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Diverse clinical compounds alter the quaternary structure and inhibit the activity of an essential enzyme

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Abstract

An in vitro evaluation of the Johns Hopkins Clinical Compound Library demonstrates that certain drugs can alter the quaternary structure of an essential human protein. Human porphobilinogen synthase (*HsPBGS*) is an essential enzyme involved in heme biosynthesis; it exists as an equilibrium of high activity octamers, low activity hexamers, and alternate dimer configurations that dictate the stoichiometry and architecture of further assembly. Reduced *HsPBGS* activity is implicated in toxicities associated with lead poisoning and ALAD porphyria, the latter of which involves hexamer-favoring *HsPBGS* variants. A medium-throughput native PAGE mobility shift screen, coupled with evaluation of hits as *HsPBGS* inhibitors, revealed twelve drugs that stabilize the *HsPBGS* hexamer and inhibit *HsPBGS* activity in vitro. A detailed characterization of these effects is presented. Drug inhibition of *HsPBGS* in vivo by inducing hexamer formation would constitute an unprecedented mechanism for side effects. We suggest that small molecule perturbation of quaternary structure equilibria be considered as a general mechanism for drug action and side effects.

Keywords

Allosterism; Inhibitors; Lyases; Morpheesins; Proteins

INTRODUCTION

Many established therapeutic agents function by binding to and altering the activity of enzymes with desirable (the intended therapeutic effect) or undesirable (a side effect) outcomes. Furthermore, drugs can bind to an enzyme active site or to an allosteric site. A recently described morpheein model for allosteric regulation (Fig. 1a) involves a dissociative equilibrium of functionally distinct alternate quaternary structure assemblies.^[1] This model is based on our studies of porphobilinogen synthase (PBGS, E.C. 4.2.1.24, a.k.a. 5-aminolevulinate dehydratase, or ALAD). This study considers possible drug side effects in terms of small molecule modulation of the quaternary structure equilibrium of this essential human enzyme (Fig. 1b). PBGS functions in heme biosynthesis by catalyzing the condensation of two molecules of 5-aminolevulinic acid to form porphobilinogen (Fig. 2). Reduced activity of *HsPBGS* in vivo leads to accumulation of 5-aminolevulinic acid, a chemical homolog of the neurotransmitter γ -aminobutyric acid, and is involved in two human diseases: the rare genetic disease, ALAD porphyria,^[2] and the much more common condition of lead poisoning.^[3] Proper function of *HsPBGS* is also essential for certain forms of photodynamic therapy.^[4] The ease with which we were able to identify drugs that can

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modulate its quaternary structure equilibrium is thought provoking. Considering that the molecular mechanism responsible for drug action is often unknown, the current work suggests that modulation of quaternary structure dynamics for toxicity as well as efficacy is feasible.

Protein quaternary structure dynamics can be considered within the context of one multimer or on a broader scale including an equilibrium of alternate multimers. In the morphoein model of allosteric regulation a homo-oligomeric protein can reversibly interconvert between alternate, functionally distinct homo-oligomers via an obligate dissociation and conformational change in the dissociated state.^[1] The quaternary structure equilibrium for human PBGS (*HsPBGS*) includes a high activity octamer, low activity hexamer, and a suite of dimer conformations (Fig. 1b).^[5] A range of sophisticated biochemical and biophysical techniques have been used to unequivocally establish this dissociative allosteric model for PBGS.^[5b, 5c] Small molecule binding to one oligomeric component of the equilibrium has been shown to exert an allosteric effect on the protein by shifting the oligomeric equilibrium toward the ligand-bound assembly.^[5b, 6] Most significantly, it is established that the change in quaternary structure is responsible for the altered function.^[5a, 5b, 7]

A recent study successfully used a computational docking approach to find small molecules that selectively bind to the *HsPBGS* hexamer and inhibit catalytic activity.^[6a] In that case the docking target was a hexamer-specific surface cavity and the library of small molecules was a structurally diverse collection of “drug-like” molecules available from Life Chemicals, Inc. In vitro methods verified hexamer stabilization and correlated this with enzyme inhibition. Unexpectedly, one of the verified hits was a close structural analog of the amebicidal drugs Clioquinol and Iodoquinol, both of which were found to stabilize the *HsPBGS* hexamer and inhibit enzyme activity. Interestingly, a documented side effect of these drugs is consistent with inhibition of the *HsPBGS* activity.^[8] This surprising result led us to question whether the oligomeric equilibrium of *HsPBGS* could be altered by other drugs, potentially leading to toxicity. Thus we report, herein results of an *in vitro* screen of 1,514 compounds from the Johns Hopkins Clinical Compound Library (JHCCL) approved for pharmaceutical use in the United States or abroad. We looked for clinical compounds that would stabilize the *HsPBGS* hexamer and inhibit *HsPBGS* activity and found 15 chemically and pharmacologically diverse compounds that stabilized the *HsPBGS* hexamer, 12 of which also inhibited catalytic activity (Fig. 3). A novel native polyacrylamide gel electrophoresis (PAGE) mobility shift screen was used to test the drugs for their ability to stabilize the *HsPBGS* hexamer, and hits were then evaluated for their ability to inhibit catalytic activity. The characterization of the effects of these drugs on *HsPBGS* is presented and discussed in the context of a correlation with mechanism-based side effects arising from perturbation of the protein quaternary structure equilibrium of *HsPBGS*.

RESULTS AND DISCUSSION

Identification of hexamer-stabilizing drugs

The JHCCL compound library was screened in duplicate using native PAGE, as detailed in the experimental procedures. This medium throughput approach allowed us to complete the screen in approximately four months time using PhastGel technology. Evaluation of alternative high throughput screening techniques did not reveal a method that could discriminate on the basis of molecular weight for mass differences less than two-fold. Native PAGE clearly resolves *HsPBGS* into octamers and hexamers, and allows quantification of each species (Fig. 4). A noticeable increase in the mole fraction of *HsPBGS* hexamer was observed for a remarkable 28 drugs, nearly 2% of the library. None of the preliminary hits was defined as a controlled substance and all were available for purchase from Sigma or Toronto Research Chemicals. When freshly purchased samples of the preliminary hits were

evaluated, hexamer stabilization could be reproduced for 15 drugs, indicating the susceptibility of this screen to false positives. False positives or false negatives could arise for any number of reasons, including chemical events during storage and handling of the microtiter plates on which the library is distributed.

HsPBGS inhibition by hexamer-stabilizing drugs

Each of the compounds that was confirmed to increase the mole fraction of *HsPBGS* hexamer was subjected to a dose-response native PAGE analysis, and to kinetic inhibition studies as detailed in the experimental section. The *HsPBGS* hexamer-stabilizing compounds are chemically and pharmacologically diverse (Fig. 3), and are detailed below. Representative kinetic inhibition, dose response native PAGE gels, and quantified native PAGE results are shown in Figure 4 for two of the compounds, nitrofurazone, which inhibited *HsPBGS* activity to 0%, and tolefenamic acid, which inhibited *HsPBGS* activity to a plateau of 41 %. All inhibition and dose-response native PAGE data are summarized in Table 1, and presented in detail in Supplemental Figures S1 and S2. Because the native PAGE experiments were performed at a high protein concentration (0.3 mg mL^{-1}), while the kinetic experiments were performed at a low protein concentration ($10 \mu\text{g mL}^{-1}$), apparent $K_{0.5}$ values estimated from the gels appear higher than the IC_{50} values calculated from the inhibition kinetics. This is consistent with the law of mass action, the equilibrium illustrated in Figure 1b, and previous studies that utilized low-protein concentration native PAGE, protein concentration-dependent chromatography, and thermodynamic analyses of the oligomeric interconversion to establish this relationship.^[5c, 9]

Nitrofurazone is a bactericidal agent used in ointments marketed under the trade name Furacin. It is not frequently used since alternative antibacterial ointments are available that are more effective and less toxic.^[10] Nitrofurazone completely inhibited *HsPBGS* activity in assays with an IC_{50} of $2.1 \pm 0.2 \mu\text{M}$ (Table 1), and yielded approximately 50% conversion to the hexamer in the native PAGE assay at the highest concentration tested (Fig. 4a).

Copper sulfate is used topically in the treatment of phosphorous burns.^[11] In the past it was used as an emetic agent, but is now considered too toxic for ingestion.^[12] Copper sulfate (insoluble in DMSO) was dissolved in H_2O , and the copper sulfate data is presented relative to a H_2O control. Copper sulfate inhibits *HsPBGS* with comparable potency to Nitrofurazone and exhibits an IC_{50} of $2.2 \pm 0.1 \mu\text{M}$ (Table 1), and yields conversion to greater than 50% hexamer in the native PAGE assay at the highest concentration tested (Supplemental Fig. S1). Based on comparable results with copper chloride (not shown) we conclude that copper is the chemical responsible for the inhibition and hexamer stabilization.

Rose Bengal is a fluorescein derivative used in diagnostic eye drops to stain and identify damaged corneal tissue.^[13] Rose Bengal labeled with I^{125} or I^{131} had been used as a radioactive tracer marketed under the trade name Robengatope, though this product is no longer in use. A formulation of Rose Bengal (PV-10) is in clinical trials as a treatment for melanoma and breast cancer.^[14] Rose Bengal completely inhibited *HsPBGS* with an IC_{50} of $6.9 \pm 0.2 \mu\text{M}$ (Table 1); however, the native PAGE assay results did not support *HsPBGS* hexamer stabilization as the likely mode of inhibition. While the band representing the octamer decreased as a function of increasing Rose Bengal concentration, the band representing the hexamer did not increase. Rather, the appearance of smears and bands running at the dye front suggests that this compound is simply destabilizing the higher-order oligomers and perhaps denaturing the protein (Supplemental Fig. S1). Of those compounds that we characterized, Rose Bengal is the only compound for which protein migrated farther than the position of the hexamer band in the gels.

2,4-dinitroanisole is a compound used in lice repellants, insecticides, and pesticides. It is metabolized to 2,4-dinitrophenol, a compound that uncouples oxidative phosphorylation and was used to promote weight loss in the 1930's.^[15] Its use as a weight-loss agent was terminated due to the side effect of dangerously high body temperature. 2,4-dinitroanisole inhibited *HsPBGS* activity with an IC_{50} of $15 \pm 1 \mu\text{M}$ (Table 1), and yielded about 50% hexamer in the native PAGE assay at the highest concentration tested (Supplemental Fig. S1).

3,5-dibromosalicylaldehyde was labeled as an antiseptic in the JHCCL library. This compound also completely inhibited *HsPBGS* with an IC_{50} of $22 \pm 1 \mu\text{M}$ (Table 1). However, it is one of the most effective compounds for stabilizing the hexamer, yielding well over 50% of the protein as hexamer in the native PAGE assay (Supplemental Fig. S1).

Five structurally-related non-steroidal anti-inflammatory drugs from the fenamate class were identified by the screen, and were found to induce hexamer stabilization and inhibit *HsPBGS* activity very similarly to each other. Tolfenamic acid is marketed under the trade name Clotam and is in use in Europe. Flufenamic acid is marketed under the trade name Arlef, and the KEGG database suggests current use in Europe and Japan. Diclofenac is widely used in the U.S. and abroad, and can be obtained over the counter in many countries. Different prescription formulations are available in the U.S. under trade names including Arthrotec, Cataflam, Pennsaid, Solaraz, Voltaren, and Zipsor. Meclofenamic acid is currently in use in the U.S. and abroad, and is available in the U.S. as meclufenamate sodium. Niflumic acid is marketed in a topical formulation under the trade name Niflugel. Data is shown for a representative compound, tolfenamic acid (Fig. 4). All of the fenamates reduce *HsPBGS* activity to a plateau of 40–50% activity with similar IC_{50} values: tolfenamic acid – $2.1 \pm 0.3 \mu\text{M}$, flufenamic acid – $2.5 \pm 0.4 \mu\text{M}$, diclofenac – $9 \pm 2 \mu\text{M}$, meclufenamic acid – $9 \pm 2 \mu\text{M}$, and niflumic acid – $13 \pm 2 \mu\text{M}$ (Table 1). These compounds also exhibit similar results in the native PAGE assay, with less than 50% conversion to hexamer observed at the highest concentration tested (Supplemental Fig. S1).

The final two compounds that conclusively stabilize the *HsPBGS* hexamer and inhibit *HsPBGS* activity are less potent than those described above (Table 1 and Supplemental Fig. S1). Oxantel is an anthelmintic agent marketed under the trade name Telopar and is utilized to treat intestinal worms. This compound inhibited *HsPBGS* to a plateau to $46 \pm 3\%$ with an IC_{50} $18 \pm 3 \mu\text{M}$, but was relatively potent in the native PAGE assay, yielding over 50% of the protein as hexamer at the highest concentration tested. Bendroflumethiazide is a diuretic and antihypertensive marketed under the trade names Naturetin and Corzide. Bendroflumethiazide, the least potent inhibitor identified in this screen, inhibits *HsPBGS* activity with an IC_{50} of $130 \mu\text{M}$, and exhibits less than 40% *HsPBGS* conversion to hexamer at the highest concentration evaluated in the native PAGE assay.

We have established that there is complex interplay of factors that determines the position of the oligomeric equilibrium of *HsPBGS*.^[5c] For instance, basic pH favors the hexamer, the presence of substrate/product favors the octamer, and the law of mass action dictates that higher protein concentration favors octamer. The studies presented herein show hexamer stabilization in an alanine based acrylamide gel at pH 8.8 at very high ionic strength (880 mM alanine, 250 mM tris), in the absence of substrate, and at a modest protein concentration (0.3 mg mL^{-1}). In contrast, enzyme inhibition assays were carried out in 100 mM bis-tris propane, pH 8.0, in the presence of substrate, and at a lower protein concentration (0.01 mg mL^{-1}). Thus, it is not surprising that hexamer stabilization and enzyme inhibition, which are done under dissimilar conditions, are not quantitatively correlated. Nevertheless, our published studies on *HsPBGS* used an activity stained native PAGE technique and provided an unequivocal correlation between *HsPBGS* hexamer stabilization and enzyme

inactivation.^[6a] Similarly, in the current study we find that clinical compounds that showed the most pronounced hexamer stabilization are not necessarily the most potent inhibitors. We also find that some clinical compounds inhibit *HsPBGS* activity completely while others inhibit to a non-zero plateau. In fact, no correlation is present between IC_{50} values and the end point of inhibition.

Clinical relevance of *HsPBGS* quaternary structure dynamics

Protein structure dynamics are increasingly appreciated as essential to protein function. Proteins that use the morphein model of allosteric regulation are denoted as morpheins (Fig. 1a) and their existence adds a quaternary structure dynamic component to the principle that protein structure changes can govern protein function. PBGS is the prototype morphein and we previously established that small molecules can modulate PBGS function by preferentially binding to one component of the quaternary structure equilibrium.^[1, 6] Here we address the possibility that the morphein model of allostery may contribute to drug side effects. In the case of PBGS, drug stabilization of the inactive hexameric assembly is predicted to potentiate the symptoms of lead poisoning and ALAD porphyria and may interfere with certain forms of photodynamic therapy. Thus, it is significant that a structure-blind screen of clinical compounds revealed twelve hexamer stabilizing *HsPBGS* inhibitors. If the inhibition of *HsPBGS* by these agents is found to translate into physiologically relevant outcomes, this would constitute an unprecedented mechanism for a drug side effect, and would suggest that these medications be contraindicated for those suffering from lead poisoning, ALAD porphyria, and other porphyrias that result in accumulation of 5-aminolevulinate. While several of the identified compounds are used topically, it is important to note that topical medications are absorbed into the body. For example, patients using topical preparations of the amebicidal Clioquinol were found to have the drug present at micromolar levels in their plasma^[16] Furthermore, those which are topical might be contraindicated for patients considering certain types of photodynamic therapy.

The connection between our observed in vitro results of drug induced *HsPBGS* hexamer stabilization and in vivo side effects remains speculative but intriguing, particularly with regard to normal allelic variations in the human population. The two predominant alleles encode *HsPBGS* exists primarily as the active octamer under physiologically relevant conditions.^[5c] However, there are eight disease associated alleles for which compound heterozygotes suffer from ALAD porphyria. Each of these alleles encodes a *HsPBGS* with an increased propensity to form the inactive hexamer.^[17] Individuals carrying these alleles would be expected to be more sensitive to porphyrogenic side effects relative to the general population. In fact, the two drugs identified from our published computational screen, Clioquinol and Iodoquinol, are documented to have side effects similar to a porphyric attack.^[8] When tested in vitro, both drugs are more potent as *HsPBGS* hexamer stabilizing agents for two porphyria-associated variants relative to wild-type *HsPBGS*.^[6a] This raises the possibility for application of results such as ours to the burgeoning science of personalized medicine.

Notable in our results is the identification of the widely prescribed anti-inflammatory agent diclofenac as a *HsPBGS* inhibitor. Diclofenac is specifically contraindicated in porphyric patients as per recommendations of the American Porphyria Foundation.^[18] In some countries, this drug is available over the counter. The current work suggests one mechanism by which this drug may be porphyrogenic. The entire family of porphyric illnesses is episodic in nature and precipitating factors are poorly understood.^[19] The drugs identified in this study, particularly the widely used diclofenac formulations, may contribute to the episodic nature of porphyric diseases.

Diversity of hexamer-stabilizing drugs that inhibit *HsPBGS*

The structural variation between agents now shown to inhibit *HsPBGS* activity by stabilizing the inactive hexamer makes it unlikely that they all interact with the protein in the same way. While identifying the specific binding sites for these agents is outside the scope of this study, we can compare the drugs identified in this study with the compounds identified in our published work, which used a computational search for hexamer-stabilizing inhibitors. These prior studies focused on identifying inhibitors predicted to bind in a hexamer-specific surface cavity of *HsPBGS*.^[6a] The drugs identified in the current in vitro screen would not have been selected from a comparable docking study because the docked poses of these compounds would not have passed our in silico selection criteria. It remains possible that these *HsPBGS* hexamer stabilizing drugs may bind anywhere on the protein. The molecules appear sterically and chemically incompatible for binding at the *HsPBGS* active site. In support of this hypothesis, it is known that active site ligand binding generally stabilizes the PBGS octamer, rather than the hexamer.^[5b, 6a, 20]

Drug stabilization of alternate oligomers of other proteins

Intriguingly, flufenamic acid and diclofenac have previously been established to stabilize a specific oligomeric form of another human protein, transthyretin, and prevent its aggregation into amyloid plaques;^[21] however, the mechanism for this effect likely differs from the hexamer stabilization that we observe for these compounds with *HsPBGS*. Transthyretin performs its biological function of binding and transporting thyroxine as a tetramer, and the binding of thyroxine to two sites at the quaternary structure interface stabilizes the tetrameric assembly. The tetramer can dissociate to monomers in which the subunits can refold to become amyloidogenic and then irreversibly assemble into fibrils. This amyloid forming process occurs more readily with transthyretin mutations associated with the human diseases familial amyloid polyneuropathy, senile systemic amyloidosis, and familial amyloid cardiomyopathy.^[22] Flufenamic acid and diclofenac are structurally similar to thyroxine, and have been shown by X-ray crystallography to occupy the thyroxine binding site suggesting that they stabilize the tetramer via similar mechanisms as the native ligand.^[21b] This is different from the hexamer stabilization mechanism we propose for these drugs with *HsPBGS*. First, the drugs have no similarity to the enzyme substrate or product (see Figs. 2 and 3) and are unlikely to bind at the *HsPBGS* active site. Second, the alternate *HsPBGS* oligomers represent a reversible equilibrium of native states, rather than a process that requires refolding and an irreversible assembly into amyloid. Despite the presumably different mechanisms, the fact that the two chemically related drugs, flufenamic acid and diclofenac, can stabilize a specific oligomer of two different proteins suggests that this class of drugs may have an affinity for protein-protein interfaces.

The morpheein concept is new enough that PBGS is the only verified example, though suggestive data exist for other homo-oligomeric proteins.^[1, 4, 6b, 23] Here we show that a significant number of drugs can modulate *HsPBGS* activity by modulating the quaternary structure equilibrium and raise the possibility that some drug side effects may be due to this effect on *HsPBGS*. Since it is unlikely that PBGS is the only protein that regulates its activity through an equilibrium of alternate oligomers, we posit that numerous other proteins could also be subject to modulation via small molecule stabilization of an alternate oligomer and that this may be the mechanism through which some drugs induce side effects. It is documented that the quaternary structure equilibrium of *HsPBGS* is affected by naturally occurring sequence variation.^[17] Other proteins that utilize the morpheein mechanism would be susceptible to similar sequence-related variability. As with PBGS, this could contribute to individual variation in drug side effects.

EXPERIMENTAL SECTION

Materials

PhastSystem electrophoresis equipment and reagents were from GE Healthcare. Identified hits (or their chemical homologs) were purchased in the highest purity commercially available from Sigma or Toronto Research Chemicals and used without further purification. All other chemicals were from Fisher or Sigma and were the highest purity available.

Protein expression and purification

HsPBGS wild type (N59/C162A) was expressed and purified as described previously.^[17]

Initial screen

The Johns Hopkins Clinical Compound Library (JHCCL) was obtained from the Fox Chase Cancer Center Translational Research Facility in 96-well plate format where each well contained 2 μL of a 10 mM solution of compound in DMSO or ddH₂O. Samples were prepared by mixing *HsPBGS* [(0.3 mg mL⁻¹, 8.3 μM subunits) in 0.1M Bis-Tris propane-HCl, pH 8.0, 10mM β -mercaptoethanol, and 10 μM ZnCl₂] (8 μL) with 10mM compound in DMSO or H₂O (2 μL). The resultant samples, which contained 20% DMSO and 2mM compound, were incubated at 40 °C for 30 min, before loading and running the gels in duplicate. Electrophoresis was performed using a PhastSystem with PhastGel native buffer strips, and 6-lane (4 μL per lane) applicators were used to load the samples. Separations were performed using 12.5% polyacrylamide gels and each gel contained a negative control (incubation with DMSO alone) and a positive control (previously identified hexamer-stabilizing inhibitor, 5-chloro-7-(dimethylaminomethyl)quinolin-8-ol).^[6a] After separation, gels were developed on the PhastSystem using Coomassie Blue stain. The native gel mobility shift evaluation was repeated for each of the preliminary hits using freshly purchased stocks prepared at 10 mM in DMSO unless otherwise noted.

Dose response native PAGE mobility shift evaluation

The 15 drugs confirmed to stabilize the *HsPBGS* hexamer were further examined by native PAGE as a function of compound concentration. Samples were prepared as described above, but with varying concentration of each compound (0, 30 μM , 100 μM , 300 μM , 1 mM, and 2 mM). The final concentration of DMSO in each sample was maintained at 20%. Samples were incubated at 42 °C for 1 h prior to resolution on 12.5% native gels as described above.

Quantification of mole fraction of hexamer in gels

Quantification of PAGE results by densitometry was carried out using the program ImageJ.^[24] Three separate determinations were made to quantify the density of each gel band. The quantified data for each gel lane is described as % hexamer, which was defined as the amount of protein present in the hexamer band relative to the total protein in the hexamer and octamer bands. For the dose response by gel shift evaluations, the data were fitted either to a simple hyperbolic equation (Equation 1), or when appropriate, an offset hyperbolic equation (Equation. 2):

$$\%Hexamer = (\%Hexamer_{\max})([I]) / (K_{0.5} + [I]) \quad (1)$$

$$\%Hexamer = \%Hexamer_o + [(\%Hexamer_{\max} - \%Hexamer_o)[I]] / (K_{0.5} + [I]) \quad (2)$$

where $\%Hexamer_{max}$ is derived from the fit as the highest fraction of hexamer observed, $[I]$ is the concentration of the inhibitor, $K_{0.5}$ is the concentration of inhibitor at the midpoint of the curve, $\%Hexamer_0$ is the fraction of hexamer in the starting sample. The fits were constrained such that $\%Hexamer_0 \geq 0$, and $\%Hexamer_0 + \%Hexamer_{max} \leq 100\%$. Gel data were fit using the program SigmaPlot®.^[25] Statistical error is reported as standard deviations.

Inhibition of *HsPBGS* catalytic activity by hexamer-stabilizing compounds

The catalytic activity of *HsPBGS* was assayed by measuring the product, porphobilinogen, colorimetrically as described previously [5b, 6a]. Inhibition was assessed using the activity assay following incubation of enzyme in the appropriate assay buffer (90 μ L) with compound solution (varied stock concentrations in DMSO) or DMSO alone (10 μ L) at 42 °C for 1 h. Following this preincubation, assay buffer comprising 0.1 M BTP-HCl, pH 8.0, 10 mM β -ME, and 10 μ M $ZnCl_2$ (800 μ L) was added and the mixture was allowed to equilibrate at 37 °C for 15 min prior to initiating the reaction with 0.1 M ALA-HCl (100 μ L). The compound concentrations reported in the text are those in the final assay volume (1 mL). Inhibition data were plotted as fractional activity relative to the absence of compound and inhibition curves were fitted to a hyperbolic decay (Equation 3), or when appropriate, a hyperbolic decay with a non-zero endpoint (Equation 4):

$$FA = (FA_{max})(IC_{50}) / (IC_{50} + [I]) \quad (3)$$

$$FA = FA_{min} + [(FA_{max} - FA_{min})(IC_{50})] / (IC_{50} + [I]) \quad (4)$$

where FA is the fractional activity, FA_{max} is the fractional activity in the absence of compound (set as 100%), FA_{min} is the minimum fractional activity derived from the titration, $[I]$ is the concentration of compound, and IC_{50} is the $[I]$ at 50% inhibition. All kinetic data were fit using the program SigmaPlot® (Systat Software, Inc., San Jose, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The morphoein model of allosteric regulation illustrated with a schematic example and with *HsPBGS*

Panel **a** depicts a homo-oligomeric protein for which the available functionally distinct multimeric states are a trimer and a tetramer, and two conformations of a monomer, one that can trimerize and one that can tetramerize. The “rule of engagement” for multimeric assembly is the association of a thick line with a dashed line. A regulator molecule (depicted as a grey wedge) has the appropriate geometry to bind only to the pro-trimer and trimer forms and shift the equilibrium in that direction, thus allosterically stabilizing the trimer and promoting its function. Panel **b** illustrates the dynamic equilibria between the quaternary structure assemblies of *HsPBGS*. The two predominant forms in solution are the high activity octamer (PDB code 1E51) and the inactive hexamer (PDB code 1PV8); interconversion between these forms requires dissociation to a dimer, which can take on alternate conformations [5b, 5c]. The interchange between dimer conformations involves alterations in a small number of ϕ/ψ angles that changes the orientation of the $\alpha\beta$ -barrel domains of each subunit with respect to each other. One significant change in ϕ/ψ angles is at residue 23, highlighted in the magnified panels.

Figure 2. The PBGS-catalyzed reaction

PBGS catalyzes the asymmetrical condensation of two molecules of 5-aminolevulinic acid (ALA) to form porphobilinogen in the first common step of tetrapyrrole biosynthesis. In humans, PBGS functions in the heme biosynthetic pathway.

Figure 3. Drugs confirmed to stabilize the *Hs*PBGS hexamer and inhibit catalytic activity
 Nitrofurazone (C₆H₆N₄O₄, CAS # 59-87-0), copper sulfate (CuSO₄, CAS# 7758-98-7), Rose Bengal (C₂₀H₄Cl₄I₄O₅, CAS# 11121-48-5), 2,4-dinitroanisole (C₇H₆N₂O₅, CAS# 119-27-7), 3,5-dibromosalicylaldehyde (C₇H₄Br₂O₂, CAS# 90-59-5), bendroflumethiazide (C₁₅H₁₄F₃N₃O₄S₂, CAS# 73-48-3), tolfenamic acid (C₁₄H₁₂ClNO₂, CAS# 13710-19-5), flufenamic acid (C₁₄H₁₀F₃NO₂, CAS# 530-78-9), meclofenamic acid (C₁₄H₁₁Cl₂NO₂, CAS# 6385-02-0), diclofenac (CAS# 15307-86-5), niflumic acid (C₁₃H₉F₃N₂O₂, CAS# 4394-00-7), and oxantel pamoate (C₁₃H₁₆N₂O, CAS# 68813-55-8).

Figure 4. Representative data for two hexamer-stabilizing drugs on the activity and oligomeric equilibrium of *HsPBGS*

Nitrofurazone (**a** and **b**) represents a typical data set for a compound that reduced *HsPBGS* activity to zero. Tolfenamic acid (**c** and **d**) represents a typical data set for a compound that reduced activity to a defined non-zero plateau. For panels **a** and **c**, a dose-response native PAGE gel ($300\ \mu\text{g mL}^{-1}$ *HsPBGS*) is inset in a plot showing the densitometry analysis of the gel (○). For panels **b** and **d**, the kinetic inhibition data ($10\ \mu\text{g mL}^{-1}$ *HsPBGS*) is shown (●). The data are fit to single hyperbolae as described in the text. Error bars representing standard deviation are shown where they exceed the size of the data point. Data for all compounds is included in Supplementary Materials.

Table 1

Parameters extracted from kinetic inhibition and hexamer-stabilization experiments.

	<i>IC</i> ₅₀ (μM)	<i>FA</i> _{min} (%)	% <i>Hexamer</i> _{max} (obs)	% <i>Hexamer</i> _{max} (calc)	<i>K</i> _{0.5} (μM)
Nitrofurazone	2.1 ± 0.2	0 [a]	50 ± 1	65 ± 3	710 ± 160
Copper sulfate	2.2 ± 0.1	0 [a]	67 ± 4	76 ± 9	100 ± 50
Rose Bengal	6.9 ± 0.2	0 [a]	100 ± 7 [b]	100 ± 10 [b]	60 ± 20 [b]
2,4-dinitroanisole	15 ± 1	0 [a]	43 ± 1	60 ± 9	1200 ± 500
3,5-dibromo-salicylaldehyde	22 ± 1	0 [a]	70 ± 1	100 ± 20	1200 ± 600
Tolifenamic acid	2.1 ± 0.3	41 ± 2	60 ± 1	63 ± 1	130 ± 7
Flufenamic acid	2.5 ± 0.4	41 ± 2	65 ± 1	67 ± 3	110 ± 20
Diclofenac	9 ± 2	35 ± 5	82 ± 3	90 ± 30	1900 ± 1300 [c]
Meclofenamic acid	10 ± 2	40 ± 4	45 ± 1	100 ± 20	600 ± 300
Niflumic acid	13 ± 2	54 ± 3	47 ± 2	45 ± 9	120 ± 100
Oxantel	18 ± 3	46 ± 3	62 ± 2	100 ± 30	1600 ± 1000 [c]
Bendroflumethiazide	130 ± 10	0 [a]	33 ± 1	[c]	[c]

The *IC*₅₀ and *FA*_{min} derive from the kinetic inhibition assays; %*Hexamer*_{max} (obs) is the percentage of hexamer observed at 2 mM compound, while %*Hexamer*_{max} (calc) and *K*_{0.5} derive from fits of the dose-response native PAGE as detailed in materials and methods. All kinetic data, gel quantifications, and original gels are available in Supplemental Figures S1 and S2.

[a] For the inhibitors where *FA*_{min} clearly approached 0, this was set as the endpoint.

[b] The native PAGE experiment for Rose Bengal revealed heavy smears and accumulation of species smaller than hexamers, suggesting promiscuous denaturing/unfolding rather than hexamer stabilization. These gels were quantified in terms of “octamer destabilization”.

[c] Several of the fits for the dose response native PAGE experiments were non optimal and yielded high errors, sometimes more than half of the extracted constant. We include these values for reference, but acknowledge the uncertainty.