Antagonistic cooperativity between crystal growth modifiers

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Ubiquitous processes in nature and industry exploit crystallization from multicomponent environments [1-5], yet laboratory efforts have focused on crystallization of pure solutes [6,7] and the effects of single growth modifiers [8,9]. Here we examine the molecular mechanisms employed by pairs of inhibitors in blocking the crystallization of hematin, which is a model organic compound with relevance to the physiology of malaria parasites [10,11]. We use a combination of scanning probe microscopy and molecular modeling and demonstrate that inhibitor pairs, whose constituents employ distinct mechanisms of hematin growth inhibition, kink blocking and step pinning [12,13], exhibit both synergistic and antagonistic cooperativity depending on the inhibitor combination and applied concentrations. Whereas synergism between two crystal growth modifiers is expected, the antagonistic cooperativity defies current crystal growth models. We demonstrate that kink blockers reduce the line tension of step edges, which facilitates both the nucleation of new crystal layers and step propagation through the gates created by step-pinners. The molecular viewpoint on cooperativity between crystallization modifiers provides guidance on the pairing of modifiers in the synthesis of crystalline materials. The proposed mechanisms suggest strategies to understand and control crystallization in natural and engineered systems, which occurs in complex multicomponent

media [1-3,8,9]. In a broader context, our results highlight the complexity of crystal-modifier interactions mediated by the structures and dynamics of the crystal interface.

Crystallization is the central process of materials synthesis in biological, geological, and extraterrestrial systems [7,14]. Nature achieves remarkable diversity of shapes, patterns, compositions, and functions of the arising crystalline structures by combining simple strategies to control the number of nucleated crystals and their anisotropic rates of growth [15,16]. To promote or inhibit crystallization in both natural and engineered environments, soluble foreign compounds are deployed that interact with the solute or the crystal-solution interface [17]. In many cases, two or more modifiers operate in tandem to alter processes of crystallization [4,18-21], yet the fundamental mode(s) of cooperative action is not well understood.

To gain molecular-level insight into the mechanisms of cooperativity between crystallization modifiers, we examine the growth of β-hematin crystals, which form in malaria parasites as a part of their heme-detoxification mechanism [22], in the presence of quinoline compounds that represent a major class of the currently employed antimalarials [23,24]. Recent work established that β-hematin crystal growth follows classical mechanisms whereby new layers nucleate on the crystal surfaces and advance by incorporation of solute molecules at the steps [12]. These studies uncovered two distinct classes of quinoline inhibition of step propagation [13]. In the first mechanism, known as "step-pinning," chloroquine and quinine (Fig. 1a) bind to flat terraces and arrest crystal formation over broad areas of the crystal surface (Fig. 1b) [25]. Alternatively, amodiaquine and mefloquine (Fig. 1a) were found to block kinks, the sites where hematin molecules incorporate into steps (Fig. 1c) [12].

Even though combinations of two or more crystal growth inhibitors are common in many drug formulations [26], a crucial gap in the understanding of interactions between inhibitor pairs that regulate hematin crystallization has been identified [27,28]. To address the molecular

mechanism of action of binary inhibitor combinations on β-hematin crystal growth we pair a step pinner, chloroquine (CQ) or quinine (QN), with a kink blocker, mefloquine (MQ) or amodiaquine (AQ). We classify the cooperativity between paired inhibitors as synergistic, additive, or antagonistic according to whether the response to a combination of two inhibitors is, respectively, stronger, equal, or weaker than the sum of the responses to individual doses [29].

Binary inhibitor combinations impose dramatic changes in the shapes and dimensions of β-hematin crystals (Fig. 1f-I and Extended Data Fig. 1). The crystal length along the *c* crystallographic axis is the result of growth in the [011] and $[0\bar{1}\bar{1}]$ directions (Fig. 1 d and e). The shorter average length enforced by both MQ and CQ than by either modifier separately indicates a strong synergistic activity of these two inhibitors (Fig. 1f). Since the crystal length is insensitive to the presence of MQ alone [13], additive cooperativity of CQ and MQ would engender crystal lengths similar to those constrained by CQ only. By contrast, the crystal lengths affected by the pairing of AQ and CQ are significantly longer than those engendered by CQ, implying an antagonistic cooperativity between these two modifiers. The addition of either MQ or AQ to CQ-containing solutions enforces greater crystal widths than those with CQ (Fig. 1g). The crystal width increases owing to growth in the ⟨010⟩ directions (Fig. 1d); thus greater widths indicate that the MQ/CQ and AQ/CQ pairs impede growth of {010} faces to a lesser extent than CQ on its own. We previously reported that MQ and AQ weakly affect the crystal width [13]; therefore, these new findings indicate antagonistic cooperativity of CQ with kink blockers MQ and AQ in inhibiting the width of β-hematin crystals. Notably, in select inhibitor concentration ranges (e.g., $C_{CQ} < 1$ μM and $C_{MQ} < 4$ μM) synergism in suppressing growth along the *c* axis accompanies antagonistic cooperativity towards growth in the *b* direction (Fig. 1f and g); the opposing responses are likely defined by the selective binding of the inhibitors to the individual crystal faces dictated by their distinct structures [17,21]. Importantly, they further weaken the synergistic cooperativity of CQ and

MQ in inhibiting hematin sequestration into crystals. Combining MQ and AQ with QN elicits mostly synergistic responses of both the crystal length (Fig. 1h) and width (Fig. 1i).

Antagonistic cooperativity between crystallization inhibitors appears counterintuitive. To understand the effects of inhibitor combinations on the molecular processes of growth of the (100) face of β-hematin crystals, we employed time-resolved *in situ* atomic force microcopy (AFM) [12,13]. We scrutinized inhibitor effects on the rate of two-dimensional nucleation of new crystal layers J_{2D} and the rate of propagation of steps v. For J_{2D}, we counted the number of new layer nuclei that grow above a critical radius R_c per unit area of the surface and unit time (Fig. 2a). We determined v from the displacement of the steps over time (Fig. 2a) [12]. The correlation between J_{2D} and the concentration of the inhibitors demonstrates that the addition of the kink blockers MQ and AQ to the step pinner CQ significantly enhances the nucleation of new layers relative to that with solitary CQ, indicating strong antagonism (Fig. 2b). The cooperativity between CQ/MQ and CQ/AQ in suppressing v is antagonistic at almost all tested inhibitor concentrations (Fig. 2c, Extended Data Fig. 2b, and Extended Data Table 1).

MQ and AQ exhibit similar transition towards stronger antagonism when combined with QN. Mefloquine (MQ), which alone does not suppress J_{2D} [13], exhibits synergistic cooperativity with QN at $C_{MQ} < 4$ μM and antagonism at $C_{MQ} > 4$ μM (Fig. 2d). Similarly, AQ, which on its own depresses J_{2D} by up to 60% [13], transitions from synergy at $C_{AQ} < 2$ μM to antagonism at $C_{AQ} > 4$ μM. Both MQ and AQ strongly inhibit the step velocity v when acting alone [13] and the similarity between velocity profiles measured in the presence of QN/MQ and QN/AQ combinations to those obstructed by QN alone (Fig. 2e) signify strong antagonism between MQ and QN and between AQ and QN. The cooperativity of inhibitor pairings can be quantified from isobolograms (Fig. 2 f and g), an established method in pharmaceutical research, in which the doses of paired inhibitors needed to inhibit J_{2D} and v by a certain percentage are compared to the sum of the responses to each inhibitor applied individually [27,29].

We establish that the antagonism between step pinners and kink blockers in inhibiting bulk crystallization and the surface processes on (100) faces is not motivated by the formation of inhibitor-hematin complexes in solution. We examined whether the constituents of an inhibitor pair formed binary complexes that do not impede crystallization. Such complexation would lower the concentration of the active inhibitor and constrain their potency. We tested the formation of CQ/MQ, CQ/AQ, QN/MQ, and QN/AQ binary complexes. Considering that the four inhibitors form complexes with hematin [13,30], we also explored whether these four combinations assemble into ternary compounds that include hematin. Results presented in the Extended Data Figure 3 demonstrate that no complexes involving both inhibitors exist in the solution, and imply that complexation between the applied inhibitors is not the source of the observed antagonistic cooperativity.

Additive and synergistic cooperativity in suppressing J_{2D} and v between a kink blocker and a step pinner can be understood within the realm of common crystal growth models. Blocking of kinks lowers the kinetic constant for growth, which works in parallel with the depression of the crystallization driving force due to step curvature enforced by step pinners (Fig. 1 a and b). We posit that the antagonism between the two types of inhibitors originates from the reduction of the step line tension γ, a thermodynamic prerequisite for the adsorption of kink blockers at steps [31]. Based on the Gibbs-Thomson relation, γ regulates the radius of the critical two-dimensional nucleus according to $R_c = \Omega\gamma/\Delta\mu$ [31] (Ω is the molecular volume; $\Delta\mu = k_BT\ln(c_H/c_e)$ is the chemical potential difference between the solution and the crystal; k_B is the Boltzmann constant; T is temperature; c_H is hematin concentration; and c_e is solubility). In turn, lower γ and R_c stimulate faster layer nucleation as $J_{2D} = J_o\exp(-\pi\gamma R_c h/k_BT)$ (h = 1.2 nm is the step height) [12] and expedite step propagation in the gaps between the adsorbed step pinners (Fig. 1b). We developed, in the Supplementary Information Sections 3 and 4 and Extended Data Figs. 5 and 6, an analytical model of combined action of step pinners and kink blockers on step propagation and analyzed the

consequences of the presence of two types of inhibitors on the nucleation of new crystal layer (SI Section 5). This examination advocates that the classical synergistic effects dominate at low concentrations of either inhibitor, whereas the proposed mechanism of antagonism mobilizes at high concentrations; stronger antagonism between step pinners and kink blockers is projected for their joint action on J_{2D} than on v (SI Section 5). Both predictions are borne by the J_{2D} and v correlations (Fig. 2 b – e).

Data on layer nucleation in the presence of MQ or AQ demonstrate that γ decreases in the presence any of these inhibitors and the measured $\Delta\gamma$ correlates with the inhibition of step motion due to association of these inhibitors to the kinks. From AFM images we directly measured R_c in the presence on 2.5 μM MQ or AQ. This parameter represents the critical size of a two-dimensional nucleus of a crystal layer below which nuclei tend to dissolve, whereas nuclei larger than R_c have a greater probability to grow (Fig. 3a). We monitored the evolution of 25 to 30 layer nuclei at each value of $\Delta\mu$ and inhibitor concentration, where $\Delta\mu$ was varied by selection of the hematin concentration c_H (Fig. 3b). The relation between R_c and $\Delta\mu$ (Fig. 3 c and d) is reciprocal, consistent with the Gibbs-Thomson relation, and reveals that the presence of MQ and AQ lowers γ from a nominal value of 25 ± 2 mJ m^{-2} to 20 ± 2 and 22 ± 1 mJ m^{-2}, respectively. In Methods, we discuss statistical tests that certify the distinction of the three γ values and relate decreasing γ to association of MQ and AQ to the kinks. We assume the two kink blockers adsorb to the steps following a Langmuir-type law. In SI Sections 1 and 2 we evaluate $-\Delta\gamma$ using the Gibbs equation of adsorption, $\Gamma = -d\gamma/d\mu_B$, where Γ is the amount of inhibitor absorbed at kinks and $\mu_B = \mu_{B0} + k_B T \ln c_B$ is the MQ or AQ chemical potential [31]. From these relations and Extended Data Figure 4 Extended Data Tables 3 and 4 we obtain $\Delta\gamma \approx -3$ mJ m^{-2} for both MQ and AQ, in good agreement with the values for these two inhibitors assessed from the $R_c(\Delta\mu)$ correlations (Fig. 3 b and c). These $\Delta\gamma$s invoke an equivalent contraction of R_c [13]. Since a 20% decrease in R_c is equivalent to a 1.44-fold (1.2^2) lowering of the surface coverage of adsorbed step pinners, and given that J_{2D} and

v are highly sensitive functions of both c_{CQ} and c_{QN}, the decrease in γ elicits a disproportionally strong response of v and J_{2D}.

In situ AFM measurements were complemented with kinetic Monte Carlo simulations to test the generality of the proposed model of antagonistic cooperativity between two classes of crystallization inhibitors. We developed a solid-on-solid model for step growth [32], in which molecules associate and dissociate from steps. For simplicity, we ignore surface diffusion on the terraces. The rate of solute association depends on the supersaturation, whereas the probability of detachment is dictated by bonds a molecule forms with its neighbors (Video 1). We assume that kink blocker adsorption and detachment are analogous to solute molecules, and that the relevant dynamics are governed by their concentration and the number and strength of bonds at an adsorption site (we assume that two of the lateral bonds are stronger and remaining two, weaker, than for the solute molecules). These assumptions lead to preferential binding to the kinks at steps (Fig. 4a) and constrained v (Fig. 4b and Video 2). We assume that step pinners bind strongly to the crystal surface, but exhibit no interactions with crystal molecules parallel to that plane. The surface is decorated with a square array of step pinners and they remain static throughout the simulation (Fig. 4c and Video 3); previous results demonstrate that the step pinner surface distribution has no effect on the step velocity [32]. Remarkably, the computed correlations between v and inhibitor concentrations are akin to those observed experimentally for the kink blockers MQ and AQ, for which v levels off at ca. 50% inhibition (Fig. 4b), as well as the step pinners CQ and QN, which induce complete growth arrest at moderate inhibitor concentrations (Fig. 4d) [13].

Combining step pinners at a concentration above the threshold for complete growth arrest (Fig. 4e) with kink blockers allows steps to advance through pinned sites, thereby reestablishing layer growth (Fig. 4 f and g and Videos 4 and 5). The simulations reveal that at the microscopic level, the antagonistic cooperativity is due the stabilization of step edge fluctuations by associating kink blockers. Steps overcome the pinner palisade by fluctuations that penetrate the gaps between

the pinners (Fig. 4c Video 5) [32]. Closely spaced pinners suppress the extent and lifetime of the fluctuations and restrain step growth. The blockers bind to the kink-rich fingers embodying the fluctuations (Fig. 4h) and increase the fluctuation lifetime. At the macroscopic level, the stabilized fluctuations manifest as a decrease in γ. Indeed, an attenuated γ enforces shorter R_c, which, in turn, allows step progress between the pinners (Fig. 4 f and g).

In summary, we put forth a mechanism of antagonistic cooperativity between crystallization inhibitors by which kink blockers attenuate the step line tension and facilitate step propagation through the palisade of step pinners. This mechanism may provide guidance in the search for suitable inhibitor combinations to control crystallization of pathological, biomimetic, and synthetic materials. In a broader context, our results highlight modifier interactions mediated by the dynamics and structures on the crystal interface as a prime element of the regulation of the shapes and patterns of crystalline structures in nature and industry.

Online Content Methods, along with additional Extended Data display items, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.

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Author information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interest. Readers are welcome to comment on the online version of the paper. Correspondence and request for material should be addressed to P.G.V. (vekilov@uh.edu) and J.D.R. (jrimer@uh.edu).

Figure Legends

Figure 1 | Cooperativity between four pairs of inhibitors in suppressing bulk growth of β-hematin crystals. a. Structures of step pinners chloroquine (CQ) and quinine (QN), and kink blockers mefloquine (MQ) and amodiaquine (AQ). **b.** Schematic of step pinning, where Δx is the separation between inhibitor molecules (shown in gold) adsorbed on flat crystal terraces and R_c is the critical radius of the 2D nucleus. Step growth is delayed if Δx is comparable to $2R_c$ and arrested if $\Delta x < 2R_c$. **c.** Schematic of inhibitors (shown in blue) inhibiting step advancement by partial blocking of access of solute molecules to kinks. **d.** SEM micrograph and schematic illustrating the β-hematin crystal habit. **e.** Preservation of the crystal shape during growth in pure solutions and inhibitor-induced suppression of crystal length ℓ or width w by interaction of inhibitors with axial and lateral crystal faces, respectively. **f – i.** Variations of the average length ℓ and width w of crystals grown in the presence of increasing concentrations of four inhibitor pairs at displayed ratios relative to ℓ_0 and w_0 reached after growth in pure citric buffer-saturated octanol (CBSO) solutions for 16 days at 23ºC. Error bars represent the standard deviation of about 30 measurements. Lines in f – i are guides for the eye. In all experiments, hematin concentration c_H = 0.28 mM and supersaturation $\sigma = \ln(c_H/c_e) \cong 0.93$, where c_e = 0.11 mM is the solubility at 23°C. The majority of the length and width data for individual modifiers are from Olafson, *et al.* [13] and are consistent with additional measurements of the effects of QN.

Figure 2 | Cooperativity of inhibitor pairs in suppressing layer generation and spreading. a. Time-resolved *in situ* AFM images showing the nucleation and growth of new layers on a (100) face at c_H = 0.28 mM and supersaturation $\sigma = \ln(c_H/c_e) \cong 0.56$, where c_e = 0.16 mM is the solubility at 28°C, the temperature in the AFM liquid cell. Arrows indicate newly nucleated islands that are counted to determine the rate of 2D nucleation, J_{2D}. The growth of the island dimensions ℓ underlies the determination of step velocity, v. The bright lines with striations at the top and bottom of some of the panels correspond to the crystal edges. **b – e.** Decrease in J_{2D} relative to that in the absence of any inhibitor, $J_{2D,0}$, in **b** and **d**, and of v relative to that in the absence of any inhibitor, v_0, in **c** and **e**, with increasing concentrations of inhibitor pairs at displayed ratios. Error bars represent the standard deviation of 15 to 25 measurements of J_{2D} and 25 to 35 measurements of v and are, in some cases, smaller than the symbol size. Lines are guides for the eye. Data for individual modifiers are from Olafson, *et al.* [13]. **f, g.** Isobolograms characterizing the inhibition of v by QN/MQ, in f, and CQ/MQ, in g. Open symbols indicate the concentrations of individual inhibitors that elicit a certain percent inhibition, sometimes referred to as inhibitory concentrations, IC. Dashed lines correspond to additive cooperativity between the paired inhibitors for certain percent inhibition. Solid symbols represent the concentrations of the paired inhibitors that evoke the same inhibition. Rightward shifts of the solid symbols from the respective dashed lines indicate antagonistic cooperativity. The corresponding Combination Index (CI) values are listed in Extended Data Table 1.

Figure 3 | Characterization of the effects of the kink blockers MQ and AQ on layer nucleation. a. Time-resolved *in situ* AFM images showing growing (I and II) and dissolving (III) islands on a (100) face at c_H = 0.28 mM and supersaturation $\sigma = \ln(c_H/c_e) \cong 0.56$. **b** and **c.** Dependences of the radius of the critical 2D nucleus R_c on crystallization driving force $\Delta\mu = k_B T \ln(c_H/c_e)$ in pure hematin solution and in the presence of MQ, in b, and AQ, in c. Error bars represent the standard deviation of 25 to 30 measurements. Solid lines are plots of the Gibbs-

Thomson relation $R_c = \Omega\gamma/\Delta\mu$ with step line tension γ = 25 mJ m^{-2} for pure hematin and 20 and 22 mJ m^{-2} for MQ and AQ, respectively. Data for pure hematin are from Olafson, *et al.* [12].

Figure 4 | Solid-on-solid kinetic Monte Carlo (kMC) modelling of the action of kink blockers and step pinners on step propagation. a. Kink blockers (magenta spheres) associate to kinks and incorporate in the crystal. **b.** Dependence of the step velocity *v* relative to that in pure solution v_0 on the concentration of kink blockers $\rho_{\text{kink blocker}}$ relative to ρ_{total}, the summed concentration of solute and kink blockers. **c.** Step pinners (gold spheres) adsorb on the terraces between steps and enforce curved steps. **d.** Dependence of the step velocity *v* relative to that in pure hematin solution v_0 on the surface density of step pinners, $\rho_{\text{step pinner}}$. Error bars in b and d represent the standard error of the simulations evaluated as discussed in Methods. **e.** Step pinners adsorbed on the surface arrest step advancement. Four numbered step pinners mark the step location. **f and g.** Addition of kink blockers stimulates the growth of a step stalled by step pinners. **h**. Magnified view of a step squeezed between stoppers 1 and 3 demonstrates kink blockers associated to kinks in the growing step segment.

Methods

Solution preparation. The following compounds were purchased from Sigma Aldrich (St. Louis, MO): Hematin porcine (≥ 98%), citric acid (anhydrous, ≥ 99.5%), sodium hydroxide (anhydrous, ≥ 98%), n-octanol (anhydrous, ≥ 99%), porcine hematin, chloroquine diphosphate (≥ 98%), quinine (anhydrous, ≥ 98.0%), amodiaquine dihydrochloride dihydrate, and mefloquine hydrochloride (anhydrous, ≥ 98.0%). All reagents were used as received. Deionized (DI) water was produced by a Millipore reverse osmosis – ion exchange system (Rios-8 Proguard 2 – MilliQ Q-guard).

Citric buffer at pH 4.80 was prepared by dissolving 50 mM of citric acid in DI water and titrating the solution, under continuous stirring, with 0.10 M NaOH to the desired pH. The buffer pH was verified before each experiment and fresh buffers were prepared every month. We placed 5 mL of citric buffer at pH 4.80 in direct contact with n-octanol at 23°C and allowed 30 min for equilibration. The upper portion of the two-phase system was decanted and denoted as citric buffer-saturated octanol (CBSO).

For this study, we used four antimalarial drugs: quinine (QN), chloroquine (CQ), amodiaquine (AQ), and mefloquine (MQ). Solid QN and MQ were added to CBSO and the solutions reached the desired concentration after 2 - 4 days. AQ and CQ were added in excess to CBSO and stored in the dark for 30 - 45 days, allowing the concentrations to approach the respective solubilities [33]. All drug solutions were filtered through 0.2 μm nylon membrane filters and the concentrations were determined by UV–vis spectrometry using a Beckman DU 800 spectrophotometer and extinction coefficients and wavelengths listed in from Ketchum, *et al.* [33].

Hematin solutions were prepared by dissolving hematin powder in 8 mL of freshly made CBSO and heating it up to 70°C for 7-9 h. The solution was filtered through a 0.2 μm nylon

membrane filter and the concentration was determined using an extinction coefficient $\varepsilon_{hematin}$ =3.1 ±0.1 $cm^{-1}mM^{-1}$ at λ=594 nm [34,35].

Characterization of the combined inhibitor effects on bulk hematin crystallization. We adopted the procedure reported by Olafson *et al.* [35,36] to produce hematin crystals from supersaturated hematin solution in CBSO. We tested crystal growth in the presence of four drug combinations, CQ/MQ, CQ/AQ,QN/MQ, and QN/AQ, with constant ratios between the two constituents of 1:4, 1:2, 1:2 and 1:2, respectively. Drug combinations were added to the hematin stock solution to achieve final total inhibitor concentrations ranging from 0 to 15 μm while maintaining a constant hematin concentration (c_H = 0.28 mM). The vials were then shaken until the solution was well mixed. A glass slide 15 μm in diameter was scratched in the center and placed at the bottom of the vial in contact with the supersaturated solution. Vials were capped and placed in an incubator at 23°C with minimal exposure to light. β-hematin crystals were observed in 1 – 2 days and reached their maximum length after ca. 2 weeks. The glass slide with attached hematin crystals was collected, washed with DI water and ethanol, dried with nitrogen gas and then coated with 10 – 20 nm gold for SEM. The length and width of about 30 crystals at each composition were measured to assess the effectiveness of inhibitor combinations.

***In situ* monitoring of the hematin crystal evolution.** We used a multimode atomic force microscope (Nanoscope IV) from Digital Instruments (Santa Barbara, CA) for all AFM experiments. AFM mages were collected in tapping mode using Olympus TR800PSA probes (Silicon nitride, Cr/Au coated 5/30, 0.15 N/m spring constant) with a tapping frequency of 32 kHz. Image sizes ranged from 300 nm to 20 μm. Scan rates were between 1 and 2.52 s^{-1}. Height, amplitude, and phase imaging modes were employed. The captured images contained 256 scan lines at angles depending on the orientation of the monitored crystal [35,37]. The temperature in the fluid cell reached a steady value of 27.8 ± 0.1°C within 15 min of imaging [37]. This value was higher than room temperature (ca. 22°C) owing to heating by the AFM scanner and laser.

Hematin crystals were grown on glass disks as described above. The density of attached hematin crystals was monitored under an optical microscope. We ensured similar crystal density for all samples to minimize potential depletion of inhibitors due to high crystal number. The glass slides were mounted on AFM sample disks (Ted Pella Inc.) and the samples were placed on the AFM scanner. Hematin solution in CBSO with a concentration of 0.28 mM was prepared less than 2 h in advance. This solution was loaded into the AFM liquid cell using 1 mL disposable polypropylene syringes (Henck Sass Wolf), tolerant of organic solvents. After loading, the system was left standing for 10 – 20 min to thermally equilibrate. The crystal edges were identified to determine the orientation and the crystallographic directions on the upward-facing (100) crystal surface. The crystals were kept in contact with the solution for 0.5 – 1.5 h to allow their surface features to adapt to growth conditions. We set the scan direction parallel to the [001] crystallographic direction and AFM images were collected for 3 – 5 h. The solution in the AFM fluid cell was refreshed every 30 min to maintain constant concentration. For studies of modifiers, growth solutions were replaced with ones containing a selected antimalarial inhibitor(s). With each modifier concentration, AFM images were collected for 2 to 4 hours, during which the solution was replenished several times. Solution without modifier was pumped in to the AFM cell and the observed crystal was allowed to grow uninhibited for about 30 min before another modifier concentration was introduced.

The evolution of the hematin crystal surface was characterized by the velocity of growing steps v and the rate of two-dimensional nucleation of new crystal layers $J_{2D.}$ To determine v, we monitored the displacements of 8 – 13 individual steps with a measured step height h = 1.17 ± 0.07 nm. Between 25 and 35 measurements were taken for each individual step and the average growth rates were reported. To determine J_{2D}, the appearance of new islands on the surface between successive images was monitored and the number of islands that grew was counted. This

number was scaled with the imaged area and the time interval between images to yield J_{2D}. From 15 to 25 measurements were averaged for each J_{2D} determination.

The goal of the AFM investigations is to establish the molecular mechanisms of synergy or antagonism between step pinners and kink blockers in inhibiting the growth of β-hematin crystals. Using AFM imaging at mesoscopic scale, we demonstrate that step pinners and kink blockers cooperate in suppressing both the nucleation of new layers and the propagation of steps on hematin crystal surfaces. The nucleation of new layers at random locations on the crystal surface requires observations at the mesoscopic lengthscale, within the range of capabilities of standard AFM techniques. Images with molecular resolution of growing steps would have provided additional insights. As shown in our previous work on hematin crystallization, imaging with resolution comparable to the size of the hematin molecule, ca. 1 nm, is possible during *in situ* AFM monitoring of flat crystal planes [38]. The presence of steps, however, disrupts the contact between the scanning tip and the crystal surface and lowers the image resolution. Strict numerical correspondence between discrete molecular-level events and the mesoscopic and macroscopic variables that characterize crystal growth and inhibition has been established in our earlier work [39-42]. This correspondence supports the molecular mechanisms based on observations at mesoscopic lengthscales.

Determination of the surface free energy of the step edge γ in the presence of MQ and AQ. We evaluate the value of γ from the correlation between the radius of the two-dimensional nucleus of new layers R_c and the supersaturation, similar to previous determinations in solutions without inhibitors carried out by Olafson and collaborators [35]. The critical radius R_c for layer nucleation is defined as the threshold size above which an island has a higher probability to grow. Islands of size $R < R_{crit}$ are more likely to dissolve. We monitored the size evolution of all newly generated islands from time-resolved sequences of *in situ* AFM images and classified the islands as growing or dissolving. The largest sizes reached by dissolving islands and the threshold,

above which all islands grew, were averaged to yield R_c. We determined from 25 to 30 independent R_c measurements at each combination of hematin and MQ or AQ concentration. Six concentrations of hematin c_H were tested in the presence of 2.5 μM MQ and seven in the presence of 2.5 μM AQ. The R_c values obtained at each concentration of the two inhibitors were averaged and plotted as a function of the supersaturation $\Delta\mu/k_BT = \ln(c_H/c_e)$, and were compared to the values of R_c in the absence of inhibitors (Fig. 3 b and c).

The Gibbs-Thomson relation $R_c = \Omega\gamma/\Delta\mu$, where Ω = 0.708 nm^3 is the molecular volume in the crystal, prescribes the values of γ corresponding to each of the $R_c(\Delta\mu)$ correlations: 25 ± 2 mJ m^{-2} in solution without inhibitors, 20 ± 2 mJ m^{-2} in the presence of MQ, and 22 ± 1 mJ m^{-2} in the presence of AQ. The standard deviations of the three γ values arise from the regression analyses of the linear correlations $R_c(\Delta\mu^{-1})$ and reveal that the confidence intervals of γ at the three tested solution compositions partially overlap.

We analyzed the similarity between the three individual values of γ by one-way analysis of variance (ANOVA), a statistical procedure, which compares the variance between two groups to the variance within each group of data. We computed individual γ values from each R_c measurement and examined the similarity between three pairs of γ data sets: no inhibitor/AQ, no inhibitor/MQ, and MQ/AQ. The ANOVA test parameters are listed in Extended Data Table 2. The three F values, corresponding to the ratio of the variances within each pair of data sets, are significantly greater than the critical values for groups consisting of 195, 177, and 297 independent measurements. The p values were of order 10^{-3}, 10^{-6} and 10^{-7}, respectively, smaller than the significance level of 0.05. These F and the p values consonantly certify that the hypothesis of equality of the three γ values is rejected.

Inhibitor-inhibitor complexation. The aim of these tests was to find out whether binary complexes between paired inhibitors form and reduce the inhibitor concentrations. Spectroscopic

characterization of solutions of the tested inhibitors reveals that the sum of the UV-vis absorbances of individual inhibitors is approximately identical to the absorbance of their combination. (Extended Data Figure 3 a –d). Moreover, no shift in absorbance peaks was observed after mixing. These results suggest that it is unlikely that complexes between two inhibitors form.

Inhibitor-hematin-inhibitor complexation. Complexes formed between hematin and antimalarial inhibitors were discussed by Egan and coworkers [43,44] and the complexation constants between hematin and antimalarial inhibitors in CBSO were reported by Olafson *et al.* [38]. Using established protocols, we tested for the complexation between hematin and four inhibitor pairs: QN/AQ, QN/MQ, CQ/AQ, and CQ/MQ. The two tested inhibitors were dissolved at equal concentrations in CBSO and 2 mL of this stock were mixed to a final concentration determined by the lower inhibitor solubility. Fresh hematin stock was diluted with CBSO to a concentration of 0.38 mM and then titrated with a solution of the inhibitor pair. At each titration step, the solution was gently stirred 8 – 10 min to complete complexation and a 350 mL aliquot was drawn for UV-vis spectrometry. The UV-vis adsorptions at 594 nm were measured for 40 titration steps and rescaled to account for the dilution. The absorbance A_i was compared with a theoretical curve calculated from the complexation constants of the two tested inhibitors.

The absorbance at around 594 nm displayed a clear shift to higher wavelengths after the addition of the inhibitor mixture, which indicates the formation of complexes. We calculated the theoretical A_{corr}/A_o values for four different models for each combination and chose the best fit from the minimal mean squared deviation between experimental and theoretical A_{corr}/A_o values. Nonzero deviations suggest the formation of complexes. The UV-Vis spectra of solutions containing two inhibitors and hematin indicate that in all four combinations, even if new complexes exist, their concentration would be limited to a level that does not appreciably attenuate the concentration of antimalarial inhibitors in solution(Extended Data Figure 3 e – i).

Therefore, the sequestration of inhibitors due to the formation of ternary inhibitor-hematin-inhibitor complexes is unlikely to be significant.

Kinetic Monte Carlo model of cooperativity between step pinners and kink blockers. We employ a standard solid-on-solid kinetic Monte Carlo (kMC) model of crystal growth. We use a surface of a Kossel crystal consisting of N_x = 50 by N_y = 100 sites occupied by N = 5000 surface molecules. In the kMC algorithm, a surface site is chosen at random and one of the possible kMC actions is performed based on the probability of the various actions; N repetitions of this act comprise one kMC time step. In the absence of inhibitors, three actions are possible at a surface site: a molecule attaches to the site, the molecular occupying the site detaches, or nothing happens (i.e. the molecule remains fixed). The probability for attachment is $dt \times \nu e^{\mu/k_B T}$, where dt is the kMC time step, ν is the inverse kMC time scale, μ is the chemical potential, T is the temperature, and k_B is Boltzmann's constant. The probability for a molecule to detach from site i is $dt \times \nu e^{E_i/k_B T}$, where E_i is the energy of the surface molecule at site i. The energy E_i is evaluated as the sum of the bond energies of the molecule with its six nearest neighbors. In a pure crystal, the bond energy is taken to be the same in all directions and is denoted ε. By expressing temperatures in the dimensionless form $k_B T/\varepsilon$ the physical value of ε is not needed. Given that a molecule in the bulk crystal has six bonds with the energy shared between it and its neighbors, the binding energy in the bulk is 3ε per molecule and so the equilibrium chemical potential is μ_{equil} = 3ε.

Inhibitors are handled in two district ways. Static inhibitors function as step pinners. They are deposited on the surface at the beginning of a simulation and do not participate in the kMC actions. When a crystal molecule is next to a static pinner, the bond energy between the two is taken to be zero. Thus, the only parameter needed to characterize the pinners is their surface density. Because they do not contribute to the binding of molecules to the crystal, the pinners disrupt and impede the growth of surface layers. For conceptual simplicity, we arrange the pinners in a square grid (Figure 4c). If the pinners are too close together (i.e. if their surface density is too

high), the step velocity is zero and crystal growth is arrested. The physics of step blocking by such inhibitors, the criterion for step pinning, and a demonstration that inhibition is independent of the physical arrangement of the step blockers has been extensively discussed in Lustsko, *et al.* [45].

A new feature of the present simulation work is the model of kink blockers. Similar to the solute molecules, the kink blockers are dynamic. In the presence of kink blockers, the pool of possible events at a crystal site is expanded to include their attachment and detachment. To block the kinks, the kink blockers must differ from the solute species and from the step pinners. We assume, for simplicity, that kink blockers do not bind to step pinners. We also assume that the kink blockers bind to the molecules in the crystal with a non-zero binding energy, otherwise, they would not exhibit a preference for kink sites. The kink blocker can only impede step growth if the bonding is weaker than the intermolecular bonds in the crystal ε. On the other hand, weakly bound inhibitors would have a low residence time at the kinks and have little or no effect on step growth [45]. To reconcile these two requirements, we assume that the kink blockers bind anisotropically. We assume that the only non-zero bonds formed by kink blockers are to in-plane crystal molecules. Furthermore, we assume that the in-plane bond strengths are not equal. Two out of the four in-plane bonding directions are randomly assigned bond strength 2ε and the other two, 0.5ε. The bond energy of a solute molecule deposited on top of a kink-blocker is ε.

The total energy of a kink blocker surrounded by crystal molecules is 6ε, equal to the crystal molecules so that the incorporation of kink blockers does not change the energetics of crystal growth. On the other hand, the asymmetry of their binding to the crystal surface modifies the kinetics of step growth. A kink blocker attached to a kink site with orientation that promotes two bonds of total energy 4ε will be bound stronger than a solute molecule bound with energy 3ε. Such kink blockers are unlikely to detach. On the other hand, the bonds that this kink blocker molecule can form with the incoming solute molecules are weak and solute molecules that deposit next to it are more likely to detach than if deposited in a free kink. These dynamics impede step growth.

A kink blocker attached to a kink in an unfavorable orientation, or adsorbed at a non-kink surface site, would have a total energy of 2.5ε or less and will tend to detach.

Our kMC model is subject to several constraints. First, the only model parameters that one can easily vary are the bond strengths in the various directions. Second, a foreign molecule acts as a kink blocker if (a) it is attracted to kink sites, (b) it inhibits step growth, and (c) it has a sufficient residency time to affect the step growth dynamics. These requirements inevitably lead to asymmetric lateral bonds with a total binding energy in a kink site equal to or greater than the energy of a crystal molecule in a kink site. Within these constraints, we do not expect our results to strongly depend on the numerical values chosen.

Errors were estimated by averaging the step velocity over windows of 1,000 surface updates thus producing a set of independent estimates of the velocity during the simulations. The arithmetic average of these values gives the overall estimate of the step velocity and the root-mean-squared deviation from the average of the averages is used to estimate its standard deviation. The error-bars reported in the figures are the standard errors of the step velocities calculated as their standard deviations divided by the square root of the number of samples.

The custom computer code used in these simulations is available upon reasonable request to James Lutsko, email: jim@lutsko.com.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.

Extended Data Figure Legends

Extended Data Figure 1 | Effects of step pinners and kink blockers on bulk hematin crystallization. a. Scanning electron micrographs of crystals grown in the presence of inhibitors at concentrations listed in each panel for 16 days at 23°C. **b and c**. Variations of the average length-to-width, *l*/*w*, aspect ratio A_{sp} of crystals grown in the presence of increasing concentrations of four inhibitor pairs at displayed ratios relative to the A_{sp} reached after growth in pure CBSO solutions for 16 days at 23°C. Lines are guides for the eye. Variations of the corresponding average crystal length *l* and width *w* are displayed in Fig. 1 f – i. **d.** Isobolograms characterizing the cooperativity of the CQ/MQ, CQ/AQ, QN/MQ, and QN/AQ inhibitor pairs in suppressing the length of β-hematin crystals. Open symbols indicate the concentrations of individual inhibitors that elicit a certain percent inhibition, sometimes referred to as inhibitory concentrations, IC. Dashed lines correspond to additive cooperativity between the paired inhibitors for certain percent inhibition and are horizontal if the inhibitor in the abscissa is inactive when applies alone. Solid symbols represent the concentrations of the paired inhibitors that evoke same inhibition. Rightward shifts of the solid symbols from the respective dashed lines indicate antagonistic cooperativity. The corresponding Combination Index (CI) values are listed in Extended Data Table 3.

Extended Data Figure 2 | Isobolograms characterizing the cooperativity of the CQ/MQ, CQ/AQ, QN/MQ, and QN/AQ inhibitor pairs in suppressing **a.** the step velocity *v*, and **b** the rate of two-dimensional nucleation rate J_{2D} of new layers. Open symbols indicate the concentrations of individual inhibitors that elicit a certain percent inhibition, IC. Dashed lines correspond to additive cooperativity between the paired inhibitors for certain percent inhibition and are horizontal if the inhibitor in the abscissa is inactive when applied alone. Solid symbols represent the concentrations of the paired inhibitors that evoke same inhibition. Rightward shifts of the solid symbols from the respective dashed lines indicate antagonistic cooperativity. The corresponding Combination Index (CI) values are listed in Extended Data Table 3.

Extended Data Figure 3 | Lack of complexation between kink blockers and step pinners in the solution. a – d. Lack of CQ/MQ, CQ/AQ, QN/MQ, and QN/AQ complexes. UV-vis absorption spectra of individual inhibitors and binary combinations indicated in the plots. The spectra of the binary solutions are nearly identical with the sum of the spectra of the individual inhibitors. **e – l**. Lack of ternary compounds that include hematin and the CQ/MQ, CQ/AQ, QN/MQ, and QN/AQ pairs of inhibitors. **e – h.** UV-vis spectra of hematin at concentrations c_H = 0.38 mM in the presence of various combinations of QN, CQ, AQ, and MQ (as indicated in the plots) at 1:1 molar ratios, where the inhibitor concentrations increase from top to bottom, as indicated by arrows. **i – l**. The relative decrease of the absorbance of a solution with initial c_H = 0.38 mM at 594 nm as a function of the concentration of the respective inhibitor pair (1:1 ratio) compared to a model assuming the presence of complexes of hematin with each of the individual inhibitors in the mixture, evaluated using the hematin-inhibitor binding constants from Olafson, *et al.* [38].

Extended Data Figure 4 | The correlation between the step velocity *v* and the inhibitor concentration in linearized coordinates $v_0'(v_0' - v)^{-1}$ and c_D^{-1} according to Eq. (7), for $c_D = [D]$ and $c_D = [H_2D]$, respectively, for (**a** and **b**) MQ and (**c** and **d**) AQ. Original data on the dependence of the step velocity on the concentration of the kink blockers MQ and AQ from Olafson et al. [38].

The values of the Langmuir constant K_{LB} determined from the slope of the straight lines are shown. The two leftmost data points for AQ, measured at C_{AQ} > 7 μM correspond to an unphysical increase in v at increasing concentration of AQ and were not considered in the regression analysis to determine K_{LB}.

Extended Data Figure 5 | The step velocity v in the presence step pinners and kink blockers, relative to that in pure solutions v_0 computed using Eq. (22). The values of ξ and K_{LB} are listed in Extended Data Table 2. γ_0 = 25 mJ m^{-2} is evaluated from the R_c determinations in Fig. 3. K_{LP} = 0.0027 μM^{-1} for CQ and 0.0013 μM^{-1} QN is evaluated from the $v(c_P)$ correlations for CQ and QN determined by Olafson, *et al.* [38] using Eqs. (14), (17), and (19). S_0= 1.12 nm^2 from the structure of β-hematin crystals [52]. **a.** The correlation between v_R and the concentrations of a step pinner (CQ or QN) c_P and kink blocker (MQ or AQ) c_B for the four listed inhibitor combinations. **b.** The step velocity v in the presence step pinners and kink blockers, relative to that in pure solutions v_0, at the listed constant ratios of kink blocker to step pinner, corresponding to experimental determinations in Fig. 2 c and e, compared to v in the presence of the listed step pinners only.

Extended Data Figure 6 | The regions of antagonistic and synergistic cooperativity in the plane of the concentrations of step pinners c_P and kink blockers c_B. Solid line represents the equation $(\partial v_R/\partial c_B)_{c_H,c_P} = 0$, where $(\partial v_R/\partial c_B)_{c_H,c_P}$ follows Eq. (28). This line corresponds to additive cooperativity and divides the (c_P, c_B) plane into fields where $(\partial v_R/\partial c_B)_{c_H,c_P} < 0$ marks that step pinners and kink blockers cooperate synergistically, and $(\partial v_R/\partial c_B)_{c_H,c_P} > 0$ indicates antagonistic cooperativity between the two inhibitors.

Extended Data Table Titles

Extended Data Table 1

The Combination Index CI for the four listed step pinner/kink blocker pairs calculated for the inhibition of crystal length in bulk crystallization experiments (corresponding to isobolograms in Extended Data Fig. 1d), the step velocity (isobolograms in Extended Data Fig. 2a), and the 2D nucleation rate of new crystal layers (isobolograms in Extended Data Fig. 2b). A classification of CI values as synergy, additivity, and antagonism is provided at the bottom.

Extended Data Table 2

The ANOVA parameters used to test the distinction between the values of the surface free energy γ in hematin solution in the absence of inhibitors and in the presence of AQ or MQ.

Extended Data Table 3

Concentrations of free hematin [H], free inhibitors [D], and kink blocker-hematin complexes [H_2B], governed by inhibitor-hematin complexation, evaluated at analytical

concentrations of hematin c_H and inhibitor C_B using complexation constants 14 and 510 mM^{-2} for MQ and AQ, respectively [Olafson, K. N., Nguyen, T. Q., Rimer, J. D. & Vekilov, P. G. Antimalarials inhibit hematin crystallization by unique drug–surface site interactions. *Proceedings of the National Academy of Sciences* **114**, 7531-7536, (2017)]. Evaluation of v_O' lowered from the step velocity in the absence of inhibitors v_0 owing to the decrease of hematin concentration from c_H to [H]. The variables $v_0'(v_0' - v)^{-1}$ and $c_B^{-1}$of the linearized form of the correlation between v and c_D, Eq. (7), for $c_B = [B]$ and $c_B = [H_2B]$, respectively.

Extended Data Table 4

The Langmuir constant for adsorption of MQ and AQ at kinks K_{LB} and the limiting fraction of occupied kinks ξ determined from the linear plots in Extended Data Figure 4 assuming that unliganded MQ and AQ are the active inhibitors and, alternatively, that the complexes H_2MQ and H_2AQ are the active inhibitors. Evaluation of [MQ] and [AQ] or [H_2MQ] and [H_2AQ] at C_B = 2.5 μM, at which the inhibitor effects on the surface free energy of the step edge γ were measured, and of the factors $\xi K_{LB}[B]$, $\xi \ln(1 + K_{LB}[B])$, $\xi K_{LB}[H_2B]$, and $\xi \ln(1 + K_{LB}[H_2B])$used in the evaluation of $\Delta\gamma$ in the presence of an inhibitor.