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Environmental contaminants perturb fragile protein assemblies and inhibit normal protein function

Sarah H. Lawrence, Trevor Selwood, and Eileen K. Jaffe

Developmental Therapeutics, Fox Chase Cancer Center 333 Cottman Avenue, Philadelphia, PA, 19111 (USA)

Abstract

The molecular mechanisms whereby small molecules that contaminate our environment cause physiological effects are largely unknown, in terms of both targets and mechanisms. The essential human enzyme porphobilinogen synthase (*HsPBGS*, a.k.a. 5-aminolevulinate dehydratase, ALAD) functions in heme biosynthesis. *HsPBGS* catalytic activity is regulated allosterically via an equilibrium of inactive hexamers and active octamers, and we have shown that certain drugs and drug-like small molecules can inhibit *HsPBGS* *in vitro* by stabilizing the hexamer. Here we address whether components of the National Toxicology Program library of environmental contaminants can stabilize the *HsPBGS* hexamer and inhibit activity *in vitro*. Native polyacrylamide gel electrophoresis was used to screen the library (1,408 compounds) for components that alter the oligomeric distribution of *HsPBGS*. Freshly purchased samples of 37 preliminary hits were used to confirm the electrophoretic results and to determine the dose-dependence of the perturbation of oligomeric distribution. Seventeen compounds were identified which alter the oligomeric distribution toward the hexamer and also inhibit *HsPBGS* catalytic activity, including the most potent *HsPBGS* inhibitor yet characterized (Mutagen X, $IC_{50} = 1.4 \mu\text{M}$). PBGS dysfunction is associated with the inborn error of metabolism known as ALAD porphyria and with lead poisoning. The identified hexamer-stabilizing inhibitors could potentiate these diseases. Allosteric regulation of activity via an equilibrium of alternate oligomers has been proposed for many proteins. Based on the precedent set herein, perturbation of these oligomeric equilibria by small molecules (such as environmental contaminants) can be considered as a mechanism of toxicity.

Keywords

ALAD; enzyme inhibition; environmental contaminants; morphine; PBGS; protein assembly

INTRODUCTION

The essential enzyme porphobilinogen synthase (PBGS, EC 4.2.1.24, also known as 5-aminolevulinate dehydratase, or ALAD) catalyzes the asymmetric condensation of two

Phone: 215-728-3695, Fax: 215-728-2412, Eileen.Jaffe@fccc.edu.

Supplementary data description: Supplemental Figure S1. Dose response of hexamer stabilization as assessed by native PAGE for all hits identified from the NTP library.

molecules of aminolevulinic acid (ALA) to form porphobilinogen in the first common step of tetrapyrrole (e.g. heme, chlorophyll, vitamin B₁₂) biosynthesis (Fig. 1). Severe inhibition of human PBGS (*HsPBGS*) *in vivo*, which can occur through varied mechanisms discussed below, plays a role in multiple disease states [1-4]. The most common disease related to *HsPBGS* inhibition is lead poisoning, which occurs *via* classic active-site inhibition [5]. Lead binds and displaces a catalytically essential zinc, crippling enzyme activity [6]. The resultant elevated levels of ALA, a chemical homologue of the neurotransmitter gamma aminobutyric acid (GABA), have been postulated as a cause for some neurologic sequelae of PBGS inhibition [3, 7-9].

HsPBGS activity is regulated, in part, by its participation in a fascinating structural equilibrium among alternate oligomeric forms. The homo-oligomeric *HsPBGS* exists in an equilibrium of high-activity octamers and low activity hexamers that interconvert by dissociating to dimers which can undergo a conformational change (Fig. 2A and B) [10]. Two alternate conformations of the dimer each support assembly to a specific oligomer. Under normal physiological conditions, the oligomeric equilibrium of wild-type *HsPBGS* favors the octamer at a mole fraction of >95% [11]. Perturbation of the equilibrium towards the hexamer decreases activity. Several point mutations to the gene encoding *HsPBGS* give rise to the disease ALAD porphyria. Each of the disease-associated *HsPBGS* variants displays an increased mole fraction of hexamer relative to wild-type, suggesting that the reduced catalytic activity resulting from perturbation of the oligomeric equilibrium causes physiologic symptoms *in vivo* [12].

The oligomeric equilibrium of *HsPBGS* makes the enzyme susceptible to allosteric (by definition, distinct from the active site) inhibition via the morpheein model of allostery [13]. The distinctive feature of the morpheein model (Fig. 2C) is a required conformational change in a dissociated state, [14]. The schematic in Figure 2C illustrates a protein that exists as an equilibrium of trimers and tetramers that interconvert by dissociating to a conformationally flexible monomer. The two conformations of the monomer each support assembly to a specific oligomer. The binding site for the allosteric regulator (shown as checkered wedges) exists only on the trimer and pro-trimer forms. Binding of the regulator prevents the conformational change in the monomer and draws the equilibrium towards the trimer, favoring the function of that form.

Our previous studies identified allosteric inhibitors of *HsPBGS* (and PBGS from other species) that function by stabilizing the inactive hexamer [15-18]. The surface topography of the *HsPBGS* hexamer is sufficiently different from that of the *HsPBGS* octamer that distinct, oligomer-specific binding sites exist on the respective surfaces (Fig. 2D and E). Several allosteric *HsPBGS* inhibitors that function by binding to and stabilizing the inactive hexamer were identified through *in silico* docking to the hexamer-specific putative binding site highlighted in Fig. 2D [17]. Subsequently, an *in vitro* screen of drugs approved for human use identified 12 drugs that also stabilize the *HsPBGS* hexamer and inhibit catalytic activity [15]. The binding sites for these compounds have not been unequivocally established; the site targeted for *in silico* inhibitor identification, and the surfaces highlighted Fig. 2E are two potential sites where significant surface cavity differences exist between the octamer and hexamer.

In the present study, we screened a collection of 1,408-compounds (environmental contaminants selected by the National Toxicology Program [19]) for compounds that shift the *HsPBGS* oligomeric equilibrium towards the hexamer and inhibit catalytic activity *in vitro*. We identify 17 functionally and chemically diverse compounds that function as oligomer-perturbing inhibitors of *HsPBGS* including drugs, metals, and myriad benzene derivatives. Evaluation of the *in vivo* effects of these compounds on *HsPBGS* is outside the scope of the current study; however, we posit that the observed inhibition of *HsPBGS* via perturbation of the oligomeric equilibrium could result in clinical symptoms similar to those observed for lead poisoning and ALAD porphyria. Furthermore, these symptoms could be exacerbated for patients with these conditions.

While *PBGS* is the first protein unequivocally established to utilize the morpheein model of allostery, numerous other proteins could be susceptible to this mode of allosteric inhibition [14]. The larger scope of enzyme inhibition via oligomer-perturbation is discussed as a putative mechanism of action for diverse small molecules.

Materials and Methods

PhastSystem electrophoresis equipment and reagents were from GE Healthcare. The collection of 1,408 compounds (herein referred to as the NTP library) was a generous gift from the National Toxicology Program/National Institute of Environmental Health Sciences. Identified hits (or their chemical homologues) were purchased 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 [12]. The Asn59-containing allele is the less common allele encoded by the human gene *ALAD2*. The C162A variant is a benign mutation that renders the protein less susceptible to intradomain disulfide formation but does not affect catalytic activity.

Initial screen

The initial screen utilized a native polyacrylamide gel electrophoresis (PAGE) approach that capitalized on the resolution of *HsPBGS* octamers and hexamers into distinct bands. The 1,408-compound NTP Library was obtained in 96-well plate format where each well contained a 10 mM solution of compound in DMSO or ddH₂O. Samples were prepared by mixing 8 μ L of protein (0.3 mg/ml, 8.3 μ M subunits) in 0.1 M Bis-Tris propane-HCl (BTP-HCl), pH 8.0, 10 mM β -mercaptoethanol (β -ME), and 10 μ M ZnCl₂ with 2 μ L of 10 mM compound in DMSO or ddH₂O. The resultant samples, which contained 2 mM compound and 20% DMSO (when DMSO was the solvent), were incubated at 40 °C for 30 min, before loading and running the gels in duplicate. PAGE was performed using a PhastSystem with PhastGel native buffer strips, and 8-lane (1 μ L 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 [17]). Following electrophoresis, gels were developed on the PhastSystem using Coomassie Blue stain. *HsPBGS* incubated with DMSO migrates predominantly as an octamer (>95%) with the hexamer comprising the only visible minor component (<5%); compounds that visibly increased the intensity of the hexamer band (relative to the negative control lane on that gel) were identified as preliminary hits. It is important to note that all compounds identified as preliminary hits may have been subject to chemical events during storage and handling of the 96-well plates.

Dose response native PAGE mobility shift evaluation

The native PAGE mobility shift evaluation was repeated for each of the preliminary hits using freshly purchased stocks dissolved in ddH₂O or DMSO for compounds insufficiently soluble in water. Thirty-two compounds 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; or 0, 300 μM, 1 mM, 3 mM, 10 mM and 20 mM). The range of concentrations used for each compound was determined by the solubility of that compound. For compounds dissolved in DMSO, 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 the mole fraction of hexamer in gels

Quantification of PAGE results by densitometry was carried out using the program ImageJ [20]. Three separate determinations were made to quantify the density of each gel band. The quantified native PAGE data is presented and discussed as “mole fraction of hexamer,” which is defined as the quantified density of protein present in the hexamer band relative to the quantified density of total protein in the lane.

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 [17, 21]. Inhibition was assessed using the activity assay following incubation of 90 μL enzyme (111 μg/mL, 3.1 μM) in the appropriate assay buffer with 10 μL of compound solution (varied stock concentrations in DMSO) or DMSO alone at 42 °C for 1 h. Following this preincubation, 800 μL of assay buffer (0.1 M BTP-HCl, pH 8.0, 10 mM β-ME, and 10 μM ZnCl₂) was added and the mixture was allowed to equilibrate at 37 °C for 15 min prior to initiating the reaction by addition of 100 μL of 0.1 M ALA-HCl, bringing the final pH to 7.8. The compound concentrations reported in the text are those in the final 1 mL assay volume. The final *HsPBGS* concentrations in the 100 μL preincubation and 1 mL activity assay solutions were 2.8 μM and 0.28 μM, respectively. The specific activity of *HsPBGS* in the absence of compound was ~40 μmoles h⁻¹ mg⁻¹. Inhibition data were plotted as fractional activity relative to the absence of compound and inhibition curves were fitted to a hyperbolic decay (Eqn. 1), or when appropriate, a hyperbolic decay with a non-zero endpoint (Eqn. 2):

$$FA = (FA_{max}) IC_{50} / (IC_{50} + [I]) \quad \text{Eqn. 1}$$

$$FA = FA_{min} + \{(FA_{max} - FA_{min}) IC_{50} / (IC_{50} + [I])\} \quad \text{Eqn. 2}$$

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).

Results

The initial native PAGE screen of the NTP library revealed 37 compounds that increased the mole fraction of hexamer by at least a factor of two; these are the preliminary hits. Five compounds, described below, were not analyzed further. Two preliminary hits were trans-cinnamaldehyde and an unknown mixture of cinnamaldehyde isomers; only all trans-cinnamaldehyde was further characterized. Two preliminary hits, cadmium oxide and p-divinyl benzene were not further characterized due to insolubility in reagents compatible with the *Hs*PBGS activity assay. Valerenic acid, as well as its acetoxo- and hydroxy-derivatives were preliminary hits. Due to availability, only valerenic acid was further evaluated. The remaining preliminary hits were purchased for further analyses, which included dose-dependent oligomer perturbation and inhibition of *Hs*PBGS catalytic activity. The 15 compounds that inhibited *Hs*PBGS activity by at least 50% at a concentration of 100 μ M (as well as two less effective, but related, compounds) are described in detail below. The structures of these compounds are presented in Figure 3, and the quantification of kinetic and native PAGE data is presented in Table 1.

For all compounds, the activity inhibition data derives from kinetic analyses performed at a protein concentration of 10 μ g/mL in the presence of 10 mM substrate, which is known to stabilize the octamer [10]. In contrast, the native PAGE analyses were performed at a protein concentration of 240 μ g/mL in the absence of substrate; therefore quantitative comparison of the kinetic and native PAGE data is unfounded. Furthermore, the position of the oligomeric equilibrium of *Hs*PBGS is governed by a complex interplay of factors [17, 22], including pH and ionic strength; the altered oligomeric equilibrium in response to pH correlates with pH dependent enzyme activity [11]. As such, a compound that appears to be a very effective hexamer stabilizer (as assessed by native PAGE performed in the absence of substrate at high ionic strength) will not necessarily inhibit catalytic activity at a lower ionic strength in the presence of substrate.

The identified hits can be divided into 8 general categories, and are clustered as such in Figure 3. The kinetic data (Fig. 4) is grouped in a similar fashion, with all of the compounds from a category presented on the same plot. For the native PAGE results, a representative gel for one compound from each category is presented in Figure 5, and all of the gels are presented in Supplemental Figure S1.

As seen in the control lane (0 mM compound) for each gel (Fig. 5), untreated *Hs*PBGS migrates primarily as an octamer (upper major band) with a small amount of hexamer (lower major band). We screened for compounds that increased the mole fraction of hexamer. Although some compounds in the initial screen increased the mole fraction of octamer and

decreased that of hexamer; these were not pursued because octamer-stabilizing compounds are not predicted to inhibit *Hs*PBGS activity. Most of the identified hits induced a gradual dose-dependent increase in the mole fraction of hexamer, as evidenced by the increasing intensity of the lower major band. Several of the compounds caused protein to appear at other positions on the gel (positions labeled on Fig. 5D). The “sample wells” are the position that the protein is loaded on the gel; protein that remains in the sample well during electrophoresis indicates formation of high molecular weight aggregates that cannot migrate into the acrylamide. The “dye front” is the endpoint of molecules migrating through the gel during electrophoresis. Protein that migrates at the dye front is a species that is not retained by the acrylamide under the experimental conditions. Two gels (Fig. 5G and H) show faint bands appearing well below the hexamer position, but above the dye front. This band most likely represents the dimeric form of *Hs*PBGS [11].

Mutagen X

The most potent *Hs*PBGS inhibitor identified in this study is 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H-furanone), commonly known as Mutagen X (Fig. 3A). Mutagen X is widely distributed in the environment; it is formed as a byproduct of the chlorination of drinking water [23-24]. The mutagenic route of Mutagen X toxicity is well-established; it is one of the most potent bacterial mutagens ever characterized [25], and has been shown to cause mutations in mammalian cell lines *in vitro* (e.g. [26]) and to act as a carcinogen to rats *in vivo* [27].

Here we identify another potential route for Mutagen X toxicity in humans as via inhibition of *Hs*PBGS; this compound is the most potent inhibitor of *Hs*PBGS ever characterized with an $IC_{50} = 1.4 \pm 0.1 \mu\text{M}$ (Fig. 4A). Native PAGE demonstrates that increasing concentrations of Mutagen X increase the ratio of hexamer to octamer (Fig. 5A); however, an additional faint band of protein also migrates at the dye front, which may represent denatured protein monomer. Because the concentration at which a small amount of protein denaturation is observed is approximately three orders of magnitude higher than the IC_{50} , we conclude that Mutagen X is not simply a promiscuous denaturing inhibitor [28].

Stabilization of the inactive hexamer likely contributes to Mutagen X inhibition of *Hs*PBGS, but may not be the only mechanism of inhibition. Due to the exquisite potency of Mutagen X against *Hs*PBGS, it was also evaluated as an inhibitor of PBGS from *Pisum sativum* (green pea), as well as the human pathogens *Pseudomonas aeruginosa*, and *Yersinia enterocolitica*; Mutagen X was found to inhibit all of these proteins with comparable potency (data not shown). This is an unexpected result for hexamer-stabilizing PBGS inhibitors as the amino acid composition of putative binding sites for oligomer-perturbing allosteric inhibitors of PBGS (see Fig 2D and 2E) varies among species, and previously discovered allosteric inhibitors have demonstrated species specificity [17-18]. Mutagen X inhibition of PBGS from multiple species suggests that it inhibits via an additional mechanism, potentially by binding at the enzyme active site. We are pursuing additional studies to identify the binding site and determine the specific mechanism(s) through which Mutagen X inhibits PBGS.

Nitrofurans

Two of the compounds identified as oligomer-perturbing *HsPBGS* inhibitors in this study, nitrofurazone and nitrofurantoin, are antibiotics from the broad spectrum nitrofuran group (Fig. 3B). While a topical formulation of nitrofurazone (Furacin) is approved for human use, internal nitrofuran use is largely limited to veterinary settings – particularly in animals used for food production. Concern about potential carcinogenicity of these drugs and a lack of information about their metabolites [29] led to bans on their use in food-producing animals by the European Union, the United States and many other countries. However, widespread misuse of the drugs continues because they are cheap and effective. Consequently nitrofuran metabolites can be found worldwide in poultry and farmed seafood [30-31].

Nitrofurazone and nitrofurantoin are among the more potent *HsPBGS* inhibitors identified from the NTP Library with IC_{50} values of 2.1 ± 0.2 and 5.9 ± 0.4 μ M, respectively. Despite the similarities in IC_{50} , nitrofurazone is a more effective hexamer stabilizer ($48.5 \pm 0.3\%$ hexamer at 2 mM nitrofurazone versus $16.0 \pm 1.0\%$ hexamer at 20 mM nitrofurantoin) as assessed by native PAGE (Fig. 5B). Notably, nitrofurazone was identified as a hexamer-stabilizing *HsPBGS* inhibitor in a previous study that screened the Johns Hopkins Clinical Compounds Library using a similar native PAGE approach [15]; nitrofurantoin, which was also in the Johns Hopkins library, was not identified as a hit during that native PAGE mobility shift screen. Such discrepancies are not uncommon in library screening results, and could result from differences in storage and handling of the libraries, age of the libraries, or discrepancies in the reported concentrations of the compounds.

Captan

Captan is a fungicide from the phthalimide family with widespread use in agriculture (Fig. 3C), and was one of the most commonly found pesticides in a recent analysis of Brazilian strawberries, pears, apples, peaches and tomatoes [32]. Captan was originally listed as a “probable” carcinogen by the United States Environmental Protection Agency, but it was later deemed unlikely to be carcinogenic at normal exposure levels. Exposure to captan dust (a risk predominantly limited to agricultural workers) causes skin, eye and respiratory tract inflammation [33]; dietary Captan exposure produced reproductive anomalies in *Drosophila* [34]. The specific targets for Captan toxicity are unknown but the compound reacts readily with thiols to form thiophosgene, a compound that is known to cause gastrointestinal lesions and malignancies [35]. Captan inhibits *HsPBGS* with an IC_{50} of 3.0 ± 0.3 μ M, and is also an effective hexamer stabilizer, shifting the oligomeric equilibrium to $72.3 \pm 0.3\%$ at 2 mM Captan (Table 1, Fig. 5C).

Cisplatin

The chemotherapeutic drug cisplatin (Fig. 3D) functions as a DNA intercalating agent. At therapeutic levels, well-documented side effects include nephrotoxicity, neurotoxicity and hearing damage. Occupational exposure to cisplatin is a documented risk for medical and pharmaceutical personnel, but the toxicity of this low-dose chronic exposure has not been well characterized [36]. Cisplatin inhibits *HsPBGS* with an IC_{50} of 17.1 ± 1.5 μ M. Cisplatin also increases the ratio of hexamer to octamer at cisplatin concentrations > 1 mM, but higher concentrations cause *HsPBGS* to precipitate, as evidenced by the absence of migrating

bands and large amounts of protein remaining in the sample wells of the native PAGE lanes containing higher concentrations of cisplatin (Fig. 5D).

Halogenated benzaldehydes

Five closely related halogenated benzaldehydes (2,6-dichlorobenzaldehyde, 3,4-dichlorobenzaldehyde, 2-chlorobenzaldehyde, 3-bromobenzaldehyde, 2,4-dichlorobenzaldehyde) were identified as *Hs*PBGS hexamer stabilizers in the screen (Fig. 3E). These compounds, and many other halogenated benzaldehydes, are common intermediates in industrial syntheses of a wide range of products including antiseptics, fragrances, dyes and paints [37]. While comprehensive human toxicology data is not available, these volatile organic compounds are generally considered toxic and irritating.

Intriguingly, these related compounds have widely varied impacts on the activity of *Hs*PBGS (Table 1 and Fig. 4E) and its oligomeric distribution (Table 1, Fig. 5E, Supplemental Fig. S1). The most potent, 2,6-dichlorobenzaldehyde, inhibits *Hs*PBGS with an IC_{50} of $15.2 \pm 1.5 \mu\text{M}$, and at 20 mM shifts the oligomeric equilibrium to 100% hexamer. 3,4-dichlorobenzaldehyde and 2-chlorobenzaldehyde display IC_{50} values of $26.6 \pm 2.0 \mu\text{M}$ and $44.6 \pm 4.9 \mu\text{M}$, respectively, and shift the oligomeric equilibrium to $30.4 \pm 2.2\%$ and $91.8 \pm 0.5\%$, respectively, at 20 mM compound. Two additional halogenated benzaldehydes, 3-bromobenzaldehyde and 2,4-dichlorobenzaldehyde, were identified as preliminary hits and confirmed to be very effective at stabilizing the *Hs*PBGS hexamer; however, these compounds were very poor inhibitors of *Hs*PBGS activity.

Other benzene derivatives

In addition to the halogenated benzaldehydes, four additional benzene derivatives were also identified as hexamer-stabilizing inhibitors of *Hs*PBGS (Fig. 3F). N-methyl-p-aminophenol sulfate, commonly known as Metol, is used in photography developing solutions, and exposure is associated with contact dermatitis [38]. N-methyl-p-aminophenol sulfate inhibits *Hs*PBGS with an IC_{50} of $13.7 \pm 1.3 \mu\text{M}$, and increases the mole fraction of *Hs*PBGS hexamer to $68.1 \pm 0.9\%$ at 20 mM compound (Supplemental Fig. S1). m-Nitrobenzyl chloride is used as a stabilizing agent in select photography developing solutions [39], and inhibits *Hs*PBGS with an IC_{50} of $27.1 \pm 2.2 \mu\text{M}$ and increases the mole fraction of *Hs*PBGS hexamer to $79.8 \pm 1.3\%$ at 20 mM compound (Supplemental Fig. S1). Diglycidyl resorcinol ether, a component of some epoxy resins that is classified as a carcinogen [40-41], inhibits *Hs*PBGS with an IC_{50} of $36.3 \pm 2.4 \mu\text{M}$, and increases the mole fraction of *Hs*PBGS hexamer to $87.4 \pm 2.7\%$ at 20 mM compound (Supplemental Fig. S1). Methyl-p-formylbenzoate is a byproduct of polyester syntheses, and has limited industrial uses as an additive to sand-casting molds used for metal casting [42]. Methyl-p-formylbenzoate inhibits *Hs*PBGS with an IC_{50} of $40.3 \pm 0.9 \mu\text{M}$, and increases the mole fraction of hexamer to $86.5\% \pm 1.4\%$ at 20 mM (Fig. 5F).

Cadmium

The heavy metal cadmium is widespread in nature arising naturally from volcanic eruptions, and as a pollutant from industrial applications including the production and inappropriate disposal of nickel-cadmium batteries [43]. Uptake of cadmium can occur by respiration,

ingestion, or topical exposure and, once in the bloodstream, cadmium is concentrated into the erythrocytes and leukocytes [44]. Cadmium is toxic to myriad organs including the kidneys, lungs, liver and endocrine system, and is classified as a carcinogen. There are likely many diverse routes through which cadmium toxicity arises; one is through the displacement of the active site metal (often zinc) from a number of essential enzymes [45].

The identification of cadmium as an inhibitor of *Hs*PBGS was unsurprising, as cadmium has previously been described as an inhibitor of PBGS from multiple species [46-47]. Furthermore, *Hs*PBGS is a zinc metalloenzyme susceptible to displacement of its active site metal. It is also interesting to note that much of the *Hs*PBGS in the body is concentrated in erythrocytes where it would be available as an *in vivo* target of cadmium. Cadmium chloride and cadmium acetate (Fig. 3G) inhibit *Hs*PBGS with IC_{50} values of $9.2 \pm 4.5 \mu\text{M}$ and $16.7 \pm 4.0 \mu\text{M}$, respectively, and the inhibition levels to non-zero plateaus (Fig. 4G). At concentrations up to 3 mM both cadmium compounds increase the mole fraction of *Hs*PBGS hexamer to > 90%, and also appear to cause a small amount of the protein to migrate as a dimer as evidenced by the band migrating just above the dye front (Fig. 5G, Supplemental Fig. S1). The aberrant migration of the protein at cadmium concentrations $\geq 10 \text{ mM}$ confounds interpretation of that data. The identification of an expected *Hs*PBGS inhibitor validates our native PAGE library screening method as a tool for identifying inhibitors of this enzyme.

Valerenic acid

Root extracts from the Valerian plant have been used as sedatives and tranquilizers in traditional medicine from many cultures, and the sesquiterpenoid valerenic acid (Fig. 3H) has been identified as one of the active components [48]. The mechanisms for the sedative effect have not been fully characterized, but valerenic acid has been identified as an allosteric regulator of certain GABA receptors in the brain that are also the targets of various anesthetics and barbiturates [49]. Commercial preparations of valerenic acid are widely available as herbal supplements that are outside the regulation of the U.S. Food and Drug Administration.

Valerenic acid is found to inhibit *Hs*PBGS activity to a minimum of $33.2 \pm 3.9\%$ with an IC_{50} of $8.2 \pm 1.7 \mu\text{M}$ (Fig. 4H). Valerenic acid increases the mole fraction of *Hs*PBGS hexamer to $64.1 \pm 0.9\%$ at 20 mM compound and, at concentrations $\geq 10 \text{ mM}$, also induces formation of trace amounts of dimer (Fig. 5H).

Discussion

The initial screen of the NTP library identified 37 compounds (2.7% of the total library) that increased the mole fraction of *Hs*PBGS hexamer. Of these preliminary hits, 15 compounds (1.1% of the total library) were confirmed to increase the mole fraction of *Hs*PBGS hexamer and also inhibit catalytic activity. These percentages are similar to those in our previous screen of the similarly sized (1514 compounds) Johns Hopkins Clinical Compound Library (JHCCL), which identified 1.8% of the compounds as preliminary hits and 0.8% confirmed hits [15]. The observation of similar hit rates for the two libraries was unexpected, as they represent dissimilar collections of molecules. The JHCCL is, unsurprisingly, enriched in

“druglike” molecules. As such, these compounds tend to: contain substructures known to have pharmacological properties; be limited in the number of hydrogen bond donors and acceptor, be soluble in aqueous solutions; have a molecular weight between 160 and 500 Da; and to have a limited polar surface area [50]. No such enrichment is expected for the NTP library and, indeed, the chemical diversity of the identified hits (both within the current study, and as compared to the JHCCL study) is remarkable. The number and diversity of identified molecules that can perturb the oligomeric equilibrium of a single protein, coupled with the large number of proteins hypothesized to utilize the morpheein model of allostery [14], suggest that molecules with this capability abound.

We have described the *Hs*PBGS octamer as a fragile assembly whose structural integrity requires maintenance of myriad factors including specific single amino acid side chains, pH, and active site ligands [10]. In the absence of these factors *Hs*PBGS assembly defaults to the hexamer, which is in equilibrium with the octamer via a dissociative mechanism. The relatively high hit rate of the current and past screens for octamer-destabilizing small molecules underscores the susceptibility of PBGS to allosteric inhibition. We posit that other proteins with a fragile active assembly might be equally susceptible to functional modulation by small molecules such as drugs and environmental contaminants.

The inhibition of an enzyme’s activity via perturbation of the quaternary structure equilibrium of alternate oligomers is proposed as a novel route through which environmental contaminants can be toxic. In the case of *Hs*PBGS, perturbation of the oligomeric equilibrium by toxins is predicted to have physiologic effects in humans similar to lead poisoning or ALAD porphyria. Furthermore, PBGS is an essential enzyme in many organisms where it has been demonstrated to participate in an equilibrium of alternate, functionally distinct multimers [16-17, 51-53]; thus, the perturbation of PBGS oligomeric equilibria by environmental contaminants such as those in the NTP library could have broader effects on plants, animals and the aquatic microbiome.

Conclusions

While PBGS is the first protein to be unequivocally demonstrated to use the morpheein model of allosteric regulation, a number of other proteins possess characteristics that are suggestive of this model [14, 54]. Some of these proteins are important to human health, and perturbation of their function would likely have physiological consequences. The identification of routes through which compounds can produce toxic effects is essential for predicting which compounds might be toxic, and determining appropriate therapies to treat exposure. The current study demonstrates that multiple small molecule environmental contaminants are capable of perturbing the oligomeric equilibrium and altering the function of an essential human protein. We propose that this mechanism of action may be widespread, and should be considered when evaluating the consequences of an environmental contaminant on human health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The function of porphobilinogen synthase. Porphobilinogen synthase (PBGS) catalyzes the asymmetric condensation of two molecules of aminolevulinic acid (ALA) to form porphobilinogen. Porphobilinogen is subsequently incorporated into all of the tetrapyrrole-containing cofactors in pathways that vary among different species. The tetrapyrrole biosynthetic pathway diverges two enzymes beyond PBGS.

Figure 2.

The oligomeric equilibrium of human porphobilinogen synthase and the morpheein model of allostery. **A)** Crystal structures illustrate the alternate homo-oligomers of human porphobilinogen synthase (*HsPBGs*). One disease-associated variant exists predominantly as an inactive hexamer allowing crystal structure determination for this normally low mole fraction assembly (PDB: 1PV8 shown as spheres with three subunits in gray and three subunits in black). The wild type protein crystallizes as the active octamers (PDB: 1E51 shown as spheres with four subunits in gray and four subunits in black). Characteristics of

the assemblies are described. **B)** The interconversion between *Hs*PBGS octamers and hexamers proceed via two conformationally distinct dimers. The hexamer and octamer are shown colored as in part **A** with one dimer of each shown as a cartoon, and the remaining subunits shown as transparent spheres. The conformational change between the pro-octamer dimer and pro-hexamer dimer is a twist at a hinge that changes the orientation of an $\alpha\beta$ -barrel domain of one monomer relative to the other. **C)** A schematic of the morpheein model for allosteric regulation shows a protein that exists in an equilibrium of tetramers and trimers, the interconversion of which occurs via conformationally distinct monomers. Multimer assembly involves the association of the dashed line with the solid line. The allosteric regulator (checkered wedge) binds specifically to the light gray forms and draws the oligomeric equilibrium towards the trimer. **D)** A hexamer-specific surface cavity (highlighted by the white circle) contains residues from three subunits (shown as surfaces): a pro-hexamer dimer (subunits A and F), and the adjacent subunit B, colored as in panel A. The remaining subunits are shown as white cartoons. The analogous site on the octamer, which contains contributions from a pro-octamer dimer (subunits A and F) and the adjacent subunit B, lacks the cavity (hashed circle). The subunits are labeled as per the PDB codes 1PV8 and 1E51. **E)** The white triangle and square highlight oligomer-specific surface cavity differences apparent from the top-view of the hexamer and octamer, respectively. For both structures, the “top” subunits are shown as surfaces colored as in panel A, and the remaining subunits are shown as white cartoons.

Figure 3.

The structures of the hexamer-stabilizing *Hs*PBGS inhibitors identified from the NTP library. **A**) Mutagen X (3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H-furanone), CAS-77439-76-0, **B**) Nitrofurazone (5-nitro-2-furaldehyde semicarbazone), CAS-59-87-0; Nitrofurantoin ((*E*)-1-[(5-nitro-2-furyl)methylideneamino]imidazolidine-2,4-dione), CAS-67-20-9), **C**) Captan ((3*aR*,7*aS*)-2-[(trichloromethyl)sulfanyl]-3*a*,4,7,7*a*-tetrahydro-1*H*-isoindole-1,3(2*H*)-dione), CAS-133-06-2, **D**) Cisplatin ((*SP*-4-2)-diamminedichloridoplatinum), CAS-15663-27-1, **E**) Halogenated benzaldehydes: 2,6-dichloro-, CAS-83-38-5; 3,4-dichloro-, CAS-6287-38-3; 2-chloro-, CAS-89-98-5; 3-bromo-, CAS-3132-99-8; 2,4-dichloro-, CAS-874-42-0, **F**) Benzene derivatives: N-methyl-p-aminophenol sulfate, CAS-55-55-0; m-nitrobenzyl chloride, CAS-619-23-8; diglycidyl resorcinol ether, CAS-101-90-6; methyl-p-formylbenzoate, CAS-1571-08-0, **G**) cadmium chloride, CAS-10108-64-2; cadmium acetate, CAS-5743-04-4, **H**) valerenic acid ((2*E*)-3-[(4*S*,7*R*,7*aR*)-3,7-dimethyl-2,4,5,6,7,7*a*-hexahydro-1*H*-inden-4-yl]-2-methylacrylic acid), CAS-3569-10-6.

Figure 4.

Dose response curves of *HsPBGS* activity inhibition. **A)** Mutagen X, **B)** Nitrofuran antibiotics, **C)** Captan, **D)** Cisplatin, **E)** Halogenated benzaldehydes, **F)** Benzene derivatives, **G)** Cadmium, **H)** Valerenic acid. Kinetic assays were performed at 10 $\mu\text{g/mL}$ *HsPBGS*. For plots containing more than one data set, the symbols are defined on the plot. Fits are to a simple hyperbolic equation, or a hyperbolic equation with a non-zero endpoint as defined in the text. Error bars representing standard deviation are shown where they exceed the size of the data point.

Figure 5.

Native PAGE analysis of *HsPBGS* hexamer stabilization by representative toxins. **A)** Mutagen X, **B)** Nitrofurazone, **C)** Captan, **D)** Cisplatin, **E)** 2,6-Dichlorobenzaldehyde, **F)** Methyl-p-formylbenzoate, **G)** Cadmium acetate, **H)** Valerenic acid. Native PAGE analyses were performed at 300 $\mu\text{g/mL}$ *HsPBGS*. Note that the range of inhibitor concentrations varies among the gels, as labeled (given in mM). The positions of the sample wells, octamer, hexamer, and dye front are labeled on **D**. The implications of *HsPBGS* migrating at positions other than octamer and hexamer are discussed in the text.

Table 1Parameters extracted from kinetic inhibition and hexamer-stabilization experiments ^a.

Compound	CAS #	IC ₅₀ (μM)	FA _{min} (%)	Mole Fraction Hexamer _{max} (obsd)
Mutagen X ^b	77439-76-0	1.4 ± 0.1	0	67.2 ± 0.6 ^c
Nitrofurazone	59-87-0	2.1 ± 0.2	0	48.5 ± 0.3 ^d
Nitrofurantoin	67-20-9	5.9 ± 0.4	0	16.0 ± 1.0
Captan	133-06-2	3.0 ± 0.3	0	72.3 ± 0.2 ^d
cis-Dichlorodiamine platinum	15663-27-1	17.1 ± 1.5	0	53.0 ± 0.8 ^e
2,6-Dichlorobenzaldehyde	83-38-5	15.2 ± 1.5	0	100 ± 0
3,4-Dichlorobenzaldehyde	6287-38-3	26.6 ± 2.0	0	30.4 ± 2.2
2-Chlorobenzaldehyde	89-98-5	44.6 ± 4.9	0	91.8 ± 0.5
N-methyl-p-aminophenol sulfate	55-55-0	13.7 ± 1.3	0	68.1 ± 0.9
m-Nitrobenzyl chloride	619-23-8	27.1 ± 2.2	0	79.8 ± 1.3
Diglycidyl resorcinol ether	101-90-6	36.3 ± 2.4	0	87.4 ± 2.7
Methyl-p-formyl benzoate	1571-08-0	40.3 ± 0.9	0	86.5 ± 1.4
Cadmium chloride	10108-64-2	6.9 ± 1.2	9.2 ± 4.5	94.9 ± 3.3 ^f
Cadmium acetate	5743-04-4	3.6 ± 0.7	16.7 ± 4.0	92.2 ± 0.6 ^f
Valerenic acid	3569-10-6	8.2 ± 1.7	33.2 ± 3.9	64.1 ± 0.9
3-Bromobenzaldehyde	3132-99-8	21.5 ± 3.5	80.8 ± 1.2	89.9 ± 3.4
2,4-Dichlorobenzaldehyde	874-42-0	107 ± 4	50.7 ± 0.3 ^g	86.2 ± 0.7

^aThe IC₅₀ and FA_{min} values derive from the kinetic inhibition assays as described in the text; %Hexamer_{max} (obsd) is the percentage of HsPBGS hexamer observed at 20 mM compound (except where indicated by footnotes).

^bMutagen X = 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H-furanone).

^cIncreasing concentrations of Mutagen X induced HsPBGS hexamer formation, but also appeared to denature the protein; the 67.2% represents the sum of the hexameric band and the protein that migrated at the dye front at 20 mM Mutagen X.

^dThe highest concentrations of Nitrofurazone and Captan were 2 mM.

^e53.0% HsPBGS hexamer was observed at 1 mM Cisplatin; higher concentrations caused HsPBGS to precipitate completely.

^fThe highest concentrations of cadmium chloride and cadmium acetate analyzed were 3 mM, and this yielded a mixture of hexamers and dimers (in a ~2:1 ratio) on the gels. The values of 94.9% and 92.2% are the sums of the hexameric and dimeric bands for each compound. Higher concentrations caused PBGS to precipitate.

^gThe activity measured at 100 mM 2,4-dichlorobenzaldehyde was 50.7 ± 0.3%; the fit suggests the activity would eventually decline to 0, but higher concentrations of 2,4-dichlorobenzaldehyde were not evaluated.