L-Cystine Diamides as L-Cystine Crystallization Inhibitors for Cystinuria

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SUPPORTING INFORMATION

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Supplemental Experimental Details

Fluorescence assay for the inhibition of L-cystine crystal formation

Formation of supersaturated solution. 21 mg of L-cystine was dissolved in 30 mL of Millipore water (~3 mM) under reflux at 100 °C for 20 min until the L-cystine was completely dissolved. The supersaturated solution was then allowed to cool slowly with stirring for 75 min.

Construction of standard curves. L-Cystine (5 mg) was dissolved in Millipore water (34.7 mL) to form a 0.6 mM solution as a stock solution. Then, L-cystine solution was diluted to 0.4, 0.3, 0.2, 0.1, 0.05 mM solution. 10 uL of each L-cystine solutions, 90 uL of 0.1 M dibasic sodium phosphate solution, and 10 uL of DTT solution (12.5 mM) were mixed at r.t. for 10 min, before the addition of 10 uL of iodoacetic acid (100 mM) and continued incubation at r.t. for an additional 15 min. This was then followed by the addition of 10 uL of OPA (100 mM in methanol) and 10 uL of NBC (100 mM in methanol). The derivatization was allowed to proceed for 3 min before 40 uL of the mixture was placed in a 384-well plate and read at Ex 355 nm/Em 460 nm. The standard curve was repeated for each set of experiments and used to calculate the concentration of L-cystine in each sample.

Determination of L-cystine concentration. All test compounds were dissolved in water to form 10 mM stock solution. Five uL of each solution was added to 500 uL L-cystine supersaturated solution. The mixtures were allowed to stand at 25 °C for 72 h. At the end of incubation, the mixtures were centrifuged at 10,000 rpm for 4 min and the supernatants were diluted 2-fold for concentration measurement. Each diluted mixture (10 uL), 0.1 M dibasic sodium phosphate solution (90 uL), and 10 uL of DTT solution (12.5 mM) were mixed at r.t. for 10 min, before the addition of 10 uL of iodoacetic acid (100 mM) and continued incubation at r.t. for an additional 15 min. Derivatization was performed by the addition of 10 uL of OPA (100 mM in methanol) and 10 uL of NBC (100 mM in methanol) for 3 min. 40 uL of the derivatized mixture was plated in a 384-well plate and fluorescence was read at Ex 355 nm/Em 460 nm to derive the concentrations of the original mixtures.

AFM crystal growth experiments

Preparation of L-cystine crystals. Hexagonal L-cystine plate crystals were formed by refluxing 70 mg of L-cystine in 100 mL of deionized water (3 mM) for 30 minutes at 100 °C while stirring. The solution was then cooled at room temperature while stirring for 70 minutes, split into two 50 mL aliquots in 100 mL beakers, and stored at room temperature for 72 hours. All crystals used for AFM crystal growth experiments were collected via vacuum filtration (Whatman Grade 4 filters, 20-25 μm pores) and air dried.

Hexagonal L-cystine plate crystals were formed using the same method described above. A 2 mM L-cystine solution for the fluid cell flow was prepared by refluxing 48 mg of L-cystine in 100 mL deionized water at 100 °C for 30 minutes. The solution was then cooled for 70 minutes and split into a 25 mL aliquot and a 75 mL aliquot. The 75 mL aliquot was used to inject into the fluid cell for L-cystine crystal growth measurements without inhibitor additives. The 25 mL aliquot was injected with **1a** or **1b** to produce 15, 30, or 45 μM inhibitor solutions. Inhibitor stock solutions were prepared by creating 2 mg/mL stock solutions in 5 mL vials. AFM samples were prepared using Norland optical adhesive (type 81) on an AFM specimen disc (15 mm diameter). The adhesive was first cured under UV radiation (λ = 365 nm) for 1 minute and then pressed against large L-cystine plate crystals that were isolated using a needlepoint and an optical microscope. The sample was then cured for 12 minutes and excess crystals were removed by lightly blowing air over the sample. All *in situ* AFM experiments were performed on a Veeco Multimode™ Scanning Probe Microscope using the "J" scanner (maximum scan size – 85-100 μm) in a fluid cell in contact mode. Solution was pushed into the cell at 10 mL/hr by a 24 mL syringe at automated rates using a Razel syringe pump. Bruker V shaped SiN AFM tips were used for measurements (model NP-10: radius of curvature = 20 nm, spring constant = 0.12 N/m). Step distances were measured using the program user interface and all step velocities and step spacing values were calculated as mean ± standard deviation.

Chemical stability measurement

Solutions of 100 μM test compounds in phosphate buffered saline were incubated at 37 °C. Aliquots (20 μL) were taken at different time intervals and frozen at -20 °C. The aliquots were diluted 5-fold with water to 100 μL prior to analysis with LC-MS/MS in positive MRM mode in the case of CDME (269.5→134.4) and **1a** (379.5→189.6) or acetylation in the case of **1b** with 1 mM Ac_2O at r.t for 30 min followed by analysis by LC-MS/MS in positive MRM mode (489.2→101.0) on an API 3000 (AB Sciex, Framingham, MA). Chromatographic separation was achieved with a 2x20 mm, 5 μm, C4 reversed phase column using a water/methanol gradient containing 0.1% formic acid (5-90%) at a flowrate of 250 μL/min. The standard curves were constructed with standards of CDME, **1a** and **1b**, respectively.

***In vivo* activity in mouse model of cystinuria**

Slc3a1 knockout mice in a mixed 129/C57BL6 background were used in the studies described here. We selected two-month old knockout male mice for treatment. Mice at this age exhibit crystalluria but very few or no bladder stones whereas approximately 50% of three-month-old mice exhibit stones, making this the ideal window to assess the effects of treatment. Most male mice over age six months have bladder stones and some have kidney stones as well. Female knockout mice have crystalluria but no stones until age over 12 months. Mouse genotypes and gender were determined by PCR amplification of tail DNA. Based on previous observations, Slc3a1 heterozygotes have no apparent phenotype so they were used in place of wild-type mice as needed. Animal studies were conducted in accordance with Rutgers University IACUC policies.

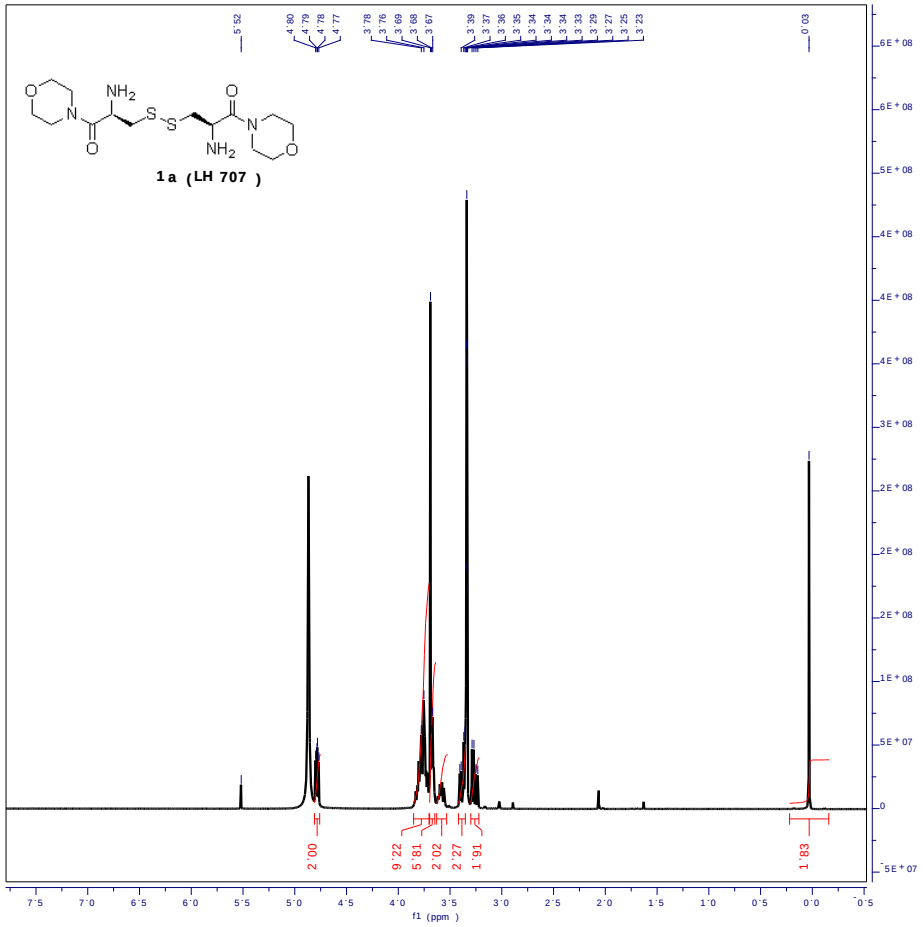
Test compound solutions were prepared fresh daily and 200 µL was administered at a dose of 29.3 µmol/Kg to *Slc3a1* knockout male mice (body weight 20 g) by gavage daily for four weeks using a 20G disposable flexible plastic feeding needle with a soft tip (Model FTP-20-30, Instech Laboratories, Inc., Plymouth Meeting, PA). The control group received daily 200 µL of water only. Urine samples (0.5 to 1 ml) were collected by placing the mice in metabolic cages for four hours or longer.

Measurement of compound concentration in urine

Levels of compounds in urine were quantified using LC-MS/MS in positive MRM mode (**1a**, 379.0→188.9; **1b**, 203.1→101.3) on a Transcend LX2 system (Thermo Fisher, Waltham, MA) coupled to an API 4000 (AB Sciex, Framingham, MA). Chromatographic separation was achieved with a 2.1x50 mm, 5 µm HILIC column (Waters Corporation, Huntingdon Valley, PA) using a water (A)/ACN (B) mobile phase system containing 0.1% formic acid (v/v). The gradient was performed at a total flow rate of 750 µL/min as follows, 95% B from 0 to 1.5 min, 95 to 5% B from 1.5 to 2.2 min, 5% B from 2.2 to 2.7 min, 5 to 95% B from 2.7 to 2.8 min, 95% B from 2.8 to 4.3 min.

Spectroscopic Data for New Compounds:

- ^{1}H NMR of L-Cystine bismorpholide (CDMOR, LH707, **1a**)



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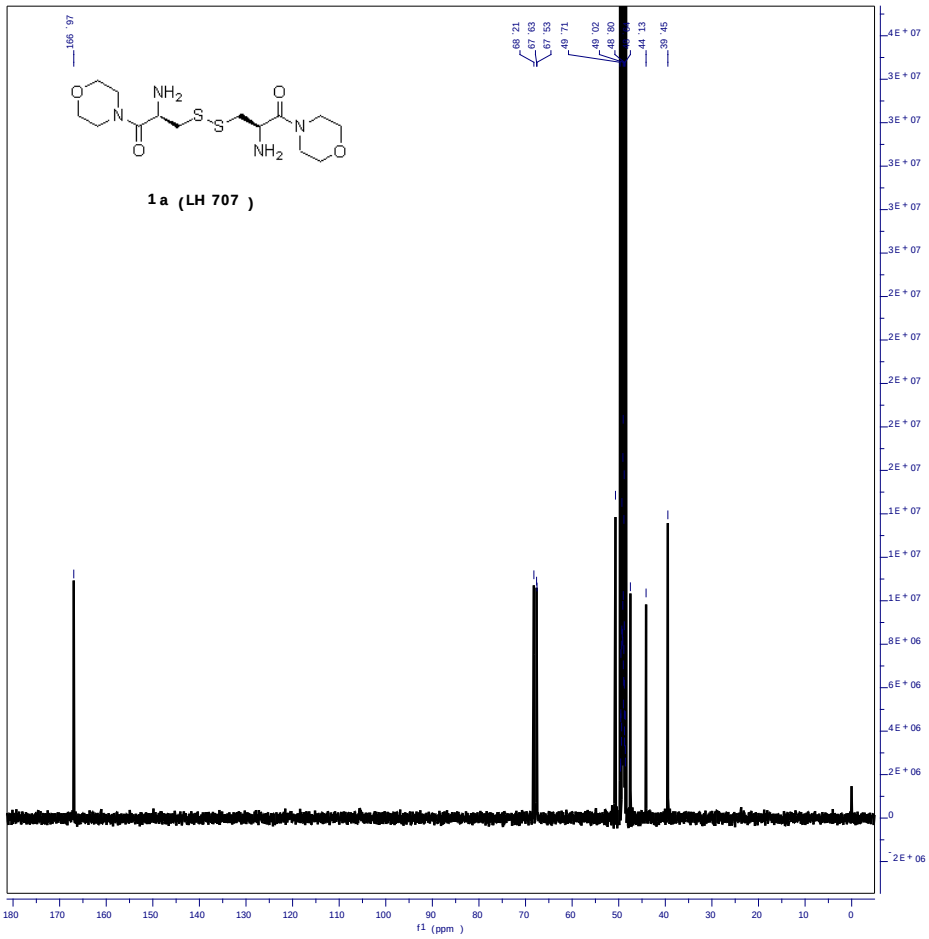
- ^{13}C NMR of L-Cystine bismorpholide (CDMOR, LH707, **1a**)



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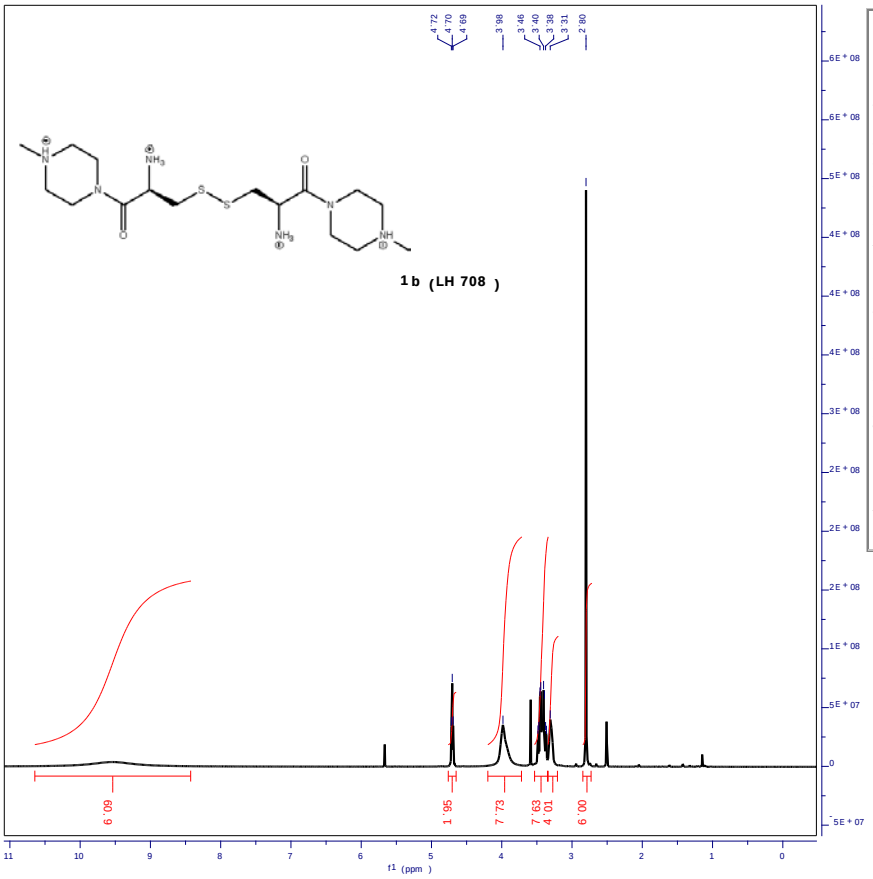
- ^{1}H NMR of L-cystine bis(N'-methylpiperazide) (CDNMP, LH708, **1b**)



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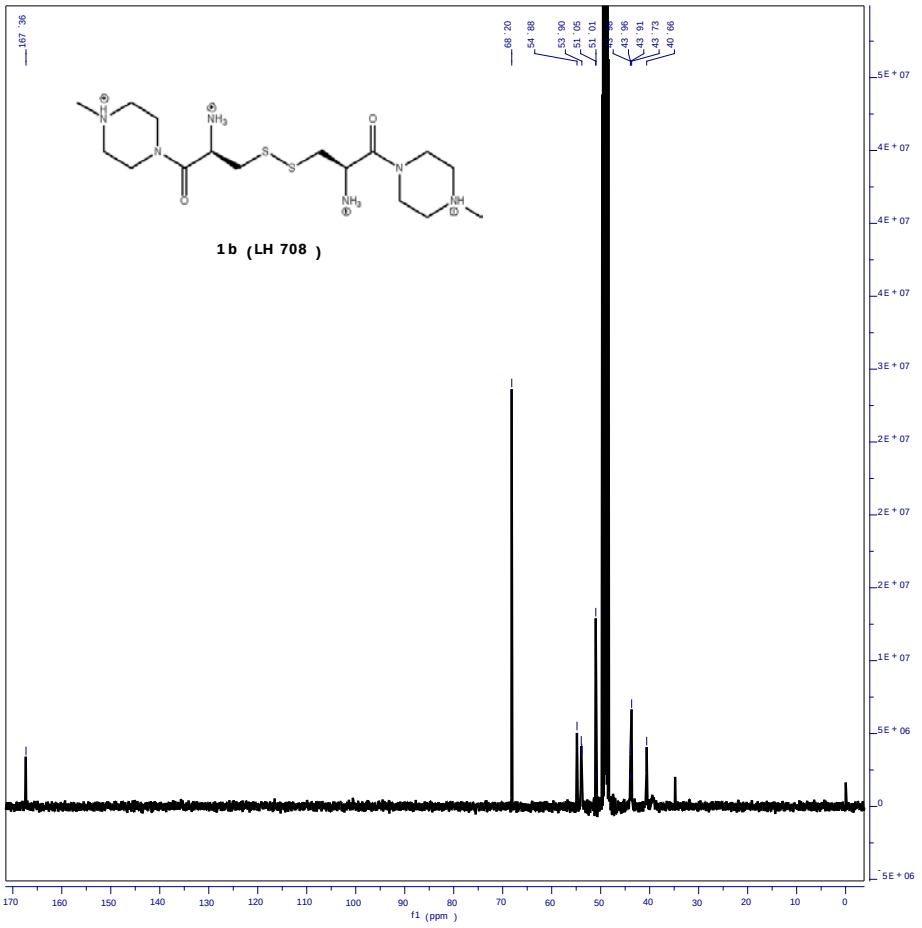
- ^{13}C NMR of L-cystine bis(N'-methylpiperazide) (CDNMP, LH708, **1b**)



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