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Letter

8-L-Cystinyl Bis(1,8-diazaspiro[4.5]decane) as an Orally Bioavailable L-Cystine Crystallization Inhibitor for Cystinuria

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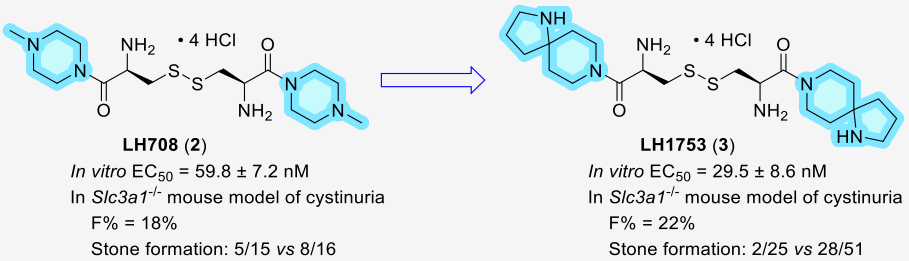

LH708 (2)
In vitro EC_{50} = 59.8 ± 7.2 nM
In *Slc3a1*$^{-/-}$ mouse model of cystinuria
F% = 18%
Stone formation: 5/15 *vs* 8/16

LH1753 (3)
In vitro EC_{50} = 29.5 ± 8.6 nM
In *Slc3a1*$^{-/-}$ mouse model of cystinuria
F% = 22%
Stone formation: 2/25 *vs* 28/51

ABSTRACT: Cystinuria, a rare genetic disorder, is characterized by defective L-cystine reabsorption from the renal proximal tubule, resulting in abnormally high concentrations of L-cystine and subsequent L-cystine crystallization in urine and stone formation in the urinary tract. Inhibition of L-cystine crystallization by L-cystine diamides such as LH708 (**2**) represents a promising new approach to prevent stone formation in patients with cystinuria. While **2** shows promising *in vivo* efficacy and a good safety profile in a *Slc3a1*-knockout mouse model of cystinuria, further structural modification of **2** led to the discovery of 8-L-cystinyl bis(1,8-diazaspiro[4.5]decane) (LH1753, **3**) incorporating a bioisosteric spiro bicyclic diamine 1,8-diazaspiro[4.5]decane for the *N*-methylpiperazine terminal groups in **2** as a promising candidate with **3** being about 120× more potent than L-cystine dimethyl ester (CDME, **1**) and about 2× more potent than **2** in inhibiting L-cystine crystallization. Furthermore, **3** demonstrated good oral bioavailability and *in vivo* efficacy in preventing L-cystine stone formation in the *Slc3a1*-knockout mouse model of cystinuria.

KEYWORDS: *Kidney stones, Cystinuria, L-Cystine diamides, Crystallization inhibition, LH708, LH1753*

Cystinuria is an autosomal recessive disorder in the transport of L-cystine and the dibasic amino acids in the renal proximal tubule.[1−7] The transporter is a heterodimer consisting of rBAT and $b^{0,+}$AT subunits. Cystinuria genotypes are classified as Type A or Type B. Type A is associated with mutations in *SLC3A1*, which encodes rBAT, while Type B is linked to mutations in *SLC7A9*, which encodes $b^{0,+}$AT. Despite the distinct genotypes, clinical symptoms within the A and B subgroups are similar. Interestingly, there is no apparent correlation between genotype and phenotype, suggesting the influence of modifying genes or environmental factors on the observed phenotype.

Globally, cystinuria has a prevalence of 1 in 7000, varying from 1 in 2500 in Israeli Jews of Libyan origin to 1 in 100,000 in Sweden.[2,3] In the US, the estimated prevalence population is 1 in 15,000. However, the actual prevalence could be underestimated, as certain individuals with the condition either do not produce stones or do so infrequently, posing challenges in diagnosis. Cystinuria, a lifelong disorder, is marked by the excessive excretion of L-cystine in the urine (>400 mg/day, compared to <30 mg/day in normal subjects) and the formation of cystine stones primarily in the kidneys, with a lesser occurrence in the bladder.[8,9] It constitutes 1−2% and 6−8% of urinary tract stones in adults and children, respectively.[10,11] The clinical consequence of cystinuria is the precipitation and aggregation of poorly soluble L-cystine crystals to form urinary tract stones. Approximately 25% of symptomatic patients form their first stone in the first decade of life, and another 30−40% form their first stone in teenage years.[11] Around 28% of male patients and 14% of female patients form calculi before the age of three.[12] While the incidence is equal between the sexes, males generally experience more severe effects.

Current clinical management of cystinuria includes high fluid intake to reduce the concentration of free L-cystine in urine or administration of citrate or bicarbonate to raise urinary pH to increase the solubility of L-cystine.[13,14] Tiopronin and D-penicillamine are the most common medications used for the treatment of cystinuria with the

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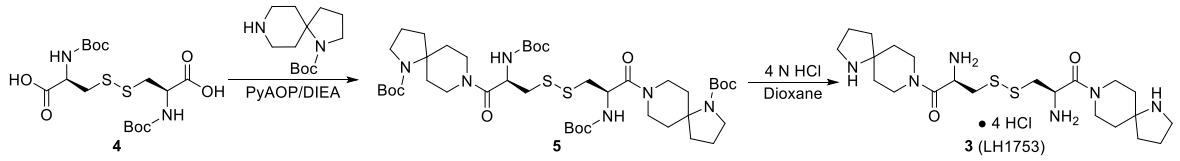
Scheme 1. Synthesis of 8-L-Cystinyl Bis(1,8-diazaspiro[4.5]decane) (3)



free thiol group undergoing a disulfide exchange with L-cystine to produce more soluble mixed disulfides.[13] However, these medications have been shown to have variable efficacy and frequent adverse events that impact patient compliance.[14,15] Therefore, there is an urgent need to develop new, safer, and more efficacious drugs for cystinuria with better patient compliance.

Leveraging molecular mimicry can be a valuable approach to identifying crystallization inhibitors. By crafting molecules that closely emulate the structure and preferred interactions between the crystallizing species, we can effectively frustrate and slow down the crystallization process. Inspired by the work of Ward and colleagues on CDME (**1**) as an L-cystine crystal growth inhibitor,[16,17] we discovered a series of L-cystine diamides, represented by L-cystine bis(*N*′-methylpiperazide) (LH708, **2**), that were more potent and more stable inhibitors of L-cystine crystallization.[18−20] Building upon the promising *in vivo* efficacy and good safety profile of **2** in a *Slc3a1*-knockout mouse model of cystinuria, we report herein the discovery of 8-L-cystinyl bis(1,8-diazaspiro[4.5]decane) (LH1753, **3**) as an orally bioavailable inhibitor of L-cystine crystallization for cystinuria. This novel candidate incorporates a bioisosteric spiro bicyclic diamine, 1,8-diazaspiro[4.5]decane, replacing the *N*-methylpiperazine terminal groups in LH708, significantly enhancing L-cystine crystallization inhibition *in vitro* and preventing stone formation more effectively in the *Slc3a1*-knockout mouse model.

CHEMICAL SYNTHESIS

Synthesis of LH1753, as shown in Scheme 1, is simple and straightforward, starting with amide bond coupling between N^{α},$N^{\alpha\prime}$-bis-Boc-L-cystine and 1-Boc-1,8-diazaspiro[4.5]decane using PyAOP and DIEA followed by Boc deprotection using 4 N HCl in dioxane with an overall yield of 73%.

CRYSTALLIZATION INHIBITION

For L-cystine crystallization inhibition assays, we employed our recently published optimized assay protocol and used multiwell plates to reliably produce apparent aqueous solubility data for the ranking of potential inhibitors.[21] Using this protocol, we were able to derive, for the most potent inhibitors, EC_{50} values as the concentration needed to inhibit L-cystine crystallization by 50% under our standardized assay conditions. As shown by the representative dose−response curves in Figure 1 and EC_{50} values listed in Table 1, LH708 (**2**) has an EC_{50} of 59.8 ± 7.2 nM and LH1753 (**3**) has an EC_{50} of 29.5 ± 8.6 nM, while CDME (**1**) has an EC_{50} of 3530 ± 360 nM. Thus, LH1753 is 120× more potent than CDME and 2× more potent than LH708 in inhibiting L-cystine crystallization.

CRYSTAL GROWTH INHIBITION USING ATOMIC FORCE MICROSCOPY (AFM)

AFM was used to inform on the mechanism of L-cystine crystallization inhibition, like we reported earlier for LH708.[18]

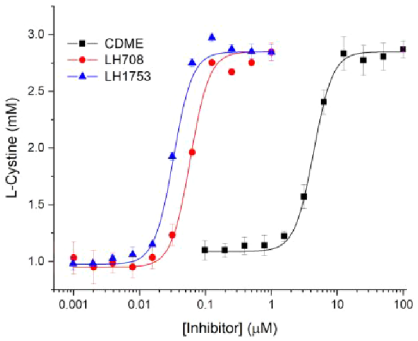


Figure 1. Representative dose−response curves for CDME (**1**), LH708 (**2**), and LH1753 (**3**) used for determination of EC_{50} values to rank inhibitor potency. A supersaturated solution of L-cystine (2.9 mM) was incubated in the presence of varying concentrations of test inhibitors for 72 h at room temperature followed by centrifugation and measurement of L-cystine concentration in the supernatant.[21]

Hexagonal L-cystine crystals were mounted on an AFM specimen disk, and real-time *in situ* AFM was performed with a Bruker Multimode AFM instrument using a Bruker MTFML-V2 fluid cell design. The mounted crystals were grown for 20 min prior to measurements in order to regenerate the crystal surface by continually flowing a supersaturated solution (2 mM L-cystine) through the fluid cell at a rate of 20 mL/h using a syringe pump. The {0001} basal plane crystal surfaces present well-defined hexagonal hillocks bounded by {1010} steps and containing a screw dislocation in the core. The {1010} step velocities, which contribute to the overall crystal growth of L-cystine, were measured in the absence of the inhibitor (V_0) and then in the presence of the inhibitor (V) at three concentrations (15, 30, and 45 μM). Figure 2 depicts an image acquired during growth on the L-cystine {0001} face in the absence of the inhibitor (A) and in the presence of 45 μM LH1753 (B). Close inspection of the image acquired in the presence of LH1753 (Figure 2B) reveals step roughening not observed in its absence (Figure 2A), signaling binding of the inhibitor to sites on the {1010} step edges.

The effects of CDME (**1**) and L-cystine diamides **2** and **3** on L-cystine step velocities are summarized in Table 1. The effect of additives is expressed by the quantity V/V_0, where V is the step velocity measured in the presence of additives and V_0 is the velocity measured initially in the absence of additives (a lower V/V_0 corresponds to greater inhibition). Like CDME and LH708, LH1753 slowed the advance of the {1010} steps, and V/V_0 decreased with increasing concentration of all three additives, as expected for the Cabrera−Vermilyea mechanism, in which adsorbed impurity particles block step propagation.[22,23] The range of V/V_0 values corresponds to measurements made on different L-cystine crystals in different 2 mM L-cystine solutions by several operators. The observation of a range of V/V_0 values reflects inherent uncertainty in the actual L-cystine concentrations when working with metastable supersaturated L-cystine solutions, as well as small uncertainties

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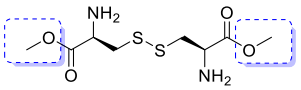
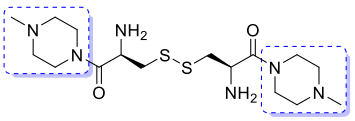
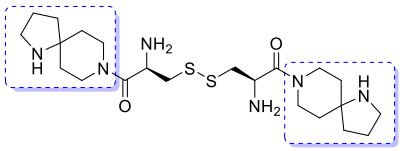
Table 1. L-Cystine Diamides LH708 (2) and LH1753 (3) as Inhibitors of L-Cystine Crystallization *in Vitro* and *in Vivo*

Compd # (ID)	Structure	Effect on L-cystine crystallization		Effect on AFM step velocity	*In vivo*	
		EC_{50} (nM)[a]	Ratio[b]	V/V_o[c] @15/30/45 μM	F%[d]	Efficacy[e] treated *vs* control
1 (CDME)		3530 ± 360	1	0.4-0.65/ 0.27-0.54/ 0.21-0.48	-	7/14 *vs* 6/11 (stones)[f]
2 (LH708)		59.8 ± 7.2	59	0.52-0.66/ 0.32-0.50/ 0.30-0.40	18% (2.3%+25%)	5/15 *vs* 8/16 (stones) 0/15 *vs* 2/16 (deaths)
3 (LH1753)		29.5 ± 8.6	120	0.62±0.01/ 0.49±0.04/ 0.40±0.01	22% (16%+27%)	2[g]/25 *vs* 28/51 (stones) 0/25 *vs* 8/51 (sac'ed[h])

[a]EC_{50} values measured in 2.9 mM supersaturated solution of L-cystine in Millipore water at room temperature for 72 h. [b]Ratio as an indication of relative potency in comparison to CDME (**1**). [c]The range of normalized step velocity (V/V_0) as obtained at three different inhibitor concentrations in aqueous 2 mM L-cystine. Data for CDME and LH708 were taken from ref 18. [d]Oral bioavailability derived after delivery of drug via gavage at 150 μmol/kg in saline as compared to tail vein injection at 30 μmol/kg; 4 *Slc3a1*-knockout male mice for each route. The percentages represent the combined oral bioavailability combining the parent and the major metabolite, while those in parentheses refer to the oral bioavailability for the parent and the major metabolite, respectively. [e]*Slc3a1*-knockout male mice were treated daily via gavage at 29 μmol/kg of CDME for 4 weeks, 150 μmol/kg of LH708 for 4 weeks, and 150 μmol/kg of LH1753 for 8 weeks. Data represents the number of mice out of the total mice treated with the drug vs water control. [f]From ref 17. [g]One of 25 mice had stones, while another mouse in the same group had a whitish streak observed indicating early stages of stone formation. [h]Mice were sacrificed early due to heavy stone burdens.

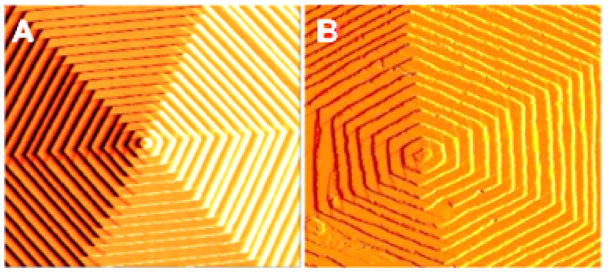


Figure 2. *In situ* AFM images of {0001} L-cystine crystal faces (2 mM L-cystine) growing in the absence of inhibitors (A) and in the presence of 0.045 mM LH1753 (B). All images were taken ~25 min after injection of the inhibitor. The area of all images is 6 μm × 6 μm.

in the very low inhibitor concentration. Nonetheless, the step roughening and dependence of step velocities on inhibitor concentration for the three inhibitors are consistent with a common mechanism. The V/V_0 values of LH708 and LH1753 are consistent with their similar EC_{50} values, but the CDME V/V_0 values suggest that CDME would be a better inhibitor than expected from its EC_{50} value. This argues that, while step growth inhibition on the {0001} face is a good indicator of inhibitor efficacy, the effect of these inhibitors on growth on other faces not accessible by AFM likely contributes to the response curves and the EC_{50} values.[24]

It should be noted that these AFM data are consistent with an increase in the apparent solubility, owing to kinetic factors. Kinetic inhibition results in a metastable supersaturated solution.[20] We have no evidence for a change in the thermodynamic factors related to a change in the chemical potential of the cystine solute that may result from complexation of the inhibitor with cystine, and we view it as unlikely given the submicromolar concentrations of inhibitor required, as measured by the EC_{50} values as well as the small concentrations for step inhibition.

ORAL BIOAVAILABILITY IN *SLC3A1*-KNOCKOUT MICE

To further evaluate the potential of LH1753 (**3**) as a therapeutic agent for the prevention of kidney stone formation in cystinuria, we measured its oral bioavailability in a *Slc3a1*-knockout mouse model of cystinuria. Because L-cystine diamides like **2** and **3** are known to undergo disulfide exchange to form mixed disulfides as a major metabolite (M1, Figure 3) *in vivo* and we have shown that the resulting mixed disulfides are also active with EC_{50} within about 2× of their parents,[25,26] we measured concentrations of both the

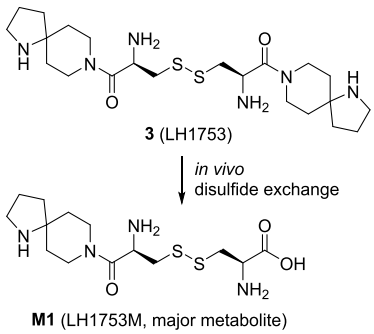


Figure 3. Major metabolic pathway of **3** (LH1753) leading to the formation of the mixed disulfide as the major metabolite (**M1**, LH1753M). M1 was chemically synthesized and used for all quantitation.

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Table 2. Pharmacokinetic Parameters Obtained for LH1753 in *Slc3a1*-Knockout Mice[a]

Compd	$t_{1/2}$ (h)	C_{max} (nmol/L)	AUC_{0-t} (nmol/L·h)	AUC_{0-t} (P + M1) (nmol/L·h)	Vz/F_obs [(μmol/kg)/(nmol/L)]	Cl/F_obs [(μmol/kg)/(nmol/L)/h]	%F (Parent)	%F (M1)	%F (P + M1)
LH1753	0.6	11381	18542	59505	0.0070	0.0080	16%		22%
LH1753M	2.4	16867	40963		0.0108	0.0032		27%	

[a]Mice were treated with a single oral dose of 150 μmol/kg or a single iv dose of 30 μmol/kg (4 mice in each group). After drug administration, approximately 60 μL of blood was collected from the saphenous vein using heparinized glass capillaries at various time points (0, 5, 15, and 30 min and 1, 4, 8, and 24 h). The blood was expelled into 0.2 mL tubes for plasma separation by centrifugation. Samples were stored at −80 °C prior to analysis by LC-MS/MS using a Sciex 6500 at Alliance Pharma. Both the parent (LH1753) and the major mixed disulfide metabolite (LH1753M) were quantitated.

parent and the active mixed disulfide metabolite when we determined the oral bioavailability of L-cystine diamides.

LH1753 (**3**) was delivered via oral gavage in a 100 μL volume in normal saline (150 μmol/kg) as well as via tail vein injection (30 μmol/kg) to four *Slc3a1*-knockout male mice for each route; blood samples were collected prior to drug administration as controls and at specific time points after drug administration. For each plasma sample, both the parent drug and the major active metabolite (M1 - mixed disulfide) were quantitated using LC-MS/MS. Table 2 summarizes the PK parameters obtained for LH1753 (**3**). As indicated, the oral bioavailability for the parent drug LH1753 is 16%, while the oral bioavailability for the major mixed disulfide metabolite LH1753M is 27% with an overall combined oral bioavailability of 22%. The AUC_{0-t} ranged from 11.4 to 16.9 μmol/L·h for the parent and major metabolite, respectively. Among the L-cystine diamides we evaluated, LH1753 (**3**) showed the most promising profile—most potent crystallization inhibition activity with EC_{50} of 29.5 nM and a good PK profile (oral bioavailability of 22%, an elimination half-life of 2.4 h for its major active M1 metabolite, and a combined AUC_{0-t} of 59.5 μmol/L·h).

URINARY CONCENTRATION IN *SLC3A1*-KNOCKOUT MICE

Oral bioavailability represents the amount of drug reaching general circulation, which is a crucial first step for an oral medication intended for kidney stone prevention. It is imperative for the drug or its active metabolite(s) to predominantly undergo renal excretion, thus ensuring a concentration in the urine that is adequate to deter the crystallization of L-cystine in patients with cystinuria. Our inhibitors have been specifically designed with this objective in mind. Consequently, we collected urine samples from the treated mice before and after a single oral dose of 150 μmol/kg or a single iv dose of 30 μmol/kg of LH1753. Because of the difficulty in collecting mouse urine samples at multiple time points, we were only able to collect mouse urine 24 and 48 h after administration. Even though we did not detect the parent drug after either oral or iv administration, we did find micromolar concentrations of the major active mixed disulfide metabolite, 2.1 ± 1.4 and 1.0 ± 0.4 μM, in the samples collected 24 h after the single oral dose of 150 μmol/kg or single iv dose of 30 μmol/kg of LH1753, respectively, suggesting that at least the major active metabolite is present at sufficiently high concentration after oral administration of LH1753.

IN VIVO EFFICACY IN A *SLC3A1*-KNOCKOUT MOUSE MODEL OF CYSTINURIA

We carried out a preliminary study of the efficacy of LH1753. Mice were generated by *Slc3a1* × *Slc3a1* mating and one pup from each mating assigned to the treatment group and one or more pups from the same litter to the control group. LH1753 was dissolved in water (18.9 mg/mL), and 100 μL was delivered by gavage daily to 6-week-old *Slc3a1* knockout male mice for 8 weeks. This is equivalent to a dose of 150 μmol/kg. Control mice were given water alone (100 μL). The average body weights in the treated and control groups were 20.3 ± 2.1 and 20.5 ± 1.2 g, respectively. Mice were sacrificed at the end of the treatment period and the kidneys and bladders weighed. Bladders were dissected, and any bladder stones were weighed and counted. It was found that only 1 out of 25 mice in the LH1753-treated group had stones and a second mouse in this group had a whitish streak observed without stones indicating early stages of stone formation (counted as two stone formers, 8%), while stones were observed in 28 of 51 mice in the water group (54.9%). Body weight increased in both groups over the study period (14.6% in the treated group and 13.0% in the water group), indicating that LH1753 has no adverse effect on growth.

In summary, a structure−activity relationship study of our previously reported L-cystine diamide, L-cystine bis(*N*′-methylpiperazide) (LH708, **2**), led to the discovery of a promising new inhibitor, 8-L-cystinyl bis(1,8-diazaspiro[4.5]-decane) (LH1753, **3**), demonstrating 1,8-diazaspiro[4.5]-decane as a bioisosteric replacement for *N*′-methylpiperazine terminal groups in LH708. LH1753 has been shown to be 120× more potent than CDME and 2× more potent than LH708 in the *in vitro* crystallization inhibition assay. AFM studies indicate that the inhibition of L-cystine crystallization is at least partially due to the inhibition of L-cystine crystal growth. LH1753 exhibited a good pharmacokinetic profile. Furthermore, LH1753 demonstrated good efficacy in the *Slc3a1*-knockout mouse model of cystinuria.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.4c00066.

Experimental details, ^{1}H and ^{13}C NMR spectra of major intermediate **5**, HRMS spectrum of major intermediate **5**, ^{1}H and ^{13}C NMR spectra of final target compound **3**, and HRMS spectrum of final target compound **3** (PDF)

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Author Contributions

L.H. conceived of the compound designs and experiments and drafted the manuscript. H.A. synthesized the compounds and performed the crystallization inhibition assay. Y.W. scaled up the synthesis and repeated the crystallization inhibition assays. M.Y. performed the animal studies under the direction of L.H. and A.S. X.Z. performed the AFM studies under the direction of M.D.W. J.Y. wrote an initial draft of the manuscript based on the dissertation by H.A. All authors discussed the experiments, commented on the manuscript, and approved of the final draft.

Notes

The authors declare the following competing financial interest(s): Some authors (L.H., A.S., and H.A.) are inventors of patents on compounds discussed in this paper.

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ABBREVIATIONS

AFM, atomic force microscopy; CDME, L-cystine dimethyl ester; DIEA, *N*,*N*-diisopropylethylamine; PK, pharmacokinetic; PyAOP, (7-azabenzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate

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