT.[7–12] AGT plays a central role in metabolizing the intermediate glyoxylate, which is generated by metabolism of glycolate, glycine, and hydroxyproline in the liver (**Figure 1**).[7] Glyoxylate is converted into either glycine by AGT or into oxalate by lactate dehydrogenase (encoded by the *LDHA* gene in the liver).[2] Reduced AGT activity leads to increased accumulation of glyoxylate and subsequent production of oxalate, the latter of which is predominantly excreted in urine and to a lesser extent via the gastrointestinal tract.

Current treatments for PH1 focus on preserving renal function by decreasing oxalate production and crystallization through increased fluid intake, intensive dialysis, oral administration of calcium oxalate crystal inhibitors, such as citrate, and high doses of pyridoxine (5–8 mg/kg/day).[13,14] Inadequate response to these therapies can be followed by removal of obstructing stones or combined liver/kidney transplant. However, these treatments are insufficient, as progressive nephrocalcinosis leads to ESRD during the first two to three decades in most patients.[13] Several novel molecular approaches to reduce toxic oxalate load have been proposed and are currently under investigation, including oxalate degrading enzyme therapy, correction of AGT mis-targeting and virally mediated *AGXT* gene therapies.[15–23] An alternate approach of substrate reduction, by which the production of glyoxylate is reduced through inhibition of glycolate oxidase (GO), can be a potentially effective therapeutic option.[24–27] Glycolate is a highly soluble small molecule and can be eliminated in the urine at high concentrations without inducing kidney damage or other toxicity. Inhibition of GO reduces the production of glyoxylate and subsequently oxalate, while increasing glycolate levels. In the accompanying paper,[28] reduction of urinary oxalate levels was observed

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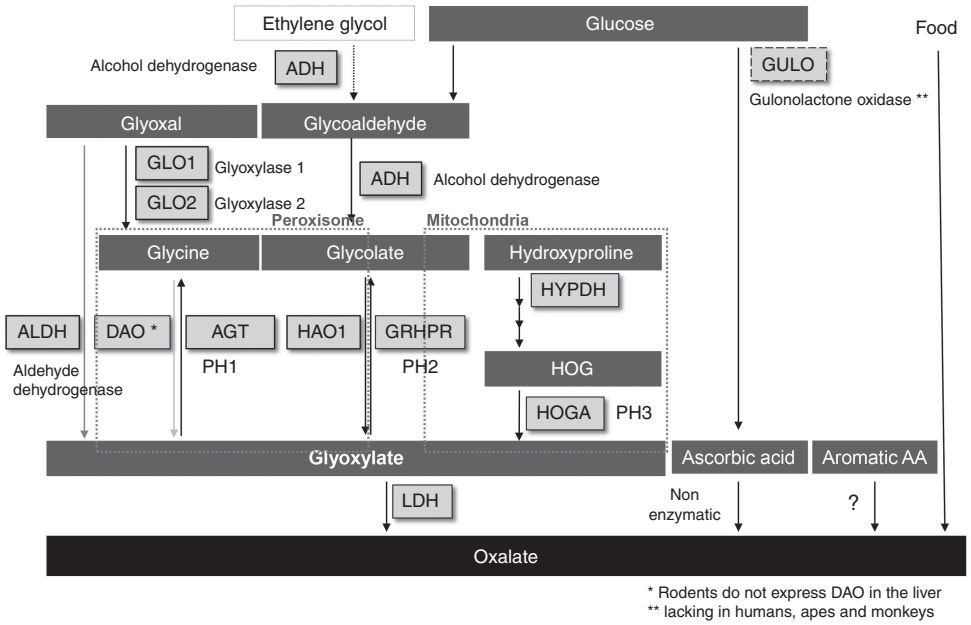

Figure 1 Oxalate metabolism pathway overview. ADH, alcohol dehydrogenase; AGT, alanine-glyoxylate aminotransferase; ALDH, aldehyde dehydrogenase; GLO, glyoxylase; GRHPR, glyoxylate reductase hydroxypyruvate reductase; GULO, gulonolactone oxidase; HAO1, hydroxyacid oxidase 1; HOG, 4-hydroxy-2-oxoglutarate; HOGA, 4-hydroxy-2-oxoglutarate aldolase; HYPDH (PRODH2), hydroxyproline dehydrogenase; LDH, lactate dehydrogenase.

when an *Agxt*-deficient (*Agxt*$^{-/-}$) mouse model, which exhibits key features of PH1 due to completely lacking of *Agxt* mRNA and GO protein, was crossed with a *Hao1*-deficient mouse model.[28] Moreover, mice with deficiencies in both AGT (*Agxt*-deficient) and GO (*Hao1*-deficient) displayed normal growth and development when compared to their heterozygous littermates. Taken together, these studies suggest GO inhibition could be a potentially effective and safe therapeutic option for PH1 patients.

In this study, we investigate RNA interference (RNAi) mediated GO enzyme reduction as a novel potential substrate reduction strategy for PH1 patients. Dicer-substrate small interfering siRNAs (DsiRNAs) have been demonstrated to specifically and effectively reduce the expression of the target mRNA and protein.[29] We have identified DsiRNAs that target the *HAO1* mRNA encoding GO protein. Here we show that administration of *HAO1* DsiRNA achieves potent and durable knockdown of *HAO1* mRNA and GO protein in mice and nonhuman primates (NHPs). In addition, we demonstrate that administration of *HAO1* DsiRNA significantly reduces urine oxalate and prevents kidney damage in the hyperoxaluric *Agxt*$^{-/-}$ mouse model of PH1. These results support further exploration of GO reduction through *HAO1*-targeting DsiRNA as a potential novel approach for the treatment of PH1.

RESULTS

HAO1-1 exhibits potent and specific activity in wild-type mice

We identified our lead *HAO1* DsiRNA by sequential screening *in vitro* followed by testing *in vivo* (**Supplementary Figure S1**). The lead *HAO1* DsiRNA formulated in a lipid nanoparticle, designated as HAO1-1, showed potent, dose-dependent reduction of *Hao1* mRNA (ED50: 0.022 mg/kg, **Figure 2a**) and GO protein (**Figure 2b**) in mouse liver. The homogeneity of *Hao1* mRNA and GO protein knockdown in mouse hepatocytes were confirmed by *in situ* hybridization and immunohistochemistry, respectively. As shown in **Figure 2c**,**d**, treatment with 0.1 or 1 mg/kg HAO1-1 resulted in significantly reduced *Hao1* mRNA and GO protein expression in nearly all hepatocytes. These results demonstrate that HAO1-1 is a potent agent that efficiently suppresses GO protein expression in murine hepatocytes.

HAO1-1 achieves rapid and durable target knockdown

In order to determine the sensitivity and durability of target knockdown, we monitored the expression of *Hao1* mRNA and GO protein for up to 60 days following a single dose of HAO1-1. HAO1-1 rapidly suppressed *Hao1* mRNA expression, achieving ~90% reduction within 12 hours after injection (**Figure 3a**). Notably, at day 45, the remaining suppression of *Hao1* mRNA observed in mice treated with 0.3 and 1 mg/kg of HAO1-1 was ~20 and 40%, respectively. We also observed that GO protein was reduced by more than 90% by 5 days post-dose, after which the levels were reduced to below detectable limits and remained undetectable until day 29 (**Figure 3b**). GO protein levels recovered and were back to baseline levels between days 45 and 60 (**Figure 3b**). In conclusion, a single treatment of HAO1-1 at a dose of 0.3 or 1 mg/kg is able to rapidly produce a sustained inhibition of GO protein expression *in vivo*.

HAO1-1 reduces urine oxalate in the *Agxt*$^{-/-}$ PH1 mouse model

To further evaluate the activity of HAO1-1 as a potential therapeutic for PH1, we employed a murine model of PH1. *Agxt*$^{-/-}$ mice have a germline mutation in the gene encoding AGT, resulting in high baseline urinary oxalate levels compared to their wild-type

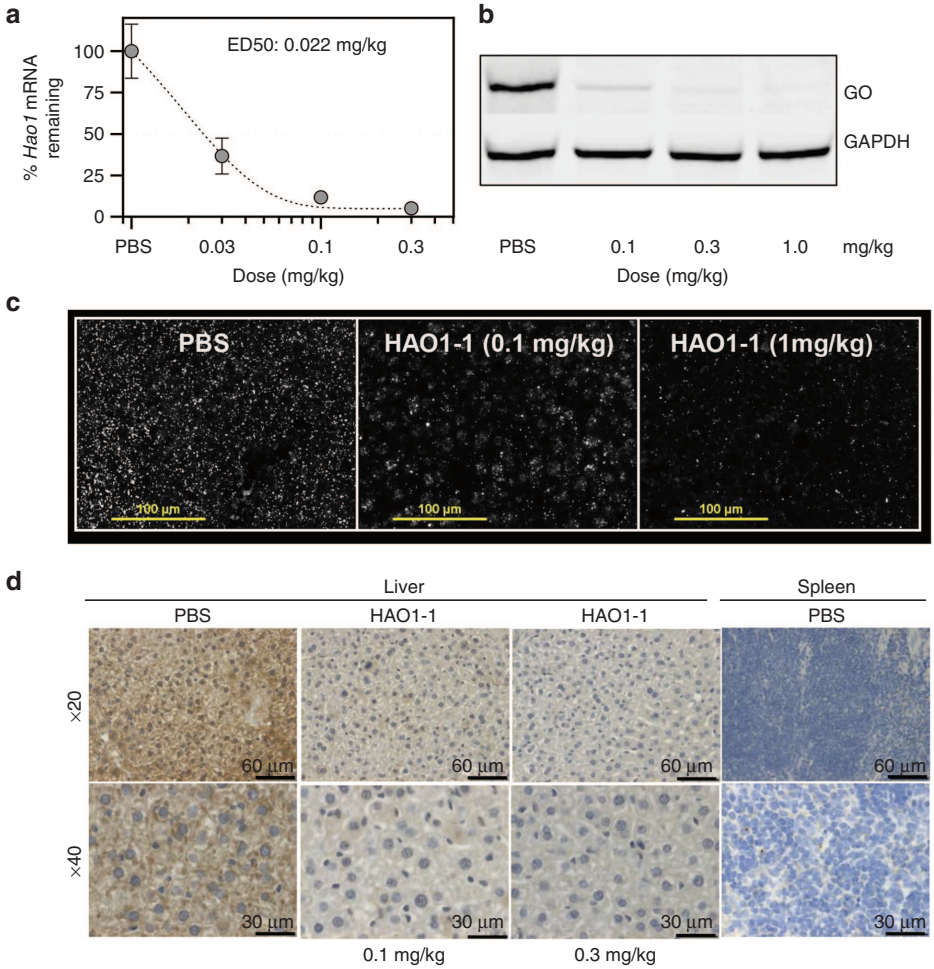

Figure 2 HAO1-1 displays potent *in vivo* activity in mice. The lead *HAO1* DsiRNA formulated in LNP (HAO1-1) was i.v. injected into wild-type mice with varying doses as indicated. Liver samples were collected for analysis of mRNA after 24 hours and for protein on day 5 after a single-dose injection. (**a**) Results of real-time reverse transcription PCR (mean ± SD) indicate that HAO1-1 inhibits *Hao1* mRNA expression with high potency (ED50: 0.022 mg/kg). (**b**) Results of western analysis show that HAO1-1 displayed potent inhibition of GO protein expression 5 days after injection. (**c**) *Hao1* mRNA was detected in liver tissues by *in situ* hybridization and showed that samples isolated from animals injected at 0.1 or 1 mg/kg have lower hybridization signals compared to PBS injected animals. Bar = 100 µm. (**d**) GO protein levels were measured by immunohistochemistry. Results showed that HAO1-1, either at 0.1 or 0.3 mg/kg, effectively and homogenously reduced liver GO protein in mouse hepatocytes, compared to PBS-injected controls. Spleen sections were used as negative controls. Bar = 30 or 60 µm. LNP, lipid nanoparticle.

counterparts.[23] Male *Agxt*$^{-/-}$ animals recevied a single i.v. dose of HAO1-1 at 0.3 mg/kg, and urine samples were analyzed for oxalate, glycolate and creatinine. Glycolate was investigated as a potential biomarker of GO inhibition, based on the assumption that suppression of GO protein would inhibit the conversion of glycolate to glyoxylate, resulting in increased uriniary glycolate concentrations. Urinary creatinine levels were used as a control to normalize for variations in urine concentration. The urinary oxalate levels normalized to creatinine were significantly reduced 5 days after HAO1-1 treatment and remained low until day 15, before slowly returning to baseline levels by day 28 (**Figure 4a**). Simultaneously, we observed that HAO1-1 treatment resulted in increased production of urinary glycolate (**Figure 4b**). These results indicate that HAO1-1 treatment reduced urine oxalate through an on-target suppression of GO expression, blocking the conversion of glycolate into glyoxylate and resulting in a subsequent elevation of urinary glycolate. Our results also indicate that urinary glycolate could be used as a pharmacodynamic marker to monitor the response to HAO1-1. Furthermore, the results that urinary oxalate levels instantly and inversely mirrored glycolate levels suggest that hepatic glycolate is a main source of glyoxylate and oxalate overproduction in the *Agxt*$^{-/-}$ hyperoxaluric animals (**Supplementary Figure S2**). The results that normalized oxalate reduction and glycolate elevation correlated well at different time points also indicate that GO is a remarkably effective target for oxalate reduction in the PH1 disease mouse model (**Supplementary Figure S2**). Urinary oxalate and glycolate levels were allowed to return to baseline levels, after which HAO1-1 was re-administered at 0.3 mg/kg (on day 33 and again on day 40). Reproducibly, oxalate reduction and glycolate elevation were detected with treatment, suggesting that the observed biological response is specifically triggered by HAO1-1. Repeated doses of control DsiRNA showed no effect on urinary oxalate in the *Agxt*$^{-/-}$ mice (**Supplementary Figure S3**). We also confirmed that HAO1-1 treatment reduced not only the oxalate concentration in the urine samples, but also the amount of excreted

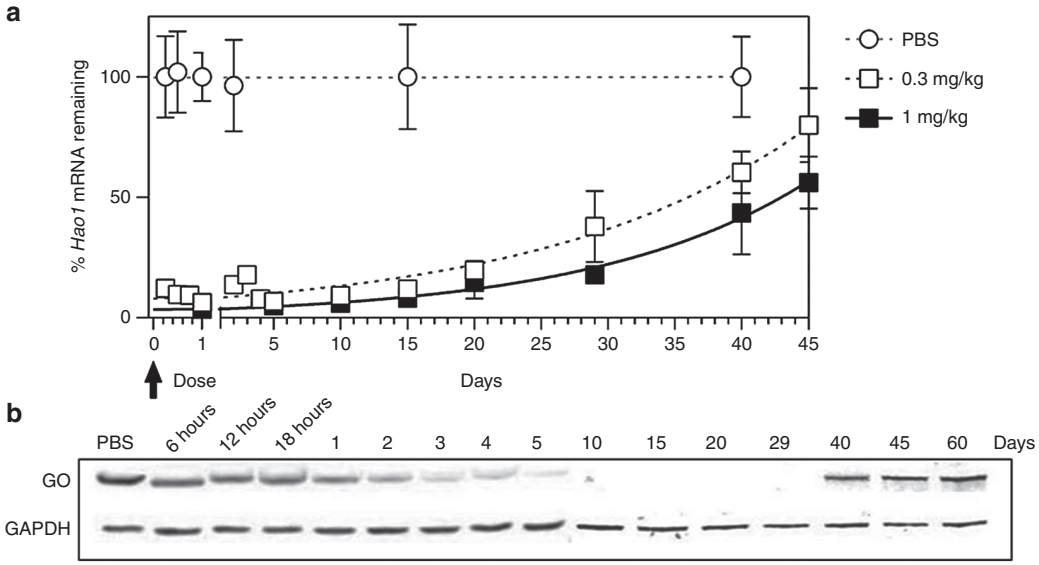

Figure 3 HAO1-1 achieves rapid and durable target knockdown in mice. Wild-type mice were injected with a single i.v. dose of HAO1-1 at 0.3 or 1 mg/kg. (**a**) At the indicated time points, five animals were sacrificed, and liver samples were evaluated for mRNA levels by real-time reverse transcription PCR. The results (mean ± SD) demonstrated that *Hao1* mRNA levels were quickly diminished and the inhibitory effect of a single dose of HAO1-1 lasted for more than a month. (**b**) Representative results of western analysis from each time point of mice injected with a single dose of PBS or HAO1-1 at 0.3 mg/kg. Results showed that GO protein gradually decreased starting 6 hours postdose, reaching levels below the detection limit by day 10 and reemerging between days 29 and 40.

oxalate over 24 hours in the treated animals (**Figure 4c**). HAO1-1 reduced GO protein expression in *Agxt*$^{-/-}$ mice to the same extent as was observed in wild-type mice (**Supplementary Figure S3**). Consistent with the results reported in the *Hao1* and *Agxt* double knockout mice,[28] our results further suggest that HAO1 could be a potential therapeutic target for PH1.

HAO1-1 protects against kidney damage elicited by ethylene glycol in *Agxt*$^{-/-}$ mice

Male *Agxt*$^{-/-}$ mice develop variable degrees of kidney calcium oxalate deposits when fed ethylene glycol (EG), a precursor of glycolate.[23] To determine whether reduction of oxalate by HAO1 treatment is sufficient to prevent kidney damage, male *Agxt*$^{-/-}$ mice were provided water containing 0.7% EG while being treated with either PBS or HAO1-1 once per week for 3 weeks. Additionally, one group of animals underwent delayed HAO1-1 treatment, receiving one dose of HAO1-1 following 2 weeks of EG administration (**Figure 5a**). Body weight and water consumption were measured during the 3 weeks of study period (**Supplementary Figure S4**). At the end of 3 weeks, animals that received only EG showed high oxalate levels (**Figure 5b**) and variable degrees of CaOx deposits in their kidneys (**Figure 5c**,**d**,**e**). Animals that started treatment with HAO1-1 simultaneously with EG administration displayed no change in urinary oxalate levels (**Figure 5b**) and showed no kidney CaOx deposition (**Figure 5d**,**e**). These results indicate that reduction of urinary oxalate levels is sufficient to prevent CaOx deposits in the kidney. Additionally, animals that received delayed HAO1-1 treatment showed minimal CaOx deposits, despite having high oxalate levels for 2 weeks prior to treatment with HAO1-1 (**Figure 5b**,**d**,**e**). Formation of kidney crystals in this model usually occurs after 2 weeks of EG administration, suggesting that not only the level of urinary oxalate but also the duration of oxalate elevation are critical for kidney CaOx depositions. Our data showed that kidney damage can be prevented by reducing the urine oxalate levels before a certain threshold point that triggers the deposition of CaOx in kidney. Taken together, our results demonstrate that utilizing HAO1-1-mediated inhibition of GO to reduce the level and/or duration of elevated urinary oxalate is sufficient to protect against kidney damage caused by prolonged hyperoxaluria in a PH1 mouse model.

Cumulative inhibitory effect through frequent administration of low-dose HAO1-1

Since PH1 is a chronic disease and requires regular treatment, we tested whether recurring treatment of HAO1-1 would yield any cumulative effects. Wild-type mice were injected with 0.03, 0.1, or 0.3 mg/kg of HAO1-1 bi-monthly and were subsequently analyzed for the expression of *Hao1* mRNA and GO protein at different time points. We observed a dose-dependent decrease in both *Hao1* mRNA and GO protein during the first month of treatment (**Supplementary Figure S5**). Notably, both *Hao1* mRNA and GO protein levels had trough points that were lower during the second cycle than the first cycle. Additionally, GO protein levels were almost indistinguishable among all three dose level groups after three doses of HAO1-1 (**Supplementary Figure S5**). These results demonstrate the cumulative effect of recurring doses of HAO1-1. We have also demonstrated that, with monthly administration of HAO1-1, there were no significant adverse effects in liver and the hematopoietic system during the course of 150 days (**Supplementary Figure S6**). Taken together, these results are consistent with the results of *Agxt* and *Hao1* double knockout mice[28] and further support that GO could be a safe and effective target for PH1.

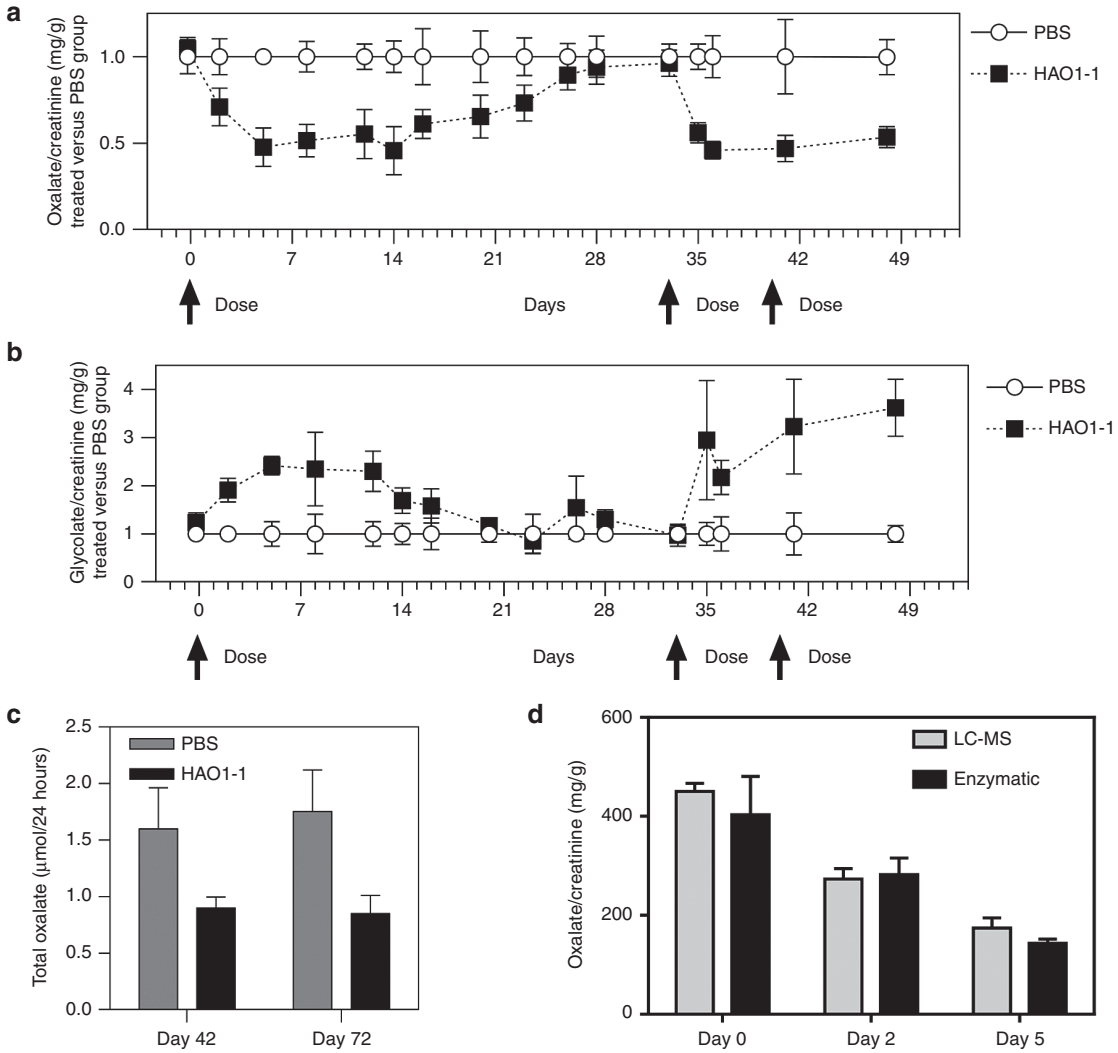

Figure 4 HAO1-1 reduces urinary oxalate and elevates urinary glycolate levels in a mouse PH1 model. Male *Agxt*$^{-/-}$ mice were injected i.v. with HAO1-1 ($N = 4$) at 0.3 mg/kg or PBS ($N = 5$) as a control on day 0, day 33, and day 40. Urine samples were manually collected on predose day –2, as well as days 2, 5, 8, 12, 14, 16, 20, 23, 26, 28, 33, 35, 36, 41, and 48, and analyzed for oxalate, glycolate, and creatinine concentrations by LC/MS. (**a**) The change in urine oxalate levels in the HAO1-1 treatment group was calculated as the urine oxalate concentration normalized to creatinine (milligram of oxalate per gram of creatinine) relative to the average normalized urine oxalate concentration of the PBS control group, where the urine oxalate (milligram of oxalate per gram of creatinine) in the PBS control group was set at 1. LC/MS analysis (mean ± SD) revealed that HAO1-1 reduced oxalate levels rapidly, and the effects lasted for up to 15 days before beginning to return to baseline levels. (**b**) Urine glycolate levels were measured, normalized to creatinine, and expressed as a value relative to the average of the PBS control group (which was set at 1, as in **Figure 3a**). HAO1-1 simultaneously induced the elevation of urinary glycolate levels and the reduction of oxalate levels. Results of these experiments with values plotted without normalization to PBS treatment group and expressed as individual animal are provided in **Supplementary Figure S2**. (**c**) Twenty-four hour urine samples were also collected using metabolic cages from day 41 to 42 and from day 71 to 72. The results (mean ± SD) indicated that oxalate excretion was substantially lower after HAO1-1 treatment compared to control (PBS). (**d**) Oxalate levels were measured by both enzymatic assay and LC/MS methods. The normalized oxalate values (milligram per gram of creatinine) determined by both methods were compared using urine samples collected on day 0, day 2 and day 5. The data (mean ± SD) indicate a good correlation between both methods and further confirm that HAO1-1 effectively reduces oxalate production. The arrows indicate the days on which the animals were treated with HAO1-1 or PBS.

HAO1 DsiRNA approach displays potent, durable, and specific activity in NHPs

To further confirm that our *HAO1* DsiRNA approach is effective across species, we evaluated *HAO1* DsiRNA in a NHP-specific formulation in male and female cynomolgus monkeys. We demonstrated that this *HAO1* DsiRNA potently suppressed mRNA and protein expression in a dose-dependent manner with an ED50 of less than 0.3 mg/kg (**Figure 6a**,**b**). Similarly, this *HAO1* DsiRNA achieved durable inhibition of *HAO1* mRNA expression in NHPs at 0.3 mg/kg, with mRNA levels remaining significantly suppressed (40% suppression) up to day 29 after a single i.v. injection (**Figure 6c**). Furthermore, as we observed in mice, the urinary glycolate levels were elevated in a dose-dependent manner in the animals that received *HAO1* DsiRNA (**Figure 6d**). Similar to urine glycolate, plasma glycolate levels displayed an equal degree of response to *HAO1* DsiRNA (**Figure 6e**), further suggesting that glycolate is a consistent biomarker for *HAO1* target inhibition. In further support of this hypothesis that knockdown of the *HAO1* target can

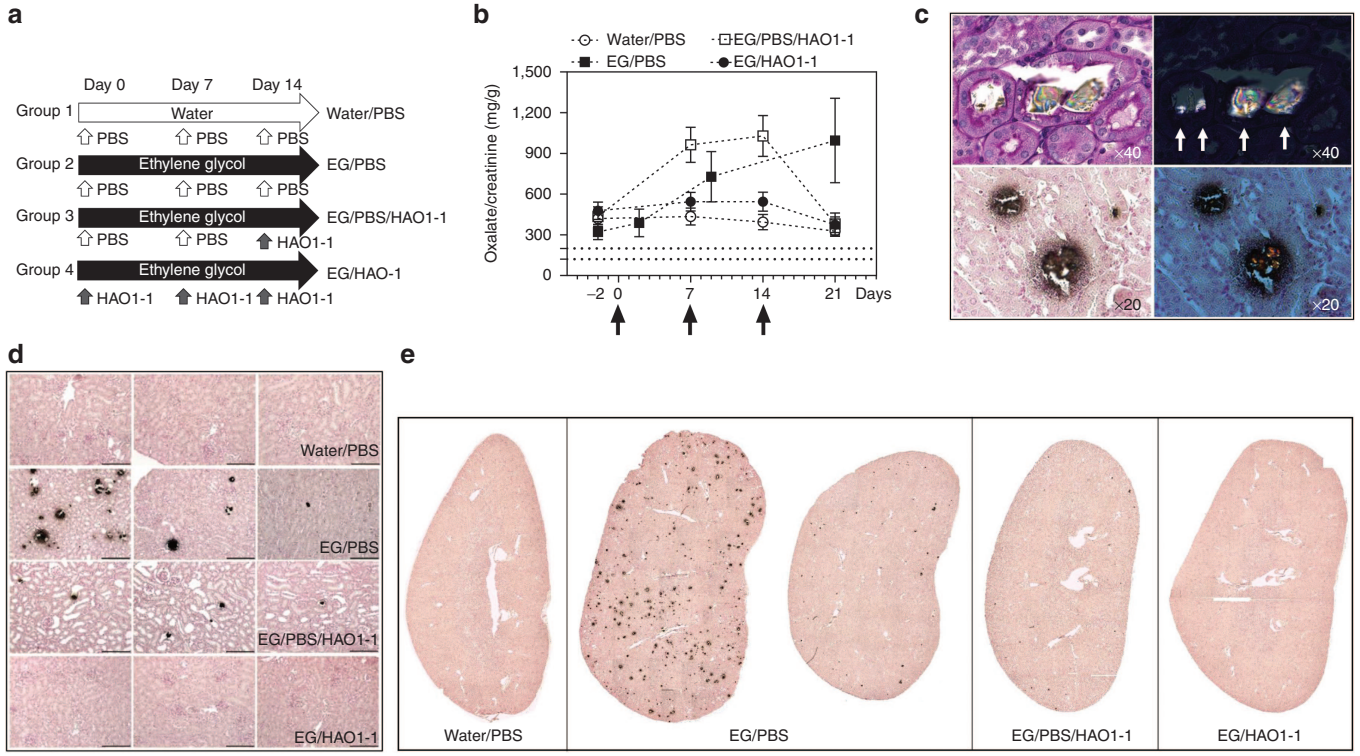

Figure 5 HAO1-1 protects against EG-induced kidney damage in a PH1 mouse model. (**a**) Male *Agxt*$^{-/-}$ mice were divided into four groups containing seven mice each and were supplied with either 0.7% EG in their drinking water (groups 2 to 4) or regular drinking water as a control (group 1). Animals of groups 1 and 2 were injected with three doses of PBS on days 0, 7, and 14. Animals of group 3 were injected with two doses of PBS on days 0 and 7 as well as a single dose of HAO1-1 on day 14. Group 4 received three doses of HAO1-1 on days 0, 7, and 14. Both PBS and HAO1-1 were given i.v.. Urine samples were collected weekly and analyzed for oxalate levels with enzymatic assay. All animals were sacrificed, and the kidneys were collected and evaluated for CaOx crystals on day 22. (**b**) Animals drinking 0.7 % of EG (EG/PBS) and treated with PBS displayed continuous elevation of urinary oxalate levels. Animals drinking EG and injected with HAO1-1 (EG/HAO1-1) exhibited urinary oxalate levels that remained at baseline concentration. Mice drinking EG and given delayed treatment of HAO1-1 (EG/PBS/HAO1-1) showed an initial elevation of urine oxalate levels (milligram per gram of creatinine) during the first 2 weeks that returned back to baseline levels following HAO1-1 treatment. Dashed lines indicate estimated range of oxalate levels of wild-type C57BL/6 mice that are not littermates and, therefore, not fully matched to the *Agxt*$^{-/-}$ mice in terms of genetic background. (**c**) Histological analysis of kidney tissue was performed to detect CaOx crystals using Pizzalato staining. Representative images of kidney sections show animals of the EG/PBS group developed CaOx crystals that exhibited characteristic birefringence under polarized light (upper panel) and positive staining using the Pizzalato's method (lower panel). (**d**) Histological analysis indicates that HAO1-1 is able to prevent the formation of CaOx crystals and protect the kidneys from further damage in the *Agxt*$^{-/-}$ mice that received EG (compare EG/PBS and EG/HAO1-1 groups). Delayed treatment of HAO1-1 also effectively reduced CaOx crystals in the kidneys, though to a lesser extent. Each picture shows a representative section from an individual animal of each group. Bar = 150 µm. (**e**) Whole kidney sections were imaged, scanned, and reconstituted with imaging software to further demonstrate that HAO1-1 prevents the formation of CaOx crystals in treated animals.

be predicted using biomarkers, we observed a notable correlation between *HAO1* mRNA knockdown and plasma glycolate elevation at steady state after multiple doses of *HAO1* DsiRNA injection (Figure 6f).

Collectively, our data demonstrate that the *HAO1* DsiRNA approach produces potent and durable responses and that these responses can be reliably monitored not only in mice but also in NHPs.

DISCUSSION

The emergence of RNAi therapeutics provides great potential to develop treatments that were previously considered impossible. The main challenges for RNAi therapies are the identification of efficacious gene targets and the successful delivery of RNAi molecules into affected cells to induce target gene knockdown. In this study, we have obtained data suggest that GO could be an effective target for treating PH1, a devastating renal disease. The lead *HAO1* DsiRNA was efficiently delivered in lipid nanoparticle to hepatocytes and durably reduced target gene expression in mice and monkeys. In *Agxt*$^{-/-}$ mice, suppression of GO protein levels, in turn, reduced the conversion of glycolate to glyoxylate, leading to a decrease in urinary oxalate levels, an expected corresponding elevation of glycolate, and elimination of CaOx deposition in *Agxt*$^{-/-}$ animals who were treated with EG, a model which recapitulates key features of human PH1. No health effects were noted in either *Hao1*-deficient mice[28] or mice that received multiple doses of *HAO1* DsiRNA over the course of 150 days. Taken together, the results support the targeted knockdown of *HAO1* by DsiRNA as a potentially safe and effective strategy for the treatment of PH1.

Potential difference in glyoxylate and oxalate metabolism in rodents, NHPs, and humans

We have demonstrated that HAO1-1 was able to reduce urinary oxalate and prevent renal damage in a PH1 mouse model

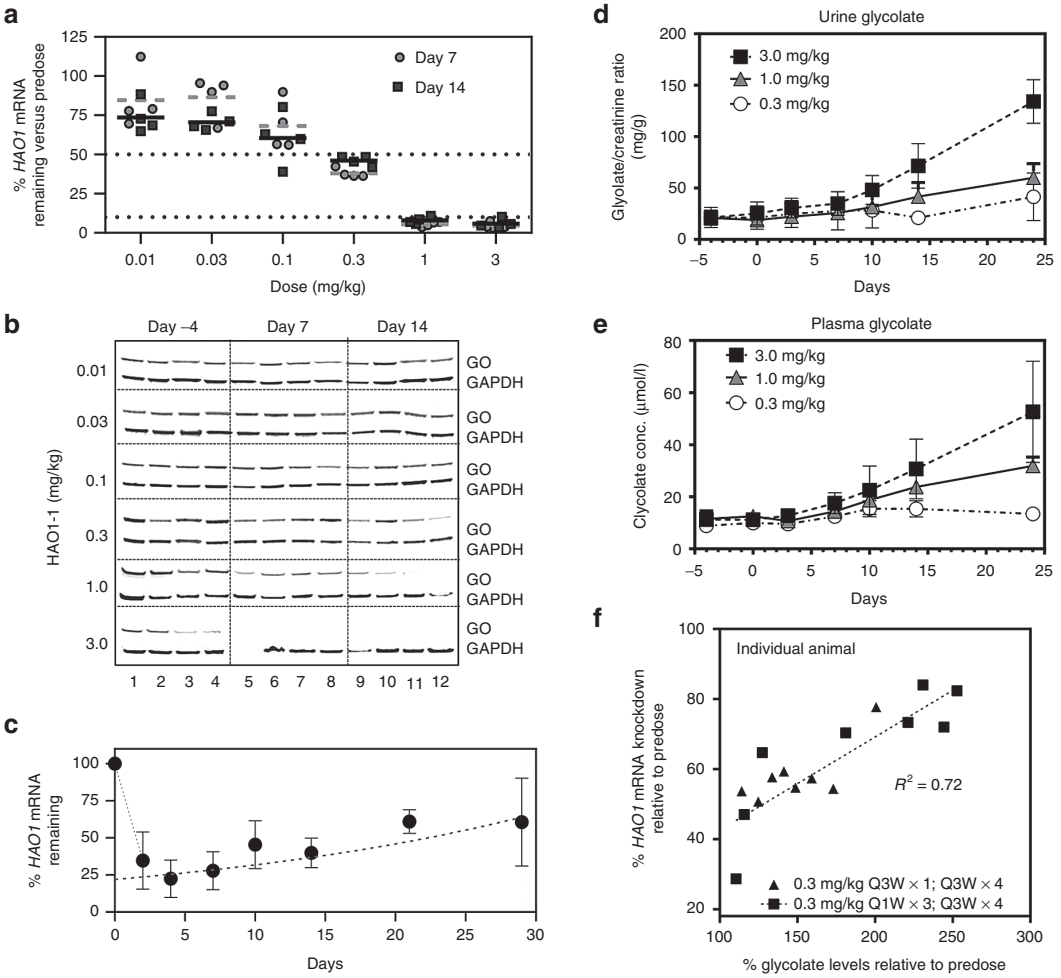

Figure 6 *HAO1* DsiRNA displays specific and potent activity in NHPs. A selected *HAO1* DsiRNA formulated in LNP, designated DCR-PH1, was i.v. injected into cynomolgus monkeys with varying doses as indicated. Liver biopsies were collected for analysis of mRNA and protein at varying time points as indicated. Urine and plasma samples were also collected at varying time points as indicated and then analyzed with LC/MS for glycolate and creatinine levels. (**a**) Results of real-time reverse transcription PCR indicated that *HAO1* DsiRNA inhibits *HAO1* mRNA expression in a dose-dependent manner. Expression of each individual animal was plotted. Each group contains four animals. (**b**) The results of western blot analysis indicated that *HAO1* DsiRNA inhibits GO protein expression on days 7 and 14. Expression in each individual animal was analyzed. Note that only three biopsies were recovered from the 3 mg/kg dose group at day 7, and lane 5 was empty with no sample. (**c**) The results of real-time reverse transcription PCR indicate that *HAO1* DsiRNA, when injected at 0.3 mg/kg durably inhibits *HAO1* mRNA expression (mean ± SD). (**d**) LC/MS analysis shows that urinary glycolate levels (normalized with creatinine) increased in *HAO1* DsiRNA-treated animals, in a dose-dependent manner (mean ± SD). (**e**) Similarly, plasma glycolate was elevated with comparable kinetics and dose dependency in treated animals. (**f**) Animals receiving *HAO1* DsiRNA at 0.3 mg/kg once every 3 weeks were analyzed for their *HAO1* mRNA expression and plasma glycolate levels at steady state. There was a notable correlation (R^2 = 0.72) between the knockdown of *HAO1* mRNA and elevation of plasma glycolate in individual animals. This indicates that plasma glycolate could be explored as a potential pharmacodynamic marker for target knockdown. LNP, lipid nanoparticle.

in which mice were treated with EG. While this result suggests that a potential reduction of urine oxalate and disease progression could be achieved in humans, there are differences in the glyoxylate metabolism pathways of mice and humans that make it challenging to fully predict the effect of *HAO1* inhibition by DsiRNA in humans (**Figure 1**). For example, D-amino acid oxidase, which converts glycine back to glyoxylate, is expressed in human but not in mouse liver (Biogps.org). Thus, it is possible that oxalate production in mice is more sensitive than that in PH1 patients to the effects of GO protein reduction. Additionally, mice, but not humans and other species, are capable of *de novo* synthesis of ascorbic acid, which can be nonenzymatically converted into oxalate.[30] These species-specific differences in oxalate metabolism indicate the limitations for translating mouse models to human responses and prompted us to verify the *HAO1* DsiRNA approach in NHPs. Our results confirm that *HAO1* DsiRNA efficiently reduces *HAO1* mRNA and GO protein levels in NHP as well as induces an increase in glycolate, a further biomarker of GO activity. However, urine oxalate levels in normal NHP are low to the point that any potential further reduction cannot be accurately detected with our current methodology.

Other potential therapeutics for PH1

Small-molecule inhibitors of GO have also been explored for treating PH1. (L)-oxothiozalidine-4-carboxylate (OTZ) was effective in a hyperoxaluric rat model[24] but showed a very limited effect on urine oxalate in PH1 patients.[25,26] Another inhibitor of GO, a novel 4-substituted-3-hydroxy-3-pyrroline-2,5-dione, has been recently

under investigation for clinical utility in patients.[27] Other potential targets for PH1 include inhibition of hydroxyproline dehydrogenase (HYPDH or PRODH2) (**Figure 1**). HYPDH controls the first step of hydroxyproline catabolism, which is hypothesized to be an important source of glyoxylate and oxalate.[31,32]

Potential therapeutics for PH2 and PH3

There are currently no effective treatments for either PH2 or PH3. In PH2 and PH3 patients, mutations in the genes encoding glyoxylate reductase (GRHPR) and 4-hydroxy-2-oxoglutarate aldolase (HOGA), respectively, underlie the disease, causing the overproduction of glyoxylate and subsequent oxalate elevation (**Figure 1**). Blocking GO protein production reduces the contribution by glycolate to the pool of glyoxylate and thus should reduce the glyoxylate pool that is available for oxalate production. Therefore, GO inhibition could also have potential as a therapeutic strategy for PH2 and PH3. However, the relative contributions of the various pathways to glyoxylate production in health and disease are not yet fully characterized and elucidating them further will be of great interest. Exploring the effect of HAO1 inhibition on oxalate metabolism in mouse models of PH2 and PH3 should provide insights to help identify new therapeutic strategies for these PH types.

Oxalate concentrations and excretion rate to predict kidney disease

The *Agxt*$^{-/-}$ mice, despite their high urinary oxalate concentrations, show very low probability of developing kidney crystals. However, male *Agxt*$^{-/-}$ mice provided with excess glycolate from EG to further increase urinary oxalate concentrations developed kidney CaOx deposits with high frequency.[23] Our results suggest that urinary oxalate levels need to reach a certain threshold level and duration for kidney deposits to occur. This is consistent with reports that, in PH patients, oxalate excretion rates at diagnosis can predict the probability of progression to ESRD.[33] In a study monitoring PH patients over a period of 30 years, those who had urinary oxalate excretion rates lower than 1.3 mmol/1.73 m^2/24 hours had a much lower chance of developing ESRD than patients with urinary oxalate excretion rates higher than 1.3 mmol/1.73 m^2/24 hours. The data also suggested that maintaining the oxalate concentration under a threshold level is sufficient to prevent ESRD. Consistent with this hypothesis, we demonstrated that HAO1-1 is able to prevent kidney damage in hyperoxaluric mice even though their oxalate levels have not been reduced to the level of wild-type animals (**Figure 5b–e**). From this, we conclude that *HAO1* DsiRNA treatment may be able to achieve a reduction of oxalate excretion rate that falls below the threshold point, thus preventing kidney crystal formation.

Relationship of *Hao1* mRNA and GO protein knockdown to oxalate reduction

We observed that the duration of oxalate reduction is shorter than that of *Hao1* mRNA and GO protein reduction (**Figures 3** and **4**). One potential explanation is that GO protein expression must be reduced to below a certain level in order to effectively prevent generation of glyoxylate from glycolate and to achieve oxalate reduction. Given the differences between mice and humans in the glyoxylate metabolism pathway, the threshold level of GO reduction in humans required to achieve changes in urinary oxalate concentrations may be different than that in mice.

Glycolate as a potential noninvasive, direct, and predictive biomarker

Hao1$^{-/-}$ mice show elevated levels of urinary glycolate without renal damage or other phenotypic consequences.[28] In addition, glycolate oxidase deficiency in humans has been shown to cause asymptomatic glycolic aciduria.[34] These findings are consistent with the concept that glycolate is highly soluble and it can be excreted by the kidneys without pathological consequences.

We observed that urinary glycolate levels correlate inversely with oxalate changes in HAO1-1-treated *Agxt*$^{-/-}$ mice. This suggests that glycolate could potentially be used as a pharmacodynamic marker to evaluate *HAO1* mRNA knockdown using easy, noninvasive procedures to monitor urinary and plasma glycolate levels.

In summary, our preclinical studies demonstrate that *HAO1* DsiRNA is a promising approach as a potential PH1 therapeutic.

MATERIALS AND METHODS

Selection of lead HAO1 DsiRNA. HAO1-1 was identified through a process of large-scale screening of siRNAs for mRNA knockdown activity *in vitro* (**Supplementary Figure S2**). RNA strands for siRNA duplexes were synthesized purified at Integrated DNA Technologies (Coralville, IA). The siRNAs in the primary screen had a 25/27-mer duplex RNA structure, with a 25-nucleotide sense strand composed of all RNA except for two 3′-terminal DNA nucleotides, annealed to a 27-nucleotide all RNA antisense strand. The siRNAs in the primary screen were partially 2′-OMe modified.

RNA preparation and real-time PCR. Sample tissues were homogenized in guanidinium thiocyanate-phenol using a TissueLyser II (Qiagen, Valencia, CA). RNA was then purified according to manufacturer instructions (Promega, Madison, WI). Transcriptor first strand cDNA kit (Roche) was used to prepare cDNA and mouse specific *Hao1* and *Hprt1* (Integrated DNA Technologies) primers were used for PCR on a CFX96 cycler-Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA).

In situ *hybridization using ViewRNA.* Manufacturer's protocol (Affymetrix, Santa Clara, CA) was followed with the exceptions noted below. Briefly, formalin-fixed/paraffin-embedded tissue sections were cut to 5 µm, baked at 60 °C for 60 minutes. Liver tissue were pretreated for 20 minutes at 95 °C and digested with protease for 15–20 minutes at 40 °C. Signal intensity and area of signal were measured using Nikon Elements software.

Western analysis of tissue samples. Tissue lysates were prepared using Qiagen Tissue Lyser with lysis buffer (20 mmol/l Tris pH 8.0, 150 mmol/l NaCl, 1% NP-40) and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). Tissue lysates were resolved by 4–15% Tris-glycine SDS–PAGE (Bio-Rad Laboratories). The blots were blocked and incubated with rabbit anti-GO primary followed by anti-rabbit-IR800 (Li-Cor Biosciences, Lincoln, NE) and with mouse anti-GAPDH or tubulin (Sigma-Aldrich) followed by anti-mouse-IR600 (Li-Cor Biosciences). The signal intensity was determined by Odyssey Infrared Imaging System (Li-Cor Biosciences).

In vivo *studies and sample collection in mice.* Mice were treated in accordance with institutional ethical guidelines of animal care, handling, and termination; all studies were approved by Dicerna's Institutional Review Board. Mice were kept in a pathogen-free facility, with free access to standard chow and water. C57BL6 mice (Harlan Laboratories, Indianapolis, IN) were used to screen the lead HAO1-1. *Agxt*$^{-/-}$ animals were used to evaluate efficacy of HAO1-1 on urine oxalate and 0.7% EG challenge experiments. All dosing with PBS or HAO1-1 was injected i.v.. Urine samples were collected manually unless stated otherwise. The urine samples were acidified before storing.

Histological analysis. Tissue were fixed in 10% neutral buffered formalin for 24 hour and then transferred into 70% ethanol and embedded in paraffin for sectioning. CaOx staining was performed on kidney sections as described.[35] The images were taken using Olympus BX51 microscope and processed using Image Pro Premier 9.1 image analysis software.

Enzymatic oxalate analysis and creatinine assay. Urine oxalate was measured using a clinical oxalate oxidase assay (Trinity Biotech, Wicklow, Ireland) modified for use with murine samples. Urine creatinine was measured using standard creatinine detection kit (Enzo Life Sciences AG, Lausen, Switzerland).

Oxalate, glycolate, and creatinine assays by LC/MS. Oxalate, glycolate, and creatinine were determined by methods based on liquid chromatography combined with mass spectrometry detection (LC/MS) on a triple-quadrupole instrument (API 5500, AB Sciex, Framingham, MA) by electrospray ionization and multiple reaction monitoring. Mouse and monkey urine samples were analyzed for oxalate, glycolate, and creatinine after sample dilution by hydrophilic interaction liquid chromatography (HILIC) and MS detection in the negative electrospray ionization mode. Glycolate was determined in monkey plasma samples by protein precipitation, followed by chemical derivatization and analysis by reversed-phase LC and MS detection in the positive electrospray ionization mode. Stable-label internal standards ($^{13}C_2$-oxalate, $^{13}C_2$-glycolate, and $^{2}H_3$-creatinine) were utilized for quantification. Calibration curves were made in surrogate matrix. Quality control samples were analyzed in triplicates at a minimum of three concentration levels in each analytical batch. The observed inaccuracy (%Bias) and precision (%CV) for quality control samples were less than 15% for all analytes.

NHP study. Treatment of the animals was conducted by a certified contract research organization in accordance with the testing facility's standard operating procedure, which adheres to the regulations outlined in the United States Department of Agriculture Animal Welfare Act (9 CFR, Parts 1–3) and the conditions specified in the Guide for the Care and Use of Laboratory Animals (ILAR publication, 1996, National Academy Press). Animals were given a single i.v. infusion over 2 minutes of lipid nanoparticle-formulated DsiRNAs. Tissue biopsies, blood and urine samples were collected for pharmacodynamics and biomarker analysis.

SUPPLEMENTARY MATERIAL

Figure S1. Results of *HAO1* DsiRNA screen to identify the lead *HAO1* DsiRNA.

Figure S2. HAO1-1 reduces urinary oxalate and elevates urinary glycolate levels at similar rates in male *Agxt*-/- mice.

Figure S3. HAO1-1 reduces urine oxalate levels in the *Agxt*-/- PH1 mouse model.

Figure S4. Body weight change and water consumption in the male *Agxt*-/- mice supplied with drinking water containing EG.

Figure S5. HAO1-1 achieves cumulative target knockdown in mice.

Figure S6. HAO1-1 long-term treatment exhibits no obvious adverse effect in mice.

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