Repurposing ciclopirox as a pharmacological chaperone in a model of congenital erythropoietic porphyria

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CONGENITAL ERYTHROPOIETIC PORPHIRIA

Congenital erythropoietic porphyria is a rare autosomal recessive disease produced by deficient activity of uroporphyrinogen III synthase, the fourth enzyme in the heme biosynthetic pathway. The disease affects many organs, can be life-threatening, and currently lacks curative treatments. Inherited mutations most commonly reduce the enzyme’s stability, altering its homeostasis and ultimately blunting intracellular heme production. This results in uroporphyrin by-product accumulation in the body, aggravating associated pathological symptoms such as skin photosensitivity and disfiguring phototoxic cutaneous lesions. We demonstrated that the synthetic marketed antifungal ciclopirox binds to the enzyme, stabilizing it. Ciclopirox targeted the enzyme at an allosteric site distant from the active center and did not affect the enzyme’s catalytic role. The drug restored enzymatic activity in vitro and ex vivo and was able to alleviate most clinical symptoms of congenital erythropoietic porphyria in a genetic mouse model of the disease at subtoxic concentrations. Our findings establish a possible line of therapeutic intervention against congenital erythropoietic porphyria, which is potentially applicable to most of deleterious missense mutations causing this devastating disease.

INTRODUCTION

Porphyrias, inborn errors of heme biosynthesis, are metabolic disorders, each resulting from the deficiency of a specific enzyme in the heme biosynthetic pathway (fig. S1) (1). This group of diseases includes congenital erythropoietic porphyria (CEP; ICD-10 #E80.0; MIM #263700), also known as Günther’s disease (2, 3), which is an autosomal recessive disorder resulting from a markedly deficient activity of the uroporphyrinogen III synthase (UROIII; EC 4.2.1.75) that leads to the specific and marked accumulation of type I porphyrins, specifically uroporphyrin I (URO I) and coproporphyrin I (COPRO I) (4). The accumulation of these porphyrins throughout the body, especially in the skin, drives the pathogenesis of the disease and leads to hemolysis, severe anemia, splenomegaly, and disfiguring phototoxic cutaneous lesions (5). A close relationship between the metabolic disturbance reflected by porphyrin excess and the severity of disease expression has been established (6). For instance, the severity of the skin manifestations varies considerably among CEP patients and is dependent on porphyrin amounts in affected tissue and the degree of light exposure (7). The prognosis for some CEP patients is severe, with death occurring in the neonatal or early life periods (8), whereas for other patients, it is devastating and debilitating due to lifelong persistence of the symptoms.

The current standard of care in most of CEP patients involves the management of disease symptoms rather than addressing the underlying pathology. Palliative care includes absolute avoidance of sun exposure, meticulous skin care, and avoiding mechanical trauma (4, 9, 10). Attempts have been made to reduce erythropoiesis and lower concentrations of circulating porphyrin via erythrocyte transfusions, but complications associated with a chronic transfusion regimen are potentially severe and include the risk of transfusion-communicated infectious disease and iron overload (11).

Splenectomy has been performed to decrease severe hemolytic anemia and to stimulate erythropoiesis and porphyrin production, thereby increasing the lifespan of erythrocytes and resulting in the reduction of cutaneous photosensitivity. However, the results of this surgical procedure are variable, and the benefit is often short-lived (4, 10–12). Bone marrow transplantation has also been used, and although there have been some reports of curative effects in CEP patients, this approach is mired with specific risks of complications including chemotherapy toxicity, immunosuppression leading to infections, transplant rejection, and demise (10, 13–15). Finally, case reports describing the success of allogeneic hematopoietic stem cell transplantation for CEP remains limited. Moreover, it is challenging to find a human leukocyte antigen–matched donor, and patients may experience acute complications after transplantation (16). Consequently, there are currently no approved pharmacological treatments for CEP, highlighting the need to identify novel therapeutic strategies that address the underlying pathology and affect the quality of life of these patients.

Most of UROIII missense mutations result in proteins that are unable to fold efficiently into their native conformation in the endoplasmic reticulum (table S1) (17). For instance, the C73R mutation in the UROS gene (present in more than one-third of reported CEP cases) produces a conformational change responsible for the decreased kinetic stability of...
the enzyme. Specifically, the in vitro activity of the UROIIIS-C73R mutant is about 15% of that of the wild-type (WT) enzyme and, more critically, the unfolding half-life of the mutated enzyme drops from 2.5 days (WT) to 15 min (C73R) at 37°C (18). In agreement with this, untagged or tagged UROIIIS-C73R is expressed in cells but rapidly unfolds and is quickly targeted for proteasomal degradation, resulting in undetectable protein levels (17). About 75% of reported missense mutations share this reduced protein stability and altered homeostasis (table S1) (17) that thus constitutes the main deleterious molecular mechanism observed in CEP patients.

Degradation of UROIIIS depends mostly on the activity of the proteasome instead of the lysosome pathway, providing a molecular mechanism for the failure of chloroquine treatment in CEP patients (21). For that reason, inhibiting proteasome activity can modulate the degradation process, as shown by the effectiveness of proteasomal inhibitors in restoring mutant UROIIIS homeostasis. In particular, MG132, a well-characterized inhibitor of the ubiquitin–proteasome degradation system, was able to restore the activity of UROIIIS in cells expressing mutant versions of the protein (19, 22). Furthermore, in vivo treatment of CEP knock-in (UROIIIS<sup>P248Q/P248C</sup>) mice with bortezomib (a proteasome inhibitor) led to a decrease in uroporphyrin accumulation in circulating red blood cells (RBCs) and urine, accompanied by the disappearance of skin photosensitivity, yet failed to improve the features of hemolytic anemia (22). Despite these promising results, efficient, long-term proteasome inhibition is difficult to maintain in vivo and may lead to serious adverse toxic effects especially in the central nervous system (23, 24). Thus, proteasome inhibitors are unlikely to constitute a safe therapeutic choice for CEP.

One attractive alternative way to potentially regulate UROIIIS proteostasis is by means of pharmacological chaperones, which are chemical substrates or modulators that usually bind to a partially folded intermediate to stabilize the protein and allow it to complete the folding process (25). These molecular chaperones have successfully reduced clinical symptoms of disease by slowing down or inhibiting the tendency of different proteins to aggregate, resulting in detectable amounts of enzyme in the cell (26). Chemical chaperones have also shown promising results in restoring several destabilized mutant proteins including heterotrimeric GTP-binding protein–coupled receptors, ion channels, adenosine triphosphate–binding cassette transporters, and lysosomal enzymes (27). Pharmacological chaperones usually target the binding site of the enzyme as reversible inhibitors that mimic the substrate’s conformation in the transition state (28). The use of allosteric chaperones, which stabilize the enzyme without competing with the substrate, is less explored.

Here, we show that the off-patent synthetic antimicrobial ciclopirox (CPX) acts as an UROIIIS pharmacological chaperone in vitro and in vivo. We selected this compound after screening several thousand candidates for their stabilization and restoration of the homeostatic properties of UROIIIS, including a thorough biophysical, biochemical, and cellular characterization of selected hits. CPX acted as an allosteric chemical stabilizer of UROIIIS, regulated heme group metabolism in multiple eukaryotic cells, and was able to revert most of the hallmark symptomatology (abnormal URO I levels in the blood, splenomegaly, and liver porphyrins, among others) in a mouse model of the disease.

RESULTS
Finding druggable allosteric sites in UROIIIS
The human isoform of UROIIIS is composed of 286 amino acids folded into two domains connected by a flexible linker (29). The active site of

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**Fig. 1.** Flow chart for the discovery of pharmacological chaperones against CEP. Analyzed compounds from each independent test (stability assay, functional assay, structural assay, and drug repurposing) are shown on the left, whereas identified hits are highlighted in green. Funnel cartoons represent the progressive reduction in the number of active compounds toward a validated hit.
UROIIIS by at least 2.5°, a substantial stabilization of the WT version of the protein ($\Delta\Delta G_{U-P} > 1$ kcal · mol$^{-1}$). The second assay targeted the L1 library in cell culture to test the ability of the compounds to raise the intracellular concentration of the defective enzyme (a functional assay). Given that the skin is one of the more damaged organs in CEP, we used skin human M1 fibroblasts stably expressing UROIIIS-C73R fused to green fluorescent protein (GFP) to monitor the intracellular degradation of the enzyme and protein expression in the presence of each compound. As previously described, the basal GFP-tagged UROIIIS-C73R protein expression was below the detection limit (19), so the observed fluorescence directly reported the intracellular protein concentration increase induced by the compound. We used this cellular model to screen the entire library, obtaining 85 compounds (3.4% of the library) that significantly increased fluorescence ($P < 0.01$) in the functional assay.

Hit compounds from each independent assay were cross-validated to yield a reduced list of 25 compounds with reported activity in both the stability and the functional assays (Fig. 1). These compounds were further characterized biochemically (cytometry in four additional eukaryotic cell lines) and by nuclear magnetic resonance (NMR) spectroscopy (structural assay) to validate and characterize the interaction site (figs. S3B and S4). On the basis of the chemical shift perturbation (CSP) analyses of the $^{1}H,^{15}N$ heteronuclear single-quantum coherence experiments, nine molecules were associated with UROIIIS (table S2), five of them in a nonspecific mode or in multiple modes, two of them (L1.27.G5 and L1.29.D6) at the enzyme’s catalytic site, and two of them (L1.17.G5 and L1.26.E3) specifically targeting the C-allosite (fig. S4). Chemical shift analysis revealed that affinities were low (50 to 150 μM), consistent with the small size of the tested fragments. Western blot analysis from human M1 fibroblasts stably expressing GFP-UROIIIS-C73R confirmed that the four molecules perturbed UROIIIS by at least 2.5°, a substantial stabilization of the WT version of the protein ($\Delta\Delta G_{U-P} > 1$ kcal · mol$^{-1}$). The second assay targeted the L1 library in cell culture to test the ability of the compounds to raise the intracellular concentration of the defective enzyme (a functional assay). Given that the skin is one of the more damaged organs in CEP, we used skin human M1 fibroblasts stably expressing UROIIIS-C73R fused to green fluorescent protein (GFP) to monitor the intracellular degradation of the enzyme and protein expression in the presence of each compound. As previously described, the basal GFP-tagged UROIIIS-C73R protein expression was below the detection limit (19), so the observed fluorescence directly reported the intracellular protein concentration increase induced by the compound. We used this cellular model to screen the entire library, obtaining 85 compounds (3.4% of the library) that significantly increased fluorescence ($P < 0.01$) in the functional assay.

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Structural analysis for drug repurposing

We used the two validated fragments targeting the C-allosite as templates for a structural comparison against a U.S. Food and Drug Administration (FDA)–approved library of 1800 drugs (L2 library). Our in silico comparison was based on chemical similarity, including chemical functionality and skeleton topology, and was normalized by molecular weight to avoid bias. We further evaluated the 15 FDA-approved molecular entities with the highest scores for their chaperone activity with UROIIIS. Specifically, the compounds were assayed in vitro for their association with UROIIIS via NMR spectroscopy and changes in the catalytic efficiency, fluorescence of GFP-UROIIIS-C73R, and IC50. Five compounds (CPX, phenylephrine, procycline, atomoxetine, and dydrogesterone) caused an increase in the accumulation of intracellular GFP-UROIIIS-C73R, but NMR analysis revealed that only CPX specifically bound at the C-allosite, whereas dydrogesterone bound at different locations of the enzyme (table S2). As a result, the best-performing compound of the L2 analysis was the fungicide CPX [6-cyclohexyl-1-hydroxy-4-methyl-2(1H)-pyridone] (Fig. 2B), which we selected for further studies.

UROIIIS homeostasis in the presence of CPX

The structural model for the association of CPX at the C-allosite of UROIIIS, based on the NMR CSP (Fig. 2D), showed that the N-hydroxypyridone moiety actively interacted with the protein pocket via Asp113, Ser95, and Tyr97, whereas the cyclohexyl group fit in a hydrophobic pocket conformed by Tyr128 (Fig. 2E). On the basis of the CSP analysis, CPX associated at low affinity [dissociation constant (Kd) ≈ 108 μM, as determined by ligand titration; Fig. 2F] but was able to stall protein aggregation in vitro (Fig. 2C, blue circles).

Modulation of UROIIIS homeostasis was also manifested in the partial restoration of the reduced apparent catalytic activity of the mutated enzyme (Fig. 2G). This likely occurred due to the increased stability of the biomolecule during the assay (Fig. 2C) and not from inhibition of the proteasome, because polyubiquitinated proteins were not more overexpressed than the control (P = 1 × 10−33; Fig. 2, H and I). The integrity of the poly(adenosine diphosphate–ribose) polymerase was not affected either (P = 2 × 10−18). Moreover, expression of EEA1, which localizes exclusively in early endosomes (32), and LAMP1, a highly glycosylated glycoprotein, was not altered, pointing to the idea that CPX does not modulate the endoplasmic reticulum pathways.

As mentioned before, M1 fibroblasts transfected with a plasmid encoding GFP-UROIIIS-C73R showed a large increase in fluorescence after treatment with CPX (Fig. 3A). Such treatment with CPX also resulted in the accumulation of GFP-UROIIIS-C73R or GFP-UROIIIS-P248Q (the second most abundant CEP-causing mutation; table S1) in different cell lines, including human immortalized myelogenous leukemia (K562) cells, human embryonic kidney (HEK) 293 cells, and murine liver progenitor (MLP29) cells, as determined by cytometry, microscopy, and Western blot analyses (Figs. 3B and 2, H and I). Together, our results are consistent with a model where UROIIIS becomes unstable upon mutation, a deleterious mechanism that is partially reverted upon direct association with CPX.

CPX and the CEP metabolic phenotype in cellular models

To investigate the effect of CPX in heme metabolism, we used clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) to transform HEK293 cells into human cellular models of CEP by replacing the endogenous UROIIIS WT with C73R or P248Q mutations, which drive the accumulation of toxic porphyrins (URO I and COPRO I) (22). WT HEK293 and mutant HEK293 UROIIIS-C73R and UROIIIS-P248Q cells were characterized for their heme group biosynthesizing properties by measuring the accumulation of toxic porphyrins in the cytosol. Flow cytometry analysis distinguished between the nonfluorescent WT HEK293 cells and the fluorescent porphyrin-filled cytosol of the UROIIIS-mutated CEP cells (Fig. 3C). In the presence of 60 μM CPX, the number of fluorescent cells dropped from 95.4% (control/no treatment) to 9.4% (CPX; Fig. 3C), sixfold more than an equivalent amount of the proteasome inhibitor (81.2%; fig. S6A). Experiments with CPX dissolved in water (fig. S6B) ruled out the possibility that the URO I reduction was due to DMSO. Porphyrins separated and quantified by high-performance liquid chromatography (HPLC) showed that the fluorescence decrease was caused by a sharp reduction in the expression of URO I (range, 1 to 6 μM), while porphyrin reduction was maintained at doses up to, at least, 250 μM (Fig. 3, D and E, and fig. S7). At higher concentrations, URO I reduction was also accompanied by URO III accumulation (Fig. 3D, inset).

These observations were reproducible in primary CEP human cell lines. Lymphocytes from a patient suffering from CEP (4 to 10% of WT UROIIIS activity) responded to treatment with 60 and 250 μM CPX (Fig. 3F), with abnormal levels of the toxic porphyrin URO I reduced by a factor of two compared to untreated lymphocytes. No effect was observed in human lymphocytes from a healthy individual (Fig. 3F).

The metabolic regulatory effect induced by CPX in CEP cells is not produced by changes in the transcriptional regulation of the genes belonging to the heme biosynthesis pathway, as demonstrated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis comparing WT and CEP cells both in the presence and absence of CPX. As expected and according to the feedback regulation described for the pathway (33), qRT-PCR showed that ALAS was up-regulated in the cellular CEP models compared to WT cells, likely to compensate for the lack of the end-product heme, whereas CPX exerted a negligible effect on the regulation of any gene in the pathway (Fig. 3G). CPX is considered an iron chelator, and this property may constitute an alternative mechanism to modulate the heme group metabolism (34), a pathway tightly regulated by iron. As expected, iron increased the amount of URO I in CEP cells, but the ability of CPX to reduce URO I levels was independent of iron concentration (Fig. 3H). Compared to other drugs that act through the chelation mechanism such as deferoxamine (35), CPX weakly coordinated iron but was unable to compete for the metal in the heme group or any of the main iron-containing prosthetic groups (fig. S8). Finally, CPX and the more common formulation CPXol produced identical results in the reduction of toxic metabolites, underscoring that the active principle is the CPX entity (Fig. 3I).

CPX and the phenotype of a mouse model of CEP

We evaluated the activity of CPXol in a transgenic murine model of CEP, which is homozygous for a mutant P248Q form of UROIIIS.
Fig. 3. The effect of CPX in cellular lines. (A) CPX induced cytosolic expression of GFP-UROIIIS-C73R in M1 cells, as monitored by fluorescence microscopy. Scale bars, 50 μm. The control and CPX images are stained using 4',6-diamidino-2-phenylindole and GFP channels, respectively. (B) A dose of 60 μM CPX increased the number of GFP-expressing cells (blue bars, left y axis) and the average fluorescence (green bars, right y axis) for a set of eukaryotic cell lines. (C) Expression analysis in HEK293 cells (WT, UROIIIS-C73R, and UROIIIS-P248Q) of the different genes of the heme biosynthetic pathway in the absence (red) and presence (blue) of 120 μM CPX. The main difference observed was the up-regulation of the ALAS gene in the cellular models of disease, as previously reported (8). Values are relative to WT UROIIIS. (D) Fluorescence-activated cell sorting (FACS) analysis of WT HEK293 cells (left), HEK293 cells with the UROIIIS-C73R endogenously introduced by CRISPR/Cas9 (HEK293 C73R+/+, centre), and HEK293 C73R+/+ cells in the presence of 60 μM CPX (right). The accumulation of porphyrines generates an intrinsic fluorescent phenotype of the cellular CEP model (fluorocytes). (E) HPLC porphyrin separation of HEK293 C73R+/+ cells, cultured in the absence (left) or presence of 60 (center) and 250 μM CPX (right). The insets correspond to a 300-fold expansion of the chromatogram. (F) CPX dose-dependent URO I reduction in HEK293 C73R+/+ cells (purple) and HEK293 P248Q+/+ cells (blue). The inset shows the URO I reduction at low concentrations of CPX. (G) Effect of CPX in human lymphocytes from a 24-year-old CEP patient (blue) and a healthy individual (purple). (H) Effect of iron chloride on the accumulation of URO I by HEK293 C73R+/+ cells and its subsequent reduction induced by CPX. All bars have been normalized to the no iron, no CPX control group (100%). (I) URO I reduction induced by 60 μM CPX (brown), 60 μM ciclopirox olamine (CPXol, purple), and 60 μM glucuronide CPX (CPXglu). All bars have been normalized to the no CPX control group (blue, 100%). **P ≤ 0.01 and ***P ≤ 0.001. a.u., arbitrary units; PE, phycoerythrin.
The CEP mouse model is a bona fide model of human CEP because it shows the metabolic defect reflected in the isomer I porphyrin accumulation in the blood and skin lesion defects upon irradiation with ultraviolet light (36). A total of 16 P248Q/I+ animals were used to evaluate the effect of CPX administered in food pellets: 7 animals were fed with CPX, whereas 9 animals had food with control diet (Fig. 4). The blood concentration of isomer I porphyrin was monitored weekly. After a basal (pretreatment) sample (day 0), treatment was started on day 2 and the first post-treatment sample was obtained on day 3, followed by weekly monitoring. Isomer I porphyrins URO I (Fig. 4A) and COPRO I (Fig. 4B) in the blood were significantly reduced in the group treated with CPX compared with the control group ($P = 0.0027$ and 0.05 for URO I and COPRO I, respectively). The reduction in porphyrins in RBCs appeared greatest in the first 20 days after treatment, after which there was a slow and steady reduction until the last evaluation (day 45). Similar results were observed for the hepta-, hexa-, and penta-isomer I porphyrins (fig. S9). Upon treatment disruption, porphyrin levels were slowly restored (fig. S9).

CPX was able to increase the concentration of protoporphyrin IX (PROTO IX), an important precursor of the heme group downstream of UROIS activity, thus demonstrating its pharmacological chaperone activity in the mouse (Fig. 4C). In addition, after 45 days of treatment, CPX reduced URO I concentrations in liver tissue by 40% (Fig. 4D) and splenomegaly (Fig. 4E), providing evidence of reduced hemolysis and decreased porphyrin deposition. Accordingly, there was a significant ($P = 2.8 \times 10^{-6}$) weight reduction in the spleen in the CPX group (mean, 410 ± 86 mg; Fig. 4E, bottom row) compared to the untreated group (mean, 985 ± 204 mg; Fig. 4E, top row). The spleen volume decrease was matched by a reduction in F4/80+ macrophages after treatment with the drug (Fig. 4, F and G).

The hematological results are also consistent with the hepatic damage evaluated by histological analysis (Fig. 4H). Liver histology showed reduced steatosis, a reduction in the cluster of erythroid cells in the sinusoids and a reduction in the porphyrin deposits (Fig. 4H). Histological quantification of collagen indicated a CPX-driven tendency to reduce fibrosis in the liver (Fig. 4F).

**Preliminary pharmacokinetics and dose-response studies**

Pharmacokinetic experiments have previously shown that CPX’s half-life in the organism is short, with strong affinity for serum albumin and fast catabolism mediated by a glucuronidation reaction (37, 38). We found that glucuronidation occurred at the hydroxyl moiety and that CPXglu was incapable of reducing porphyrins in the CEP cellular models (Fig. 3I), consistent with the predicted stabilizing role of the OH group in its interaction with the protein (Fig. 2G). To quantify the effective concentration of CPX administered within the pellets, we have developed an NMR-based method for the analysis of the active and glucuronide forms of CPX in serum and urine (see Materials and Methods) and cross-validated it against the most conventional HPLC-based method (38). The average concentration of active CPX in serum was CPX = 2.79 ± 0.6 μM and CPXglu = 12 ± 1.2 μM, which is equivalent to an intake (gavage) of 300 mg/m² and close to the toxicity limit for the drug (39). A small fraction of the animal population consistently displayed bowel inflammation, a feature also observed in a clinical trial of CPX in humans at equivalent doses. In a second independent experiment with WT mice ($n = 8$), CPXglu was determined by NMR spectroscopy with CPX doses in the range of 3 to 300 mg/m² ($n = 2$ for each dose) (table S3). This species always peaked in concentration ($C_{max}$) at 1 hour after administration (with $C_{max}$ concentrations ranging between 1 and 15 μM), with a 30% of remaining substance in serum after 6 hours of administration. Glucuronidation of the drug increased its solubility, favoring its excretion as evidenced by a peak of CPXglu in the millimolar concentration range in urine within the first 24 hours after administration. Finally, serum analysis by NMR spectroscopy showed that the ratio of CPXglu/CPX was higher ($P = 1.7 \times 10^{-2}$) in WT mice (with 94% of glucuronide species) than in CEP P248Q mice (with 84% of glucuronide species) (table S4).

Finally, to determine the minimum effective dose (MED) and the maximum tolerated dose (MTD), we administered CPX to 30 CEP mice by oral gavage for 35 days (control, 3, 15, 30, and 75 mg/m²; $n = 6$ per dose) (Fig. 4G). Three milligrams per square meter showed no effect as compared to control, and we infer the MED was <15 mg/m² because, at this dose, a 22% reduction in the URO I content was observed. A similar effect was observed at the higher dose (75 mg/m², 28% reduction), so we conclude that 75 mg/m² < MTD < 300 mg/m². Thus, CPX acts as a pharmacological chaperone in CEP mice in a concentration range below the toxicity limit of the drug.

**DISCUSSION**

CEP is a multisystem pathology in which hematological, cutaneous, hepatic, ocular, and skeletal manifestations contribute to the severity of the disease. Therapeutic interventions for the treatment of CEP are either palliative or do not adequately address the underlying mechanistic pathogenesis of the disease such as continual abnormally high levels of toxic porphyrins throughout the body, particularly in the skin (8). Here, we have demonstrated a CPX-induced reduction in porphyrin levels in cell-based models of CEP (UROIS-C73R and UROIS-P248Q homozygous) and in human lymphocytes derived from a patient with CEP. The medical plausibility of CPX-based treatment for CEP was further suggested by our experiments using a relevant animal model (a UrosP248Q/P248Q knock-in mouse) (22) that replicated the features of CEP in humans. Our results showed that treatment with CPX (i) reduced the levels of porphyrins (URO I and COPRO I) in RBCs, the liver, and urine; (ii) increased the levels of the downstream heme precursor PROTO IX in RBCs, which is an indirect measure of normal homeostasis restoration of the heme pathway; (iii) decreased splenomegaly (a direct consequence of the CEP phenotype hemolytic anemia), which is an indirect measure of a reduction in circulating porphyrins; and (iv) had a therapeutic effect on damaged tissues (liver, spleen, and kidney). From this, we anticipate that toxic porphyrin accumulation in tissues and organs, the hallmark of CEP, is likely to decrease upon treatment with CPX in patients with CEP.

We found that the mode of action of CPX in relation to CEP is as a pharmacological chaperone that binds UROIS at the C-allosite and stabilizes its folded conformation, reducing its homeostatic instability back toward normal levels, thereby increasing UROIS-specific activity and reducing the levels of toxic porphyrins. This is in contrast to pharmacological chaperones that select for molecules that bind at the catalytic site or at a given mutation site, mimic the transition state of the enzyme, and target only the active site. This more canonical approach has shown promising results in Fabry, Tay-Sachs, and Gaucher diseases (26), but because this methodology targets the enzyme binding site, it can also conflict with intrinsic catalytic activity. Instead, our strategy successfully pinpointed an allosteric site of the protein that minimally affected enzyme activity while adequately acting on the protein’s thermodynamic stability.
CPX weakly interacted with UROIII, but this does not preclude its effect in cell lines or in vivo because the intracellular concentration of UROIII is also very low: In erythroblasts derived from bone marrow progenitor cells (the main heme producer cells), the average UROIII production rate is about 25 pmol of URO III/min·ml of RBC (10 to 15 nM). On the other hand, the CPX concentration in all the intracellular UROIII concentration falls in the low nanomolar range (10 to 15 nM). Because UROIII has a $k_{cat}$ of about 2240 molecules min$^{-1}$ (30), the intracellular UROIII concentration falls in the low nanomolar range (10 to 15 nM). On the other hand, the CPX concentration in all the cellular and murine experiments was always within the micromolar range and, therefore, in large excess.

Here, we repurposed CPX, a drug with proven efficacy for topical treatment of cutaneous fungal infections, vaginal candidiasis, seborrheic dermatitis, and onychomycosis (41). In addition, a phase I study administered CPX orally in patients with advanced hematological malignancies. The data showed that the oral dosing was well tolerated in patients at low (20 mg/m$^2$) and medium (40 mg/m$^2$) doses, although intestinal toxicity was observed at doses above 80 mg/m$^2$, and it exhibited a sustained pharmacodynamic effect (a decrease in the amount of survivin mRNA) and resulted in hematological improvement and/or disease stabilization in two of three patients (39). Mechanistically, the anticancer activity of CPX has been attributed to intracellular iron chelation, which in turn disrupts iron-dependent pathways such as Wnt signaling and ultimately suppresses expression of the antiapoptotic gene survivin. Iron also regulates the heme pathway, and strong iron chelators such as deferoxamine have been investigated for potential use against porphyrias (34). However, our experimental evidence suggested that CPX does not affect the regulation of the heme group because mRNA expression of the heme biosynthesis pathway remained unaltered in the presence of CPX in a CEP cell line. The CPX-induced URO I reduction observed in cells remained unaltered in the presence of a large excess of iron III chloride. This is likely the case because CPX is only a weak binder of iron and it stabilizes the heme group instead of competing with it for the metal chelation.

Several studies have addressed the potential toxicology of CPX at the regulatory level. For instance, 3-month repeat-dose toxicity studies have been performed in rats (60 mg/m$^2$ per day) and dogs.
(200 mg/m² per day) with no observed adverse effect, demonstrating no toxic effects or changes in electrocardiography (37). Moreover, preliminary toxicity studies using cell lines or a single oral gavaged dose in mice did not find evidence that CPX is mutagenic (table S5), and CPX showed no increase in drug-related neoplasms as compared to control in a 104-week dermal study in mice (https://ndclist.com/ndc/). However, intestinal toxicity has been observed at medium to high doses (>80 mg/m²), possibly due to the limited absorption of the compound in the intestine after oral administration (39).

From a pharmacokinetic point of view, CPX has a short lifetime in the organism, because it is highly affected by circulating serum albumin and has an efficient and simple catabolic pathway constituted by a glucuronidation reaction followed by urine excretion. The glucuronidation reaction occurs in the liver and is more efficient in WT than in CEP mice, probably due to poor liver function in porphyria patients. Such catabolic dependence on the liver sets a frame for the exploration of alternative pharmacological formulations to defer the glucuronidation reaction to try to optimize the effect of the active compound.

In the context of the potential therapeutic value, the described CPX effect on heme metabolism regulation faces several limitations. First, the mechanism is only suitable for 75% of all reported missense pathogenic mutations (although it includes the most frequent ones), and it should have no effect on patients carrying intronic mutations or splicing defects. Moreover, we have not yet conducted studies in humans or in human tissue, so it is unclear whether the observed reduction in animal models will be sufficient to significantly alleviate patient symptomatology. Finally, toxicity of CPX has not been extensively studied when orally administered, and this may represent a serious limitation for the use of the drug in CEP, particularly given the likely necessity of chronic treatment.

In summary, we have demonstrated that CPX is active against CEP at subtoxic concentrations, although the lowest concentration at which we observe activity (15 mg/m²) represents a limited therapeutic range that may require further development before its therapeutic application, including dose adaptation depending on the mutation carried by the patient. In this context, we believe that the main problem is low bioavailability and therefore a continuous high concentration of drug in the gastrointestinal tract. Whether the use of CPX prodrugs or drug delivery systems result in an improved pharmacodynamic profile remains to be tested.

MATERIALS AND METHODS

Study design

To search for pharmacological chaperones able to stabilize UROIIIIs, we performed several assays on a fragment compound library (L1 library, 2500 compounds) with enhanced chemical diversity and optimal solubility properties (>1 mM in aqueous media). The stability assay was designed to identify compounds able to produce stabilizing interactions with the protein. A functional assay in M1 cells selected for compounds that increased the cytosolic concentration of a GFP-tagged enzyme. NMR spectroscopy and computational methods were used throughout the screening to identify the protein-binding site (the C-allosite) and to provide mechanistic information. For the drug repurposing studies, we used the two most validated fragments that targeted the C-allosite as templates for a structural comparison against a library of 1800 drugs approved by the FDA (L2 library). Our in silico comparison was based on chemical similarity, including chemical functionality and skeleton topology. The size of the equivalent fragment was normalized by the molecular weight of the FDA-approved drug to avoid bias. The validated hits were further characterized using biophysical and biochemical methods and in a murine model of the disease (CEP knock-in mice, Uros<sup>P248Q/P248Q</sup>) (22) for proof-of-concept experiments, and dosage studies were performed on Uros<sup>WT/WT</sup> mice. All work performed with animals was approved by the competent authority (Diputación de Bizkaia) following European and Spanish directives. The CIC bioGUNE Animal Facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Statistical analysis

All experiments were performed using triplicate repeats unless otherwise indicated, and data are presented as means ± SD. Statistical significance was tested using analyses of variance (ANOVA), and P values are reported as *P < 0.05, **P < 0.01, and ***P < 0.001. In the stability assay, an F test versus an in plaque internal control was used to minimize the number of false positives. Statistical tests were carried out using in-house built scripts in MATLAB.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Heme group biosynthetic pathway.
Fig. S2. Computational docking on UROIIIS.
Fig. S3. Thermal denaturation and fluorescence microscopy for some positive compounds (hits).
Fig. S4. CSPs.
Fig. S5. Cell viability assays and IC<sub>50</sub> determination.
Fig. S6. FACS analysis of HEK293 cells and DMSO controls.
Fig. S7. FACS analysis of HEK293 cells with UROIIIIS-P248Q endogenously introduced by CRISPR/Cas9.
Fig. S8. CPX affinity for the heme group.
Fig. S9. Amounts of hepta, hexa, and penta porphyrines in mouse RBCs.
Table S1. Stability properties of the reported CEP pathogenic mutants and their potential correction using pharmacological chaperones.
Table S2. Biochemical and biophysical characterization of the selected compounds.
Table S3. Serum concentrations of CPXglu in mice.
Table S4. Serum concentrations of free CPX and CPXglu after 17 days of food intake.
Table S5. Summary of the genotoxicity assays developed with CPXol and CPX.
Table S6. Primers used for transcript expression analysis.

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Drug repurposing helps iron out porphyria
Porphyria is an inherited incurable disorder resulting from the buildup of heme precursors throughout the body. Urquiza et al. showed that ciclopirox, already approved as an antifungal, allosterically stabilized a mutated biosynthetic enzyme (uroporphyrinogen III synthase or UROIIIS) that leads to this condition. Oral ciclopirox administration increased UROIIIS activity and reduced clinical symptoms in a mouse model of porphyria. Further work will be needed to show whether ciclopirox is suitable for chronic treatment. The authors’ drug repurposing pipeline could potentially be co-opted to investigate therapies for other enzyme mutations that cause metabolic disease.