Role of Molecular Recognition in L-Cystine Crystal Growth Inhibition

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Supporting Information

ABSTRACT: L-Cystine kidney stones—aggregates of single crystals of the hexagonal form of L-cystine—afflict more than 20,000 individuals in the United States alone. Current therapies are often ineffective and produce adverse side effects. Recognizing that the growth of L-cystine crystals is a critical step in stone pathogenesis, real-time in situ atomic force microscopy of growth on the (0001) face of L-cystine crystals and measurements of crystal growth anisotropy were performed in the presence of prospective inhibitors drawn from a 31-member library. The most effective molecular imposters for crystal growth inhibition were L-cystine mimics (aka molecular imposters), particularly L-cystine diesters and diamides, for which a kinetic analysis revealed a common inhibition mechanism consistent with Cabrera–Vermilyea step pinning. The amount of inhibitor incorporated by L-cystine crystals, estimated from kinetic data, suggests that imposter binding to the {0001} face is less probable than binding of L-cystine solute molecules, whereas imposter binding to {1010} faces is comparable to that of L-cystine molecules. These estimates were corroborated by computational binding energies. Collectively, these findings identify the key structural factors responsible for molecular recognition between molecular imposters and L-cystine crystal kink sites, and the inhibition of crystal growth. The observations are consistent with the reduction of L-cystine stone burden in mouse models by the more effective inhibitors, thereby articulating a strategy for stone prevention based on molecular design.

INTRODUCTION

Kidney stones comprising L-cystine account for nearly 2% of all stones, affecting more than 20,000 U.S. patients. Unlike the vast majority of calcium oxalate monohydrate (COM) stones, L-cystine stones, which are caused by a genetic disorder, tend to be larger, recur more frequently, and are more likely to cause chronic kidney disease.1 The formation of L-cystine stones is a consequence of excessive levels of L-cystine in the urine due to a defect in the reabsorption of L-cystine.2 This condition is exacerbated by the low solubility of L-cystine,3 which provokes the formation of crystals that aggregate into millimeter-sized stones. Current treatments for L-cystine stone prevention include dilution through high fluid intake,4 increasing urine pH through ingestion of alkalinizing potassium or sodium salts,4 and administering thiol-containing drugs such as D-penicillamine and α-mercaptopropionylglycine (Thiola). These drugs react with L-cystine to generate more soluble asymmetric disulfides, decreasing the concentration of L-cystine in urine,5 thereby suppressing—but not completely preventing—stone formation. Additionally, these drugs can have an unpleasant odor, can cause adverse side effects such as nausea, fever, fatigue, and skin allergies,4 and must be accompanied by high fluid intake to achieve an acceptable cystine excretion rate.4 These undesirable side effects prompted the exploration of an alternative approach for the treatment of L-cystine kidney stones based on crystal growth inhibition using molecular imposters.

Structural mimics of crystallizing solute molecules (aka tailored auxiliaries or molecular imposters) have been demonstrated to regulate crystallization through specific binding at crystal surfaces, driven by molecular recognition, providing an effective means to inhibit crystal growth and influence morphology.5−12 Effective molecular imposters preserve essential structural features of the crystallizing solute that enable binding to a crystal site, thereby perturbing solute attachment owing to the presence (or absence) of substituents that either block or eliminate key interactions of the solute with the crystal surface. Crystal growth typically is disrupted along specific crystallographic directions, often with associated changes in crystal morphology. For example, whereas S-
asparagine crystals exhibit a prismatic habit, a small amount of S-aspartic acid—which differs from S-asparagine by the replacement of the terminal amino group with a hydroxyl group—promotes the formation of {010} plates. This behavior has been attributed to stereoselective binding of S-aspartic acid to the {010} faces, which slows growth rates along the [010] directions. Adipic acid crystals grow more slowly along the [01] direction in the presence of n-alkanoic carboxylic acids, which substitute for adipic acid in the crystal lattice such that their alkyl tails protrude from the {010} face, thereby blocking association of adipic acid solute molecules with the crystal surface. The addition of amino acid auxiliaries has been reported to inhibit growth of β-glycine along its polar axis by stereospecific and enantioselective binding to the {010} faces at the crystal tips, transforming needle-shaped crystals to plates.

Using real-time in situ atomic force microscopy (AFM), our laboratory has demonstrated that L-cystine crystal growth is characterized by six interlaced spirals emanating from a single dislocation, each spiral corresponding to {101} steps of a highly dissymmetric layer of L-cystine molecules. In the presence of L-cystine dimethyl ester (L-CDME) and L-cystine methyl ester (L-CME), the step velocities on the {0001} face decrease significantly. This effect can be explained by the stereospecific binding of the internal backbone of these molecular imposters to the {101} steps, such that the terminal methyl groups block the approach of incoming solute molecules to adjacent crystal sites. The reduction of the step velocity, the presence of a “dead zone” (the range of supersaturations for which step velocity is zero), and an increase in step roughness were consistent with the Cabrera–Vermilyea step pinning mechanism for inhibition.

The AFM observations were mirrored by a reduction in the total mass of L-cystine crystals obtained from bulk crystallization and a thousand-fold reduction in the volume of crystals, accompanied by a change in crystal habit from large {0001} plates to small hexagonal {0001} rods. Notably, L-CDME reduced the size and occurrence of L-cystine stones in a knockout mouse model study.

Herein we describe an investigation using a library of L-cystine mimics and related compounds (Charts S1–S3, Table S1), with an aim toward design of additional clinically effective inhibitors. A combination of real-time AFM and optical microscopy reveals the critical structural features responsible for inhibition. Specifically, L-cystine inhibition is extraordinarily sensitive to the terminal substituents, the presence of internal disulfide groups, and commensurate match between the additive and the L-cystine crystal growth site. The most effective inhibitors were subject to a comprehensive kinetic analysis, with a particular focus on relating crystal morphology and growth kinetics to incorporation of molecular imposters. A recent study with a knockout mouse model demonstrated that L-CDME afforded a 50% reduction in overall stone mass and size, but esterase-catalyzed hydrolysis of L-CDME in vivo is not unlikely. This prompted a study of two imposters with improved hydrolytic stability—L-cystine bis(methyl piperazide) (L-CDMOR) and L-cystine bis(N′-methylpiperazide) (L-CDNMP)—known to sustain a higher supersaturation of L-cystine compared with L-CDME and suggesting more effective crystal growth inhibition. Curiously, the effect of L-CDME, L-CDMOR, and L-CDNMP on {1010} step velocities was comparable, prompting examination of growth inhibition on the {1010} faces as well as {0001}. Molecular imposter incorporation into the corresponding growth sectors and molecular modeling of additive binding to kink sites provided further insight into the inhibition mechanism. Collectively, the data suggest that the most potent imposters bind to a single site on the {1010} surface and inhibit crystal growth through the same mechanism. These observations also suggest that therapies for L-cystine stones will likely be limited to a narrow set of inhibitors due to highly constrained stereospecific binding at the active crystal growth sites.

### EXPERIMENTAL SECTION

#### Materials

L-Cystine (99%), L-cystine dimethyl ester dihydrochloride (≥95%), L-cystine dibenzyl ester ditosylate, L-cysteine methyl ester hydrochloride (98%), L-cysteine (97%), L-homocystine (≥98%), triethylamine (≥99%), L-NN′-diboc-L-cystine (L-Cys-Boc), S-methyl-L-cysteine, dithert-butyl dicarbonate (≥99%), hydrochloric acid (37%), sodium hydroxide (reagent grade), methanol (reagent grade), ethanol (reagent grade), isopropanol (reagent grade), tert-butanol (reagent grade), hexane (reagent grade), dichloromethane (reagent grade), ethyl acetate (reagent grade), N,N′-dicyclohexylcarbodiimide (DCC) (≥99%), and Grubbs’s second generation catalyst were obtained from Sigma-Aldrich (MO, USA) and used without further purification. Deuterated chloroform (≥99.8 atom % D) and deuterated water (D2O; 99.99 atom % D) were obtained from Cambridge Isotope Laboratories (MA, USA). Hydrochloric acid in ethyl acetate and allylglycine hydrogen chloride salt were obtained from TCI (OR, USA) and used without purification. Aqueous solutions were prepared using deionized water (18.2 MΩ) purified with a Direct-Q, 3 Millipore purification system.

#### Preparation of Hexagonal L-cystine Crystals

All crystallization experiments were performed in deionized water at pH ≈ 7, in which L-cystine crystallizes as the hexagonal polymorph. The hexagonal form was crystallized from a supersaturated L-cystine solution prepared by adding 70 mg of L-cystine to 100 mL of deionized water (κ = 3 mM) and heating under reflux at 100 °C for 30 min with stirring to completely dissolve L-cystine. The resulting solution corresponds to a relative supersaturation (σ = c/cΩ = 1) of ∼4.3, based on the reported solubility (cΩ = 0.7 mM at pH 7, 25 °C). The solution was then allowed to cool slowly with stirring for 75 min, after which 30 mL aliquots were transferred to separate glass containers, which were then sealed (to prevent evaporation and exposure to airborne particulates) and stored for 72 h at room temperature without stirring. Single crystals were collected by vacuum filtration (Whatman grade 1 filters, >11 μm pores) and air-dried prior to AFM experiments. The {0001} face of the hexagonal plates rested on the filter surface.

Bulk crystallization was performed according to the same procedure used for preparation of crystals for AFM measurements. L-Cystine crystals were typically grown from aqueous solutions (100 mL) containing 3 mM, but a limited number were grown from 2 and 2.5 mM solutions. The crystallization times ranged from 2 h to 7 days, but typically crystallization was performed for 6 days. The crystals were retrieved by vacuum filtration, weighed to determine the yield, and examined with a polarized light optical microscope to identify the polymorph and measure the aspect ratio.

#### In Situ Atomic Force Microscopy

Real-time in situ atomic force microscopy (AFM) was performed in a cell designed to contain liquids with a Digital Instruments (Santa Barbara, CA) Nanoscope IIIa Multimode system by acquisition of sequential images of growing crystals. In Situ Atomic Force Microscopy (AFM) was performed in a cell designed to contain liquids with a Digital Instruments (Santa Barbara, CA) Nanoscope IIIa Multimode system by acquisition of sequential images of growing crystals. In Situ Atomic Force Microscopy (AFM) was performed in a cell designed to contain liquids with a Digital Instruments (Santa Barbara, CA) Nanoscope IIIa Multimode system by acquisition of sequential images of growing crystals.

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The equilibrium morphology was calculated for L-cystine using either imposters were determined once they achieved a steady state after the same area of the crystal. Step velocities in the presence of additives were preceded by measurements in their absence to obtain a baseline and verify native growth rates (i.e., without additive). Advancing along a given direction. Step velocities in the presence of additives were calculated from the spatial coordinates of the intersection of a step edge, and the crystallographic direction along which the step velocity was measured in consecutive deflection images of the same scan direction \((x_1, y_1)\) and \((x_2, y_2)\), corresponding to a periodic interval of \(-21\) s (images acquired at a rate of 24.4 Hz at 256 samples/line). Step velocities were calculated using pixel arithmetic, similar to a previously reported approach.\(^3\)

The standard deviation was calculated based on the average of three separate measurements of step velocities for five different steps advancing along a given direction. Step velocities in the presence of additives were preceded by measurements in their absence to obtain a baseline and verify native growth rates (i.e., without additive). Subsequent images in the presence of an additive were obtained on the same area of the crystal. Step velocities in the presence of imposters were determined once they achieved a steady state after the introduction of the imposter to the AFM cell.

Scanning Electron Microscopy. Air-dried L-cystine crystals were lightly pressed onto a double-sided carbon tape to mount on the aluminum stubs for scanning electron microscopy (SEM) and coated with 4 nm of iridium. The images were acquired with a Zeiss Merlin field emission scanning electron microscope using a secondary electron detector and operating at a voltage of 1.5 kV and probe current <120 pA.

Computations. Computational results were obtained using software programs from Dassault Systems Biovia Corp. Geometry optimizations, and single point energy calculations were performed using the Forcite molecular mechanics tool in Materials Studio v. 8.0.\(^{19}\) The Smart optimization algorithm was used for all optimizations and convergence criteria for optimizations and energy calculations were set to better than \(1.0 \times 10^{-4}\) kcal/mol for the energy and \(5 \times 10^{-7}\) kcal/mol Å for the forces. The energy of the hexagonal L-cystine unit cell was minimized using the COMPASS,\(^{20}\) pcf, and cwf\(^{21}\) force fields with force field-assigned partial charges and nonbonding energies calculated using the Ewald summation method.\(^{22}\) Each force field optimized the unit cell dimensions to within 5% of their original values, indicating that all three force fields were appropriate for this structure.\(^{23}\) The cwf force field within a dielectric constant of 80 was chosen for subsequent geometry optimizations.

Surface Energies. Surface energies for the \(0001\) and \(10\overline{1}0\) surfaces were obtained from morphology predictions based on the equilibrium morphology prediction method and implemented using the Morphology Module in Materials Studio. This module determines the equilibrium morphology of a crystal from the surface energies of different surfaces and represents the morphology using a Wulff plot. The equilibrium morphology was calculated for L-cystine using either the cwf force field or the COMPASS force field with a dielectric constant of 80 in both cases. Both methods allow calculation of the \(0001\) and \(10\overline{1}0\) surface energies and the distribution coefficient, \(K_d\) (eq 11), which describes the amount of inhibitor incorporated into L-cystine crystals during growth.

Molecular Volumes. The volume of the molecular imposters was calculated using the Materials Studio Atom Volumes & Surfaces tool, which calculates solvent volumes, selecting a van der Waals scale factor of 1.0 Å, an initial solvent radius of 1.4 Å (corresponding to water), and a maximum solvent radius of 2.0 Å. The geometry of each molecular imposter was optimized prior to calculation of the solvent volume. The volume of each imposter substituent was calculated from the difference in the molecular volumes of the imposter and L-cystine.

Adsorption to Flat L-Cystine Surfaces. Adsorption simulations were conducted using the Adsorption Locator module to perform simulated annealing calculations to identify the lowest energy adsorption configuration for the adsorption of L-cystine, L-CDME, L-HCME, L-CDPE, L-CDMOR, and L-CDNMP to flat \(0001\) and \(10\overline{1}0\) L-cystine faces. The simulated annealing calculation consists of Monte Carlo searches of the substrate–adsorbate as the temperature is slowly decreased. This calculation was performed for each substrate–adsorbate combination (L-cystine flat surfaces as the substrates and molecular imposters as the adsorbates) with 100,000 loading steps, 3 heating cycles with 50,000 steps per cycle, and automated temperature control. A geometry optimization of the adsorbate was performed following each heating cycle. A flat \(0001\) L-cystine surface was built using the Surface Builder function to cleave the L-cystine hexagonal unit cell along \(0001\) to a fractional depth of \(1.75\) (10 L-cystine molecules; a thickness of 96.468 Å and dimensions of \(5.42875 \times 5.42875 \times 5.42875 \) Å). A supercell with dimensions of \(6a \times 1v\) (32.5725 Å × 32.5725 Å, or \(6 \times 6\) molecules) was generated, a vacuum slab of 100 Å was inserted above the surface, and three-dimensional (3D) boundary conditions were applied to simulate an infinite surface. A flat \(10\overline{1}0\) L-cystine surface was built using the Surface Builder function to cleave the L-cystine hexagonal unit cell along \(10\overline{1}0\) to a fractional depth of \(20\) (20 L-cystine molecules; a thickness of 94.029 Å and dimensions of \(5.42875 \times 5.521243 \) Å). A supercell with dimensions of \(6a \times 1v\) (32.5725 Å × 55.1243 Å, or \(6 \times 6\) molecules) was generated, a vacuum slab of 100 Å was inserted above the surface, and 3D boundary conditions were applied to simulate an infinite surface. Following the simulated annealing calculation, the lowest energy configuration was selected, and a geometry optimization was performed with only the adsorbate allowed to relax, with all other molecules comprising the L-cystine surface constrained. All molecules were then unconstrained, and single-point energy calculations were performed \((E_{\text{surface+adsorbate}})\). The adsorbate molecule was manually deleted, and single-point energy calculations were performed \((E_{\text{surface}})\). Isolated adsorbate molecules were optimized \((E_{\text{adsorbate}})\).

Adsorption energies of adsorbate molecules to flat \(0001\) and \(10\overline{1}0\) L-cystine surfaces were calculated by eq 1.

\[
E_{\text{adsorption}} = E_{\text{surface+adsorbate}} - (E_{\text{surface}} + E_{\text{adsorbate}}) \tag{1}
\]

Kink Binding Energies. Flat \(0001\) L-cystine surfaces were built using the Surface Builder function to cleave the L-cystine hexagonal unit cell along \(0001\) to a fractional depth of \(2.167\) (13 L-cystine molecules; a thickness of 119.436 Å and dimensions of \(5.42875 \times 5.42875 \) Å). Kinked surfaces for steps oriented along \(10\overline{1}0\) were generated by building supercells with dimensions of \(8u \times 8v\) (43.4300 Å × 43.4300 Å, or \(8 \times 8\) molecules), followed by manual deletion of molecules to generate the unique kink site at each of the six unique \(10\overline{1}0\) step edges—the “clockwise” and “counterclockwise” kink sites—a total of 12 kink sites on the \(0001\) face. Flat \(10\overline{1}0\) L-cystine surfaces were built using the Surface Builder function to cleave the L-cystine hexagonal unit cell along \(10\overline{1}0\) to a fractional depth of \(12\) (12 L-cystine molecules; a thickness of 56.417 Å and dimensions of \(5.42875 \times 55.1243 \) Å). Kinked surfaces for steps oriented perpendicular to \(0001\) and \(10\overline{1}0\) were generated by building supercells with dimensions of \(12u \times 2v\) (65.1449 Å × 110.249 Å, or \(12 \times 12\) molecules), followed by manual deletion of molecules to generate the 12 unique kink sites along each of the \(0001\) and \(10\overline{1}0\) step edges for a total of 24 kink sites on the \(10\overline{1}0\) face. A vacuum slab of 100 Å was inserted above each kinked surface, and 3D boundary conditions were applied to simulate an infinite surface. The thickness of the vacuum slab was chosen to be greater than the cutoff distance of the force field to prevent surface molecules from interacting with the image of the bottom surface of the crystal.

The binding energy of an L-cystine molecule to a particular kink site was calculated subsequent to geometry optimizations of the molecule at the kink site, in which only the L-cystine molecule at the kink site was allowed to relax with all other molecules constrained. All molecules were then unconstrained, and single-point energy calculations were performed \((E_{\text{kink+L-cystine}})\). The L-cystine molecules at kink sites were manually deleted, and single-point energy...
calculations were performed \((E_{\text{L-cystine}})\). An isolated l-cystine molecule was optimized \((E_{\text{l-cystine}})\). Binding energies of l-cystine molecules to kinks on step edges perpendicular to the \([1010]\) growth directions on the \([0001]\) face and the \([0001]\) and \([1120]\) growth directions on the \([1010]\) face were calculated by eq 2.

\[
E_k = E_{\text{kink} + \text{l-cystine}} - (E_{\text{kink}} + E_{\text{l-cystine}})
\]

(2)

To calculate the binding energies of additive molecules to kink sites, the kinked surfaces with l-cystine at the kink site were modified by adding substituents to the hydroxyl moiety of l-cystine or deleting part of the l-cystine molecule. This was followed by optimization of the surface with only the additive molecule at the kink site allowed to relax. All molecules were unconstrained and single-point energy calculations were performed \((E_{\text{additive}})\). The additive molecules at the kink sites were manually deleted, and single-point energy calculations were performed \((E_{\text{kink}})\). Isolated additive molecules were optimized \((E_{\text{additive}})\). Binding energies of additive molecules to kinks on step edges perpendicular to the \([1010]\) growth directions on the \([0001]\) face and the \([0001]\) and \([1120]\) growth directions on the \([1010]\) face were calculated by eq 3.

\[
E_k = E_{\text{kink} + \text{additive}} - (E_{\text{kink}} + E_{\text{additive}})
\]

(3)

## RESULTS AND DISCUSSION

### General Features of L-Cystine Growth

l-Cystine crystals exhibit two polymorphic forms, hexagonal\(^{33}\) and tetragonal.\(^{34}\) l-Cystine stones are aggregates of thin hexagonal plates with large \{0001\} basal surfaces, as large as 400 \(\mu\)m wide, bound by six equivalent \{1010\} faces. The typical thickness of these crystals ranges from 10–30 \(\mu\)m. The crystal structure of the hexagonal polymorph (space group \(P6_22\), \(a = 0.5422\) nm; \(c = 5.6275\) nm) reveals l-cystine molecules organized as a helix about the \(6_1\) screw axis such that six cystine molecules span the \(\sim 5.6\) nm unit cell length along the \(c\)-axis. The l-cystine molecules exhibit intermolecular \(\text{NH}_3^+\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\))}\) hydrogen bonding along the \(6_1\) screw axis (Figure 1C, I), intermolecular \(\text{S}\cdots\cdot\cdot\cdot\text{S}\) interactions in the \{1010\} planes (Figure 1C, II), \(\text{NH}_3^+\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\))}\) hydrogen bonding and \(\text{CH}_2\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\))}\) (Figure 1D, III–VII) between adjacent helices in the \(ab\) \(\{0001\}\) plane. The \(\text{S}\cdots\cdot\cdot\cdot\text{S}\) interactions on each \{1010\} face occur at intervals of \(c/2\). The tetragonal polymorph (space group \(P4_1, a = 0.6710\) nm; \(c = 2.173\) nm), which is generally regarded as the less preferred form and is not observed in vivo, can be crystallized from a physiological pH (6 \(\leq\) pH \(\leq 8\)).\(^{35}\) Acidification of basic l-cystine solutions to neutral pH,\(^{36}\) or gradual cooling of solutions supersaturated with l-cystine,\(^{37}\) at neutral pH, l-cystine crystallizes as hexagonal plates with large \{0001\} basal surfaces, as large as 400 \(\mu\)m wide, bound by six equivalent \{1010\} faces. The typical thickness of these crystals ranges from 10–30 \(\mu\)m. The crystal structure of the hexagonal polymorph (space group \(P6_22\), \(a = 0.5422\) nm; \(c = 5.6275\) nm) reveals l-cystine molecules organized as a helix about the \(6_1\) screw axis such that six cystine molecules span the \(\sim 5.6\) nm unit cell length along the \(c\)-axis. The l-cystine molecules exhibit intermolecular \(\text{NH}_3^+\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\))}\) hydrogen bonding along the \(6_1\) screw axis (Figure 1C, I), intermolecular \(\text{S}\cdots\cdot\cdot\cdot\text{S}\) interactions in the \{1010\} planes (Figure 1C, II), \(\text{NH}_3^+\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\))}\) hydrogen bonding and \(\text{CH}_2\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\))}\) (Figure 1D, III–VII) between adjacent helices in the \(ab\) \{0001\} plane. The \(\text{S}\cdots\cdot\cdot\cdot\text{S}\) interactions on each \{1010\} face occur at intervals of \(c/2\). The tetragonal polymorph (space group \(P4_1, a = 0.6710\) nm; \(c = 2.173\) nm), which is generally regarded as the less preferred form and is not observed in vivo, can be crystallized from a slowly cooled supersaturated ammonium hydroxide solution, or from aqueous solutions containing effective inhibitors of the hexagonal phase (vide infra).

Crystal growth near equilibrium is commonly described by the terrace-ledge-kink model,\(^{38}–^{41}\) in which faces are atomically flat and attachment of molecules to the surface occurs at kink sites on step edges. New steps can be generated either by screw dislocations\(^{39}\) or by 2D nucleation.\(^{40}\) Crystal growth can be visualized directly by real-time in situ AFM,\(^{15,44}\) enabling direct and quantitative measurements of step velocities, step roughness, and kinetic coefficients along different crystallographic directions, whether in the presence of pure solute or solutions containing additives, including growth inhibitors. In this respect, our laboratory reported that in situ AFM performed on the \{0001\} face of hexagonal l-cystine crystals in the presence of supersaturated l-cystine solutions (0.6 mM < \(c < 3.5\) mM) revealed spiral hillocks resembling a pinwheel emanating from screw dislocations.\(^{16,18}\) Consecutive AFM images during l-cystine crystal growth revealed a clockwise rotation of the pinwheel at the dislocation core (a left-handed screw) accompanied by continuous generation of new step edges (Figure 1B). Under these conditions the \{0001\} surface displayed hexagonal growth hillocks that resembled stacks of islands. Each island was approximately 5.6 nm high, corresponding to the \(c\) unit cell length (\(c = 5.6275\) nm). The hexagonal space group \(P6_22\), affords six equivalent molecular layers within the \(c\) unit cell length (labeled C1–C6 in Figure 1A).

![Figure 1](https://www.herrninglab.com/)

**Figure 1.** (A) Scanning electron microscopy image of an L-cystine stone consisting of aggregated hexagonal crystals (from Herring Laboratory, [http://www.herringlab.com](http://www.herringlab.com)). (B) Atomic force microscopy image of spiral hillocks emanating from a single dislocation. (C) The crystal structure of hexagonal l-cystine, illustrating adjacent helices of l-cystine molecules as viewed perpendicular to one of the \{1010\} planes. The helices wind about a \(6_1\) screw axis, which coincides with the \(c\) axis. The l-cystine molecules are labeled C1–C6 along the helix. Intermolecular amine-carboxylate hydrogen bonds exist along the helix (I, \(d_{\text{NH}_3^+\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\))}} = 1.89\) Å). Intermolecular \(\text{S}\cdots\cdot\cdot\cdot\text{S}\) interactions between the adjacent helices (II, \(d_{\text{S}\cdots\cdot\cdot\cdot\text{S}} = 3.47\) Å) are observed at C2 and C5. Identical \(\text{S}\cdots\cdot\cdot\cdot\text{S}\) interactions occur at symmetry-related sites on the other five \{1010\} planes. (D) Intermolecular amine-carboxylate hydrogen bonds in the \{0001\} plane are denoted by the dotted lines (III \(d_{\text{CH}_2\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\))}}\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\)) = 2.48\) Å}, IV, \(d_{\text{CH}_2\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\)) = 2.44\) Å}, V, \(d_{\text{CH}_2\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\)) = 1.80\) Å}, VI, \(d_{\text{CH}_2\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\)) = 1.91\) Å}, VII, \(d_{\text{CH}_2\cdots\cdot\cdot\cdot\text{O(\(\text{C} = \text{O}\)) = 2.64\) Å}). Atom color code: carbon (gray), oxygen (red), nitrogen (blue), sulfur (yellow), hydrogen (white). (E) Schematic illustration of hexagonal l-cystine, denoting the Miller indices.
(1C), each related to the next by a 60° rotation and an elevation of c/6 in accordance with the screw axis. The screw axis inherent to the space group symmetry is evidenced by a pinwheel of six equivalent minor steps on the hillock terraces, successively rotated clockwise around the c axis by 60°, which spin out from each island, intersecting the edges of the island below. This surface micromorphology is a consequence of six interlacing spirals corresponding to individual molecular layers related by the screw axis. The measured height of these minor steps is approximately 1 nm, equivalent to c/6, the thickness of one of the six symmetry-related molecular layers in the crystal structure and equivalent to the length of one L-cystine molecule. The magnitude of the Burgers vector normal to the {0001} face is c = 5.6275 nm,33 and the observation of the c/6 steps is tantamount to step splitting.16,45 The taller 5.6 nm steps form as a consequence of bunching of the minor steps along each direction, which in turn is a consequence of the inherent anisotropy in each molecular layer that affords different minor step velocities along the six directions of a molecular layer. One of the steps advances more slowly than the other five, retarding their advancement and resulting in the formation of step bunches flanking the perimeter of each hillock.

The velocity of the {1010} step bunches in the measurements described below was determined by measuring the location of the step in consecutive frames during growth in solutions supersaturated with L-cystine (see Materials and Methods). These measurements were restricted to hillocks surrounding a single dislocation center sufficiently remote from other hillocks to minimize interference. The L-cystine concentration was adjusted to 2 mM in order to achieve growth rates that could be measured readily from consecutive images acquired at 10 second intervals. The average {1010} step bunch velocity was V0 = 11.4 ± 1.5 nm/s (ca. ~50 molecules/nm²·s) and was equivalent along all six {1010} directions, as expected for the hexagonal symmetry.

### L-Cystine Crystal Growth Inhibition by the Molecular Imposters

In situ AFM performed on the {0001} face of hexagonal L-cystine crystals in the presence of the additive L-cystine dimethylester (L-CDME, 2-L in Table 1) revealed a...
reduction in the velocities of \{10\overline{1}0\} steps on \{0001\} L-cystine faces compared with growth at the same supersaturation in the absence of the additive.\textsuperscript{17,18} The AFM observations were mirrored by reduced crystal yield and crystal size.\textsuperscript{18} Kinetic analysis of \{10\overline{1}0\} step velocities over a range of L-cystine concentrations revealed that the inhibition by L-CDME was consistent with the Cabrera–Vermilyea step pinning mechanism,\textsuperscript{19,20} in which adsorbed impurity particles block step propagation. Analysis of the anomalous birefringence in the \{10\overline{1}0\} growth sectors of L-cystine crystals grown in the presence of L-CDME, combined with computational modeling, supported a high fidelity of stereospecific binding of CDME, in a unique orientation, exclusively at one of the six crystallographically unique projections of L-cystine on the \{10\overline{1}0\} plane.\textsuperscript{17} These results demonstrated that L-CDME served as an ideal example of a tailored growth inhibitor, or “molecular imposter”, as it reduces crystallization rates through stereoselective binding at a specific crystallographically unique step site.

Tailored additives consist of a binder moiety that emulates a critical structural element of the solute that attaches to a specific crystal site and a perturbing moiety that obstructs the approach of additional solute molecules to neighboring sites, pinning step motion. L-CDME contains the core backbone of the L-cystine molecule, whereas the methyl substituents provide the perturbing moieties. Several functional groups on the L-cystine molecule with identifiable intermolecular interactions can be modified as well; however, the effect of position, size, or chemical character of perturber moieties on the inhibition mechanism has not yet been explored comprehensively. This challenge was addressed by characterizing the inhibition of L-cystine crystal growth by a library of 31 prospective L-cystine molecular imposters (Charts S1–S3), which can be divided into four groups: (i) replacement of the terminal hydroxyl group; (ii) modification of the L-cystine backbone core; (iii) L-cysteine mimics; (iv) common cystinuria therapeutic compounds, aimed at addressing whether the therapeutic action of currently prescribed compounds is related to crystal growth inhibition.

The effect of different L-cystine molecular imposters on \{10\overline{1}0\} step velocities was determined using real-time in situ AFM in the presence of these additives during growth in supersaturated solutions of L-cystine. The step velocities in the presence of additives were normalized to the step velocity $V_0$.

### Table 4. Normalized Step Velocities ($V/V_0$) for Cystinuria Therapeutic Compounds or other FDA-Approved Drugs\textsuperscript{a}

| Compound   | No. | Chemical Structure | $V/V_0$ |
|------------|-----|--------------------|---------|
| NAC        | 25  | ![NAC Structure](image) | 1.02    |
| NACME      | 26  | ![NACME Structure](image) | 0.95    |
| L-pen      | 27-L| ![L-pen Structure](image) | 0.85    |
| D-pen      | 27-D| ![D-pen Structure](image) | 0.81    |
| DiD-pen\textsuperscript{a} | 28  | ![DiD-pen Structure](image) | 0.90    |
| DiNAC\textsuperscript{a} | 29  | ![DiNAC Structure](image) | 0.74    |
| Thiola     | 30  | ![Thiola Structure](image) | 0.79    |

\textsuperscript{a}Structural modifications of L-cystine indicated in red. $c = 2.0$ mM; $c_\text{d} = 0.030$ mM except those marked with \*, in which case $c_\text{ad} = 0.015$ mM.

### Table 5. Definitions of Concentration Symbols

| symbol | definition |
|--------|------------|
| $c$    | L-cystine concentration in solution |
| $c_\text{eq}$ | L-cystine equilibrium concentration in solution (thermodynamic solubility) |
| $c_\text{td}$ | minimum L-cystine concentration required for nonzero step velocity (dead zone width) |
| $c_\beta$ | L-cystine concentration at which the step velocity increased linearly with a slope similar to that in the absence of additives |
| $c_\text{ad}$ | concentration of the additive in solution |
| $c_\text{ad(tetr)}$ | concentration of the additive, above which only the tetragonal polymorph crystallizes |

Figure 2. (A) Step velocities, $V$, measured for \{10\overline{1}0\} steps on \{0001\} L-cystine crystal surface with no additive and with 0.056 mM L-CDME, L-HCME, L-CDPE, L-CDMOR, and L-CDNMP. The absence of growth at concentrations $c_\text{eq} < c < c_\text{td}$ is the dead zone. Step velocities increase slowly initially as $c$ is increased, eventually exhibiting an abrupt change at concentration $c_\beta$, at which step velocities increase quickly and linearly with increasing $c$. The slope is proportional to the kinetic coefficient, $\beta$, and is nearly identical for L-CDME, L-HCME, L-CDPE, L-CDMOR, and L-CDNMP. Lines are provided as a guide to the eye. (B) Step velocity, $V$, measured for \{10\overline{1}0\} steps on the \{0001\} L-cystine crystal surface as a function of L-CDME, L-CDMOR, and L-CDNMP concentrations for L-cystine concentrations $c = 2$ mM (lower data) and 3 mM (upper data).
without inhibitor, that is, $V/V_0$, where $V$ and $V_0$ are the step bunch velocities, which equate to the step velocity of the slowest advancing minor step in the presence and absence of the additive, respectively. Smaller values of $V/V_0$ reflect greater inhibition and $V/V_0 \approx 1$ corresponds to negligible inhibition.

Replacement of Terminal Hydroxyl Groups: L-Cystine Diesters and Diamides (Table 1). The inhibition of L-cystine crystal growth and {101̅0} step advancement on {0001} surfaces by L-CDME was attributed previously to its binding at crystal sites on the {101̅0} step planes, thereby blocking attachment of L-cystine solute molecules to adjacent crystal sites and pinning the steps. This prompted an investigation of the effect of other substituents at the terminal position, including L-cystine diesters with alkyl substituents of various size and character, specifically methyl (L-CDME, 2-L), ethyl (L-CDEE, 3), i-propyl (L-CDIE, 4), t-butyl (L-CDTE, 5), and phenyl (L-CDPE, 6). These substituents span volumes from ~25 Å³ (methyl) to 90 Å³ (phenyl). L-Cystine methyl ester (L-CME, 7), investigated previously by our laboratory, is also included in Table 1 for comparison. In situ AFM of the {0001} surface in the presence of L-cystine diester additives at a concentration of $c_{ad} = 0.015$ mM revealed that all the additives reduced the {101̅0} step velocity, accompanied by an increase in step roughness, consistent with the Cabrera–Vermilyea pinning mechanism reported previously for CDME. The values of $V/V_0$ in Table 1 reveal that the inhibition efficacy decreases with increasing size of hydroxyl substituents, suggesting steric effects hinder binding of the additives at the {101̅0} step sites. L-CDPE, equipped with terminal phenyl substituents, is an exception to this trend, reducing the {101̅0} step velocities more effectively than any of the alkyl diesters suggesting stronger binding to the crystal sites despite its larger size. The simple diamide imposter L-cystine diamide (L-CDA, 8) reduced the {101̅0} step velocity $V/V_0 = 0.53 \pm 0.04$, a somewhat weaker effect than most of the alkyl diesters and the L-CDPE. The values of $V/V_0$ in the presence of L-cystine bismorpholide (L-CDMOR, 9), and L-cystine bis(N′-methyl-piperazide) (L-CDNMP, 10) revealed inhibition comparable to L-CDA despite the larger substituent sizes (L-CDA ≈ 9 Å³, L-CDMOR ≈ 86 Å³, L-CDNMP ≈ 112 Å³ per substituent). Overall, the trends for inhibition for compounds 2–10, as measured by the reduction in {101̅0} step velocity, reveal that steric effects and chemical character contribute.
Motivated by the inhibition observed for the L-cystine diamides and the need to design effective therapeutic agents with improved pharmacokinetics and stability in vivo, L-cystine peptide analogues Ala-Cys-Ala (11), Val-Cys-Val (12), and Phe-Cys-Phe (13) were investigated. Although the volume of the terminal peptide groups does not differ significantly from many of the diester and diamide imposters, these compounds did not reduce the \( \Delta V/V_0 \) step velocities, possibly signaling disruption of the L-cystine core by the hydrogen-bonding groups of the terminal amino acids.

The stereochemical specificity for inhibition was examined by comparison of the \{10\overline{1}0\} step velocities in the presence of d-CDME or L-CDME. The values of \( V/V_0 \) in the presence of 0.15 mM d-CDME was somewhat higher than observed for L-CDME at this concentration, but the \( V/V_0 \) values did not decrease significantly at higher concentrations, unlike L-CDME. Likewise, the values of \( V/V_0 \) for d-cystine crystals in the presence of 0.15 mM d-CDME were somewhat lower than for L-CDME, but d-CDME reduced the \{10\overline{1}0\} step velocities much more effectively than L-CDME at higher concentrations.

Modifying the L-Cystine Backbone (Table 2). The role of intermolecular interactions between disulfide groups of imposters and L-cystine crystal sites was examined by replacement of the sulfur atoms with carbon. l-CCcys (14) and its dimethyl ester l-CCcysDME (15) did not reduce \( V/V_0 \), and the alkene analogue of the dimethyl ester (16) afforded a modest reduction of \( V/V_0 \). These observations suggest that intermolecular S···S interactions between the imposter and L-cystine molecules at crystal surface are essential for binding of the imposter. The absence of inhibition also is consistent with a poor commensurate match between the length of these imposters and the L-cystine crystal sites on the \{10\overline{1}0\} faces.

### Table 6. Threshold Additive Concentration, \( c_{adb} \), for the Exclusive Formation of the L-Cystine Tetragonal Polymorph

| Inhibitor | Lowest \( c_{adb} \) at which only tetragonal form sometimes crystallizes (mM) | Lowest \( c_{adb} \) at which only tetragonal always forms (in mM) |
|-----------|------------------------------------------------------------------|--------------------------------------------------|
| l-HCME    | 0.010                                                           | 0.010                                           |
| l-CDME    | 0.007                                                           | 0.007                                           |
| l-CDPE    | 0.003                                                           | 0.004                                           |
| l-CDMOR   | 0.0008                                                          | 0.002                                           |
| l-CDNMP   | 0.00008                                                         | 0.0015                                          |

*The concentration of L-cystine is \( c = 3 \) mM. \(^{a}\)Four of 18 experiments with CDNMP were performed at \( c = 2.5 \) mM.*

Figure 5. Representative SEM images of L-cystine crystals grown (A) without and (B−F) with molecular imposters. Crystals grown in the presence of (B) 0.003 mM l-CDME, (C) 0.007 mM l-CDME, (D) 0.0005 mM l-CDMOR, (E) 0.0015 mM l-CDMOR, and (F) 0.002 mM l-CDMOR reveal that a much higher concentration of l-CDME (0.007 mM) is required for formation of tetragonal l-cystine compared with l-CDMOR (0.002 mM). (G−I) Images acquired by optical microscopy using cross-polarizers for L-cystine crystals grown in the presence of (G) 0.0025 mM l-CDPE and (H) 0.0003 mM l-CDMOR reveal that different molecular imposters bind to L-cystine crystals at the same binding site, as described previously for l-CDME. \(^{17}\) (I) Tetragonal L-cystine crystals formed by l-cystine crystallization in the presence of 0.005 mM l-CDME also reveals sectoral zoning.
as the backbone lengths of imposters 14 and 15 are shorter by approximately 0.75 Å compared with l-cystine. The small, but measurable, effect of the even shorter 16 may reflect an inherent advantage for more rigid imposters in the absence of S···S interactions, but nevertheless the inhibition is not substantial. The effect of imposter length was examined further with imposters 17 and 18, homocystine and its dimethyl ester, respectively. Whereas 17 actually afforded a small increase in $V/V_0$, imposter 18 exhibited a measurable effect comparable to some of the poorly inhibiting l-cystine diesters, suggesting a lack of commensurism between the imposter and crystal site can be mitigated when S···S interactions are available. The flexibility of the homocystine backbone may also play a role. The observation of an increase in $V/V_0$ in the presence of 17 is surprising, but a similar effect has been attributed to a change in the solvation at the growth interface due to attachment of additives.46–48 Cystamine (19), commensurate with the binding site but absent the terminal carboxylic group, is an effective inhibitor with $V/V_0 = 0.60 ± 0.04$. Collectively, these results indicate some tolerance for different imposter lengths, but a more essential role for intermolecular S···S interactions between the imposter and crystal site. Notably, a recent computational study suggested that the S···S interaction between adjacent l-cystine molecules in the crystal is stronger than equivalent interactions between small molecules, highlighting the significance of the S···S bond.

### Table 7. Calculated Inhibitor Concentration in Crystal, $x$ (mmol/mol), and Distribution Coefficients, $K_d$, in (0001) and (1010) Growth Sectors of l-Cystine Crystals

|          | (0001) growth sector | (1010) growth sector |          | (0001) growth sector | (1010) growth sector |
|----------|----------------------|----------------------|----------|----------------------|----------------------|
|          | $x$, mmol/mol        | $K_d$                | $x$, mmol/mol | $K_d$                | $x$, mmol/mol | $K_d$                |
| l-CDME   | 0.66                 | 0.029                | 1.2       | 0.029                | 2.0       | 1.7                  |
| l-HCME   | 0.53                 | 0.024                | 0.8       | 0.024                | 2.0       | 1.5                  |
| l-CDPE   | 0.84                 | 0.038                | 1.7       | 0.038                | 1.7       | 1.7                  |
| l-CDMOR  | 0.66                 | 0.029                | 2.0       | 0.029                | 5.0       | n/h                  |
| l-CDNMP  | 0.64                 | 0.028                | 2.1       | 0.028                | 5.1       | n/h                  |

n/h indicates no hexagonal crystals observed.

### Table 8. Adsorption Energies to Flat {0001} and {1010} l-Cystine Crystal Surfaces

|          | absolute (kcal/mol) | relative to l-cystine (kcal/mol) |
|----------|---------------------|----------------------------------|
|          | (0001) | (1010) | (0001) | (1010) |
| l-cystine| −87    | −261   | 0      | 0      |
| l-CDME   | 69    | −496   | 156    | −235   |
| l-HCME   | 6     | −269   | 93     | −8     |
| l-CDPE   | 65    | −526   | 152    | −265   |
| l-CDMOR  | 71    | −515   | 158    | −254   |
| l-CDNMP  | 61    | −524   | 148    | −263   |

n/h indicates no hexagonal crystals observed.

Figure 6. Schematic representation of the step edges to which solute molecules can attach on the (0001) and (1010) l-cystine surfaces. The colors denote the unique orientations of the l-cystine molecule projected at the (1010) surface and correspond to the labels in Figure 1C, where C1 = green, C2 = red, C3 = cyan, C4 = pink, C5 = yellow, and C6 = blue.
Table 9. Binding Energies of L-CDME, L-HCME, L-CDPE, L-CDMOR, and L-CDNMP to Kink Sites on L-Cystine Crystal Surfacesa

|        | C1 | C2 | C3 | C4 | C5 | C6 |
|--------|----|----|----|----|----|----|
| L-CDME | 35 | 38 | 32 | 67 | 100 | 53 |
| L-HCME | 30 | 107| 27 | 34 | 76  | 136 |
| L-CDPE | 16 | 22 | 40 | 28 | 136 | -22 |
| L-CDMOR| 43 | 15 | -14| 29 | 135 | 79 |
| L-CDNMP| 14 | 19 | 41 | 25 | 117 | 70 |

Kinks on (0110) Step Edges on (0001) Face

L-CDME: -365 -381 -129 -169 n.c. n.c. 302 446 -381 -306 -269 -206
L-HCME: -74 -140 -40 -7 166 160 279 337 -138 -44 174 -62
L-CDPE: -401 -396 -145 -233 199 -360 291 424 -390 -327 -197 -225
L-CDMOR: -397 -369 -124 -249 113 167 387 -401 -413 -332 -292 -232
L-CDNMP: -386 -375 -181 -245 83 206 366 -400 -412 -354 -289 -236

Kinks on (0001) Step Edges on (1010) Face

L-CDME: -387 -388 -272 -204 -283 56 -363 282 -351 -329 -161 -235
L-HCME: -148 -90 31 -73 -20 138 -102 273 194 -31 27 25
L-CDPE: -396 -399 -234 -231 -343 101 -439 257 -346 -343 -69 -275
L-CDMOR: -423 -387 -301 -225 -300 -196 -407 391 232 -373 -165 -195
L-CDNMP: -416 -392 -285 -231 -302 -167 202 376 215 -335 -197 -237

Kinks on (1100) Step Edges on (1010) Face

L-CDME: -387 -388 -272 -204 -283 56 -363 282 -351 -329 -161 -235
L-HCME: -148 -90 31 -73 -20 138 -102 273 194 -31 27 25
L-CDPE: -396 -399 -234 -231 -343 101 -439 257 -346 -343 -69 -275
L-CDMOR: -423 -387 -301 -225 -300 -196 -407 391 232 -373 -165 -195
L-CDNMP: -416 -392 -285 -231 -302 -167 202 376 215 -335 -197 -237

aAll values are in kcal/mol relative to binding of L-cystine to the same site. Values in bold represent those that stayed in the kink site during optimization, and non-bold values indicate that the molecule moved out of the kink site.

\{1010\} step velocities. These compounds, which do not resemble the structure of L-cystine in a manner that would suggest an impostor role, did not reduce V/V_0 appreciably, even when the concentration of the inhibitor was doubled to 0.30 mM. Recently, compound 25 was reported to inhibit the formation of L-cystine crystals, but the inhibition was weak, below the detection limit by the AFM method used here. The absence of inhibition by 26, the methyl ester of L-cysteine but with an acetylated amine, suggests a critical role for the amine groups in hydrogen bonding to L-cystine growth sites. L-pen and D-pen (27-L and 27-D) and DiD-pen (28) were ineffective, illustrating the important role of the terminal ester groups and a likely negative impact by substituents on the L-cysteine or L-cystine backbones. Likewise, DINAC reduced V/V_0 only slightly. Thiola, which is among the most commonly prescribed therapeutic, was not substantially active, arguing against growth inhibition as a mode of action. In the presence of L-CDME, the dead zone was widened above the detection limit by the AFM method used here.

As the concentration of L-cystine was increased above c_{td}, the {10\overline{1}0} step motion, the step velocities in the presence of inhibitors would merge with those in the absence of inhibitors at high concentrations, indicating a greater sustainable L-cystine supersaturation. Expanding on our previous investigation, kinetic data were acquired in the presence of the imposters deemed most effective, in addition to L-CDME: L-HCME (21), L-CDPE (6), L-CDMOR (9), and L-CDNMP (10). The kinetic curves for these imposters were remarkably similar to that observed for L-CDME. For a common additive concentration of c_{ad} = 0.056 mM, the value of c_{ad} was falling in the range 1.2 ≤ c_{ad} ≤ 1.5 mM for all imposters except L-CDPE, which exhibited a c_{ad} value exceeding 2 mM (Figure 2A). Using the c_{ad} values, which correspond to complete inhibition of {10\overline{1}0} step motion, the effectiveness of the imposters decreases in the order L-CDPE > L-CDMOR ≈ L-CDMOR ≈ L-CDME ≥ L-HCME.

As the concentration of L-cystine was increased above c_{td}, the {10\overline{1}0} step velocity, V, increased slowly until reaching a threshold, c_{eb} above which the step velocity increased more steeply and linearly in a manner consistent with Cabrera–Vermilyea step pinning. This slowly increasing step velocities in the range c_{td} < c < c_{eb} indicate an intermediate regime with a kinetic coefficient β = V/[(c - c_{ad})ω] ≥ 0.03 mm/s, where ω = 142 cm^3/mol is a molar volume (β is determined from the slopes in Figure 2A). The upper limit of this regime, c_{eb}, ranges from 2 to 2.3 mM for the various molecular imposters. At c > c_{eb} the kinetic coefficient is essentially identical to that measured for pure L-cystine, β ≈ 0.07 mm/s (Figure 2A). The intermediate regime at c_{ad} < c < c_{eb} with its smaller kinetic coefficient, is a departure from the classical Cabrera–Vermilyea step pinning mechanism. This discrepancy may reflect a short residence time for adsorbed impostor molecules or random distribution of imposters over the crystal face.

A similar Cabrera–Vermilyea behavior predicts that the step velocities in the presence of inhibitors would merge with those in the absence of inhibitors at high concentrations, at
which the driving force is sufficient to overcome the energy associated with curvature of the step moving through the arrays of inhibitor stoppers. The data in Figure 2 illustrate that the upper limit of the supersaturation range explored here is not sufficiently high for observation of this effect.

The overall kinetic behavior argues for a common mechanism of inhibition for the five imposters despite their structural differences. This was corroborated by step roughening for each imposter observed by in situ AFM during dislocation-actuated growth on the (0001) L-cystine surface (Figure 3), as well as the comparable dependence of the {1010} step velocity on inhibitor concentration at two different L-cystine concentrations (2 or 3 mM, Figure 2B).

**Growth of {1010} L-Cystine Surfaces.** The small size of the {1010} L-cystine crystal faces and their high step density have precluded the observation of step motion on these faces by in situ AFM thus far. The effect of molecular imposters on the growth of {1010} surfaces, however, can be estimated from the AFM data acquired for the {1010} steps on the {0001} L-cystine surface and the height (H) to width (W) aspect ratios of L-cystine crystals, \( k = H/W \), obtained in the course of bulk crystallization experiments and which equates to the ratio of the corresponding growth rates (eq 4).

\[
k = \frac{R_{\{0001\}}}{R_{\{1010\}}}
\]

The effect of molecular imposters on the growth of a particular crystal surface can be inferred by the growth rate normal to the face normalized to the growth rate in the absence of molecular imposters, \( R_0 \). This normalized rate can be determined from the measurement of the bulk crystal aspect ratio given by eq 5, in which \( k_0 \) corresponds to aspect ratio measured in the absence of imposters. For L-cystine crystals, \( k_0 = 0.065(16) \) when grown from a 3 mM L-cystine solution (averaged over 20 experiments).

\[
\left( \frac{R}{R_0} \right)_{\{1010\}} = \frac{k_0}{k} \left( \frac{R}{R_0} \right)_{\{0001\}}
\]

Assuming that the molecular imposters do not change the slope, \( p \), of the {0001} growth hillocks significantly, as was observed previously for L-CDME\textsuperscript{17} as well as the other molecular imposters investigated here, the growth rate reduction for {0001}, \( R/R_0 \), can be replaced by the ratio of step velocities as given by eq 6.

\[
\left( \frac{R}{R_0} \right)_{\{0001\}} \approx \left( \frac{pV}{p_0V_0} \right)_{\{0001\}} \approx \left( \frac{V}{V_0} \right)_{\{0001\}}
\]

The available data (Figure 2B) clearly show that for imposter concentrations, \( c_{ad} \), at which {1010} faces do not grow, \( c_{ad} < 10 \mu M \), inhibition of {0001} faces is not significant, \( (V/V_0)_{\{0001\}} \approx 1 \). Thus, the normalized velocities of {1010} face can be obtained from eq 7.

\[
\left( \frac{R}{R_0} \right)_{\{1010\}} = \frac{k_0}{k}
\]

The aspect ratios of bulk L-cystine crystals grown in the presence of L-CDME, L-HCME, L-CDPE, L-CDMOR, and L-CDNMP, spanning a concentration range of 0.2–10 \( \mu M \), were used to deduce \( R/R_0 \) which corresponds to the reduction of the growth rate normal to the {1010} surfaces (Figure 4A,B).

As the inhibition becomes strong and the {1010} growth rate approaches zero the hexagonal L-cystine polymorph is replaced with the tetragonal one. Using the concentration of inhibitor, above which only tetragonal polymorph crystallizes, \( c_{ad(tet)} \) as a benchmark, the effectiveness of the imposters was found to decrease in the order L-CDNMP > L-CDMOR > L-CDPE > L-CDMEE > L-CDME > L-HCME (Tables 6 and S2). The efficacy of different molecular imposters also can be compared from the...
dependence of $R/R_0$ on $c_{ad}/c_{ad(tet)}$, which describes the effectiveness of an imposter on growth rate reduction within the window where the hexagonal form appears. Figure 4C reveals coincidence of the data for the five imposters, signaling a common mechanism of growth inhibition for growth on the \{10\bar{1}\} face.

The ability of the imposters to suppress growth at high supersaturations is a kinetic effect. Crystallizations performed over long times, therefore, can be expected to produce mass yields that correspond to the thermodynamic solubility of a given form. Crystallization of L-cystine from 3 mM aqueous solutions in the absence of molecular imposters produced a mass yield of $92 \pm 9\%$ (average of 12 experiments), calculated based on the solubility of hexagonal L-cystine (0.7 mM). Crystallization from 3 mM aqueous solutions of L-cystine in the presence of molecular imposters at concentrations $c_{ad} < c_{ad(tet)}$ resulted in a similar mass yield of $88 \pm 8\%$ (average of 22 experiments), with a concomitant decrease in L-cystine concentration and an increase in crystal size (Figure S1). At imposter concentrations exceeding threshold for formation of only tetragonal polymorph, $c_{ad(tet)}$, the mass yield after crystallization for 7 days was $43 \pm 12\%$ (average of 24 experiments). Using the highest yield observed as a conservative measure (55%), these yields suggest that the solubility of the tetragonal phase is no less than 1.6 mM, more than twice the solubility of the hexagonal phase. This indicates that the suppression of hexagonal L-cystine crystallization can result in formation of tetragonal form only if L-cystine concentration in a solution is sufficient. Higher solubility of the tetragonal phase is consistent with its assignment as the less stable polymorph and its absence when the hexagonal form is generated in reduced amounts at imposter concentrations below $c_{ad(tet)}$. Consequently, imposters with lower values of $c_{ad(tet)}$ would be expected to be more effective therapeutics.

The morphologies of bulk crystals grown in the presence of molecular imposters reflect the relative efficacy of the five imposters studied in detail. For example, L-cystine crystals grown in the presence of L-CDME (Figure S8–C) exhibit a morphology similar to that observed when grown in the presence of smaller L-CDMOR concentrations (Figure S8–F). Additionally, hexagonal L-cystine crystals formed in the presence of L-CDMOR and L-CDNMP exhibit less tapering than crystals formed in the presence of L-HCME and L-CDME.

**Adsorption and Incorporation of Imposters.** The \{10\bar{1}\} face growth data reveals that significantly lower imposter concentrations are needed to achieve growth inhibition compared with those for the \{10\bar{1}\} steps on the \{0001\} surface. Notably, these quantitative kinetic data can be used to estimate the incorporation of imposters into the \{10\bar{1}\} and \{0001\} growth sectors. The growth inhibition of the \{0001\} L-cystine faces follows the Cabrera–Vermilyea step pinning mechanism, such that step velocities can be described by the classical equation \cite{9,20} adapted for high supersaturations (eq 8), where $\gamma$ is the surface energy, $\omega$ is the molar volume of L-cystine, $\Theta$ is the inhibitor surface coverage, $s$ is the average size of a growth unit attached to the crystal plane, $R_s$ is the universal gas constant, $T$ is the absolute temperature, and $c_{eq}$ is the equilibrium solubility (0.7 mM for L-cystine). \cite{32}

$$V = V_0 \sqrt{1 - \frac{\exp(2\gamma \sqrt{\Theta}/sR_sT) - 1}{c/c_{eq} - 1}}$$

Although the small areas of the \{10\bar{1}\} faces preclude direct observations, it is reasonable to suggest that step pinning also is responsible for inhibition on the \{10\bar{1}\} face as the \{10\bar{1}\} face growth ceases at very small imposter concentrations and step pinning is the only mechanism that allows such strong inhibition by small additives. Molecules that “pin” a step are also likely to be incorporated into the crystal as the crystal overgrows the binding site. We assume that all molecules working as stoppers get incorporated into a crystal volume. The unit volume concentration of additive in the corresponding growth sector becomes equal to the surface coverage, $x \equiv \Theta$, given by eq 9, and the distribution coefficient, $K_{dc}$, can be calculated by eq 10, which assumes that $c \gg c_{ad}$.

$$x = \left(\frac{c_{ad}}{c + c_{ad}}\right)_{\text{crystal}} \equiv \theta$$

$$K_{dc} = \left(\frac{c_{ad}}{c + c_{ad}}\right)_{\text{crystal}} \approx \left(\frac{c_{ad}}{c + c_{ad}}\right)_{\text{solution}} = \frac{xc}{c_{ad}}$$

From eqs 8 and (10) $K_{dc}$ can be calculated by eq 11.

$$K_{dc} = \frac{xc}{c_{ad}} = \frac{c}{c_{ad}} \left(\frac{sR_sT}{2\gamma\omega}\ln\left[1 + \frac{c - c_{eq}}{c_{eq}}\left(1 - \frac{V^2}{V_0^2}\right)\right]\right)^2$$

The value of $V/V_0$ for the \{0001\} surface can be calculated directly from the in situ AFM measurements of step velocities using eq 6. The $R/R_0$ ratios in eq 7 can be used for the \{10\bar{1}\} surfaces if the step density of the growth hillock is not appreciably affected by the inhibitor, such that $V/V_0 \approx R/R_0$. The bulk crystallizations used to determine the normalized aspect ratio ($K_d/k$) using eq 7 were performed at $c = 3.0$ mM; however, the value of $c$ declined sharply after 2 days of crystallization (Figure S1). A value of $c = 2.5$ mM was used as a benchmark concentration to calculate the distribution coefficient ($K_d$) as the crystal morphology typically became fixed at this concentration. $K_d$ was then calculated for L-CDME, L-HCME, L-CDPE, L-CDMOR, and L-CDNMP using $c_{eq} = 0.7$ mM, $T = 25$ °C, growth unit sizes of $s = 0.54$ nm for \{0001\} and $s = 0.71$ nm for \{10\bar{1}\}, the latter calculated as an average of unit cell dimensions $a$ and $c/6$. The surface energy ($\gamma$ in eq 11) of flat \{0001\} and \{10\bar{1}\} faces can be calculated using a cvff force field with a dielectric constant of 80, affording values of 0.22 and 0.68 J/m$^2$ for, respectively. Alternatively, a COMPASS force field produced values of 0.31 and 1.11 J/m$^2$, respectively. Our laboratory, however, demonstrated \cite{17} that calculation of the step energy corresponding to the slowest moving step edge on the \{0001\} face was $\gamma = 0.15–0.25$ J/m$^2$ for the \{0001\} face; this approach was regarded as more realistic (because crystal growth occurs by step motion) and it produced better agreement with experimental data.\cite{17} No data are available for the \{10\bar{1}\} face and we assume it is comparable to \{0001\}, using a value of $\gamma = 0.2$ J/m$^2$ for both faces.

Table 7 provides the calculated distribution coefficients and inhibitor concentrations in the crystal. The values reveal a low probability for inhibitor binding to \{0001\} growth sector ($K_d < 0.04$) compared with the much larger value for the \{10\bar{1}\} growth sector ($K_d = 1.5$ to 5.1). This conclusion could have been deduced qualitatively without calculating $K_d$ from the
greater sensitivity of the {1010} growth rate on inhibitor concentration. The calculated $K_d$, however, suggests that the {1010} surface does not discriminate substantially between L-cystine and an efficient imposter, and that the {0001} surface does not bind imposters readily.

Our laboratory previously reported that incorporation of CDME was evident from the anomalous sector-zoned birefringence when L-cystine crystals grown in the presence of CDME was evident from the anomalous sector-zoned CDNMP, and L-CDMOR in L-cystine crystals. CDME > L-HCME. The adsorption energies of L-CDPE, L-CDNMP, and L-CDMOR with the S–S bond vector parallel to each of the six {1010} growth sectors. Birefringence also is observed here for hexagonal L-cystine grown in the presence L-CDPE, L-CDNMP, L-CDMOR, L-CDEE and L-HCME, as well as for tetragonal crystals grown in the presence of L-CDME (Figure 5 G,H,I; the small size of tetragonal crystals grown in the presence of the other inhibitors precluded analysis of birefringence). H NMR substantiates incorporation of L-CDME (ε = 2.5 mM, $c_{ad}$ = 0.019 mM), revealing concentrations of 3.9 ± 0.9 mmol additive/mol L-cystine, equivalent to $K_d$ (whole crystal) = 0.62. eq 11, when applied under the same conditions, affords $x_{(0001)} = 0.08$ and $x_{(10-10)} = 2.05$ mmol/mol. Averaging over the crystal volume (the volume of two {0001} growth sectors is equal to 1/3 of the volume of the entire crystal) affords $x = (2x_{(10-10)} + x_{(0001)})/3 = 1.44$ mmol/mol and $K_d$ (whole crystal) = 0.19. Although this $K_d$ (whole crystal) is 3.3 times less than that expected from the 1H NMR measurements, the values are within reasonable agreement. Some discrepancy in the $K_d$ values are observed at different additive concentrations, but this can be attributed to assumptions in the model or the nonequilibrium nature of the distribution coefficient.

**Binding of Molecular Imposters to L-Cystine Crystal Surfaces.** The adsorption of L-CDME, L-HCME, L-CDPE, L-CDMOR, and L-CDNMP to flat {0001} and {1010} surfaces was modeled using simulated annealing calculations in Biovia Materials Studio to explore the differences in imposter binding to the {1010} and {0001} surfaces (Table 8, Figures S2 and S3). The adsorption energies, which can be used to compare binding strength of the additive for the relevant surfaces, are negative on the {1010} face for all imposters. The adsorption energies for {0001} are all positive, corroborating the experimental observations of preferred additive binding to {1010}. The adsorption energies for additives on {1010} faces decline in the order L-CDPE > L-CDNMP > L-CDMOR > L-CDME > L-HCME. The adsorption energies of L-CDPE, L-CDNMP, and L-CDMOR are similar, and they indicate stronger binding compared with L-CDME and L-HCME, in agreement with the calculated distribution coefficients (Table 7) and anticipating greater incorporation of L-CDPE, L-CDNMP, and L-CDMOR in L-cystine crystals.

Molecular modeling of adsorption to flat crystal surfaces, however, ignores the binding of the imposter to kink sites, a critical feature of the Cabrera–Vermilyea mechanism that is substantiated by the kinetic data on the {0001} surface. Assuming the Cabrera-Vermilyea mechanism is operative for the {1010} surface as well, kink sites on the {0001} and {1100} steps of the {1010} surface must be considered as well as kink sites on {1010} steps on the {0001} surface. The projection of the L-cystine unit cell on the {1010} surface results in six unique projections of the L-cystine molecule at the surface by symmetry (labeled C1–C6 in Figure 1C), as denoted by colors corresponding to each of the projections (C1 = green, C2 = red, C3 = cyan, C4 = pink, C5 = yellow, C6 = blue in Figures 1, 6, S4, and S5). This results in six unique {1010} step edges on the {0001} face, as well as six unique binding positions along {0001} and {1100} step edges on the {1010} faces. For each binding position, the handedness of the kink, distinguishable by a clockwise (+) or counterclockwise (−) twist about the axis perpendicular to the surface, results in two unique kink sites on each of the six unique {1010} step edges for a total of 12 unique kink sites on the {0001} L-cystine surface (Figure S4). There are six unique binding positions and 2 possible kink configurations (clockwise or counterclockwise) for each of the {0001} and {1100} step edges, a total of 24 unique kink sites on the {1010} L-cystine surface (Figure S5). Binding energies were calculated for the incorporation of L-cystine, L-CDME, L-HCME, L-CDPE, L-CDMOR, and L-CDNMP to all 36 unique kink sites (Table 9). The kinked surface was fixed for each configuration but the molecule at the kink site was allowed to relax, identical to a procedure reported by our laboratory for calculating kink binding energies at highly disymmetric crystal surfaces. The kink-imposter binding energy was determined from the difference between the sum of the separate energies of the kink site and binding molecule and the energy of the surface with the imposter docked at the kink site.

The binding energies of additives to kink sites on the L-cystine crystal are provided in Table 9, where the nonbolded values correspond to examples in which the additive molecule moved out of the kink position, indicating that the kink site is unfavorable for imposter binding. Considering only the bolded values, the lowest energy binding site for each additive to {1010} step edges on the {0001} face are C1(+) for L-CDME, L-CDPE, and L-CDNMP and C2(+) for L-HCME and L-CDMOR. The lowest energy binding site for each additive to {0001} and {1100} step edges on the {1010} face are one of the two C1 binding positions (Figure 7). This is consistent with previous work in which replacement energies calculated for L-CDME at the flat {1010} surface revealed that the C1 L-cystine position was the most favorable binding site. These computational results corroborate the observation of anomalous birefringence reported for L-CDME as well as for the L-CDPE and L-CDMOR crystals in Figure S5G,H. The observed birefringence pattern indicate the incorporation of molecular imposters through uniform attachment to the same crystallographically unique binding site in each growth sector, with high fidelity.

**CONCLUSION**

This investigation of L-cystine crystal growth inhibition by an expanded number of L-cystine mimics, as well as compounds reported to exhibit some degree of therapeutic efficacy, has revealed the essential structural features for binding to L-cystine crystal sites and the associated suppression of crystal growth. Measurement of step velocities on the {0001} surface and growth rates perpendicular to the {1010} surfaces demonstrate that effective inhibition relies on strict stereochemical recognition between an inhibitor and specific L-cystine crystal sites, supporting further the molecular imposter principle. The most effective imposters were L-cystine diesters and diamides, which exhibited comparable reductions of step velocities on the {0001} surface. L-Cystine imposters with morpholine and piperazine terminal groups, however, were much more effective inhibitors of crystal growth on the {1010} faces compared with the diesters, suggesting stronger binding of these additives at step and kink sites on the {1010} faces. Comprehensive kinetic analyses revealed a common inhibition mechanism for the
diesters and diamides, but with stronger adsorption of the diamides on the \{1010\} surfaces, consistent with the higher in vitro supersaturations attainable with the morpholine and piperazine diamides (\textit{i}-CDMOR and \textit{i}-CDNMP, respectively) compared with \textit{i}-CDME, despite comparable step velocity reductions on the \{0001\} surface. Moreover, this observation may explain the greater efficacy of \textit{i}-CDNMP toward the suppression of stone formation in mouse model studies. The effectivness of the cystine imposters toward the growth inhibition of hexagonal \textit{l}-cystine, the crystal form responsible for stone formation, is also revealed by the formation of the tetragonal polymorph. The imposters likely inhibit the growth of incipient nuclei of the hexagonal phase, and the small amounts of the tetragonal form observed in vitro would not be evident in vivo can be attributed to its higher solubility. The incorporation of the additives was evident from anomalous birefringence in the \{1010\}, which was consistent with a high fidelity of stereospecific binding of CDME, in a unique orientation, exclusively at one of the six crystallographically unique projections of \textit{l}-cystine on the \{1010\} plane, providing further evidence of strict molecular recognition of imposters at crystal sites. The distribution coefficients estimated from the kinetic data demonstrate that \{1010\} faces do not discriminate \textit{l}-cystine and imposters, whereas \{0001\} faces are strongly biased against imposter incorporation. Importantly, these studies reveal that commonly prescribed therapeutic agents, which rely on thiol–disulfide exchange to form asymmetric disulfides that are less prone to crystallization, were ineffective as crystal growth inhibitors. Overall, these observations implicate a mechanism for reduction of \textit{l}-cystine stone burden by imposters in knockout mouse model studies that differs dramatically from currently prescribed therapies.

**ASSOCIATED CONTENT**

3 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.cgd.7b00236.

Charts S1–S3, Syntheses of molecular imposters, Tables S1 and S2, and Figures S1–S5 (PDF)

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Notes

The authors declare no competing financial interest.

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