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Pharmacologic rescue of an enzyme-trafficking defect in primary hyperoxaluria 1

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Primary hyperoxaluria 1 (PH1; Online Mendelian Inheritance in Man no. 259900), a typically lethal biochemical disorder, may be caused by the AGT^{P11LG170R} allele in which the alanine:glyoxylate aminotransferase (AGT) enzyme is mistargeted from peroxisomes to mitochondria. AGT contains a C-terminal peroxisomal targeting sequence, but mutations generate an N-terminal mitochondrial targeting sequence that directs AGT from peroxisomes to mitochondria. Although AGT^{P11LG170R} is functional, the enzyme must be in the peroxisome to detoxify glyoxylate by conversion to alanine; in disease, amassed glyoxylate in the peroxisome is transported to the cytosol and converted to oxalate by lactate dehydrogenase, leading to kidney failure. From a chemical genetic screen, we have identified small molecules that inhibit mitochondrial protein import. We tested whether one promising candidate, Food and Drug Administration (FDA)-approved dequalinium chloride (DECA), could restore proper peroxisomal trafficking of AGT^{P11LG170R}. Indeed, treatment with DECA inhibited AGT^{P11LG170R} translocation into mitochondria and subsequently restored trafficking to peroxisomes. Previous studies have suggested that a mitochondrial uncoupler might work in a similar manner. Although the uncoupler carbonyl cyanide m-chlorophenyl hydrazone inhibited AGT^{P11LG170R} import into mitochondria, AGT^{P11LG170R} aggregated in the cytosol, and cells subsequently died. In a cellular model system that recapitulated oxalate accumulation, exposure to DECA reduced oxalate accumulation, similar to pyridoxine treatment that works in a small subset of PH1 patients. Moreover, treatment with both DECA and pyridoxine was additive in reducing oxalate levels. Thus, repurposing the FDA-approved DECA may be a pharmacologic strategy to treat PH1 patients with mutations in AGT because an additional 75 missense mutations in AGT may also result in mistrafficking.

Primary hyperoxaluria [PH1; Online Mendelian Inheritance in Man (OMIM) no. 259900] is an autosomal recessive disease that results from mutations in alanine:glyoxylate aminotransferase (AGT; EC 2.6.1.44). PH1 is caused by an inability to efficiently metabolize glyoxylate in liver, leading to the accumulation of calcium oxalate in the kidney and urinary tract (1). Subsequent chronic kidney failure leads to accumulation of calcium oxalate deposits throughout the body. AGT is a pyridoxal phosphate-dependent liver-specific enzyme that resides in the peroxisome and catalyzes the transamination of glyoxylate to glycine (2). In PH1, AGT deficiency results in glyoxylate diffusion through the peroxisomal membrane into the cytosol where glyoxylate is subsequently converted to oxalate by lactate dehydrogenase.

The evolution and trafficking of AGT are unique. AGT is primarily found in mitochondria in carnivores, peroxisome in herbivores (including human), and both peroxisome and mitochondria in rodents (3). The diverse localization for the single gene is caused by two transcription and translation start sites (4, 5). Expression from the first site (in carnivores and rodents) reveals a strong mitochondrial targeting sequence whereas expression from the second start site in herbivores, human, and rodents reveals a weak mitochondrial targeting sequence. The C terminus contains a variant of the canonical peroxisomal C-terminal targeting sequence (the tripeptide SKL) and a second peroxisomal targeting sequence, PTS1A (3). In humans, WT AGT localizes to the peroxisome (3). Thus, AGT is an example of a dual localized protein, with targeting sequences to direct it to two locations in cells; localization to two compartments is a common theme with mitochondrial proteins (6).

Numerous mutations in AGT lead to PH1 (3, 7), but the molecular basis varies. A prominent polymorphism (P11L) is common; alleles with P11L are referred to as the minor allele, and 5% of the mutant protein localizes to mitochondria instead of peroxisomes (3). The P11L mutation likely increases the strength of the mitochondrial targeting sequence, but 95% of the AGT pool still localizes to the peroxisomes, presumably because it quickly folds and dimerizes, allowing peroxisome assembly 5 (Pex5) to direct it to peroxisomes (3). In the P11L background, the mutation G170R results in a functional AGT that localizes exclusively to mitochondria (8, 9); AGT^{P11LG170R} accounts for one third of all PH1 cases. It is suggested that the G170R mutation might impair folding in the cytosol, facilitating translocation to the mitochondrion. Alternatively, the charged residue may increase the visibility of the mitochondrial targeting sequence for chaperones or receptors on the mitochondrial outer membrane.

Current treatments for PH1 include pyridoxine supplementation and, later, liver or liver/kidney transplantation (10–12). However, the success has been limited (13); and the recommendation ultimately is an organ transplant, which has obvious complications. Seventy-five additional mutations in AGT have been identified that lead to PH1 (7) so problems in protein trafficking may be an underlying cause of PH1 in additional cases. In a similar vein,

Significance

The lethal disorder, primary hyperoxaluria 1 (PH1), is caused by mutations in peroxisomal-localized alanine:glyoxylate aminotransferase (AGT). AGT contains a C-terminal peroxisomal targeting sequence, but mutations generate a strong N-terminal mitochondrial targeting sequence that directs AGT to mitochondria. Although mutant AGT is functional, the enzyme must be in the peroxisome to detoxify glyoxylate and prevent oxalate accumulation. We have identified a Food and Drug Administration-approved drug, dequalinium chloride (DECA), from a chemical genetic screen to identify probes that attenuate mitochondrial protein import. DECA treatment restores trafficking of mutant AGT from mitochondria to peroxisomes with a subsequent reduction in oxalate levels. Thus, repurposing DECA has potential in therapeutic strategies for PH1 because current clinical trials have not produced an effective treatment, short of organ transplant.

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The authors declare no conflict of interest.

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mistargeting of mutant enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase (EHHADH) from peroxisomes to mitochondria results in another kidney disease, inherited renal Fanconi's syndrome (14).

Given that AGT is mistargeted to mitochondria, a strategy that may form a platform for developing therapeutics is the application of small molecules that restore trafficking from mitochondria to peroxisomes. To this end, we have developed a screen to identify small-molecule modulators that attenuate mitochondrial protein translocation, based on mistargeting of Ura3. Here, we show that one Food and Drug Administration (FDA)-approved small molecule, dequalinium chloride (DECA, also referred to as MitoBloCK-12/MB-12) restores trafficking of $AGT^{P11LG170R}$ from mitochondria to peroxisomes in a cell model, with a subsequent reduction in oxalate production.

Results

An in Vivo Screen for Inhibitors of Mitochondrial Protein Translocation Reveals an FDA-Approved Compound That Inhibits Protein Import. To identify small-molecule modulators of the translocase of the outer membrane/translocase of the inner membrane-23 (TOM/TIM23) translocation system, we adapted the genetic screen that resulted in the identification of import components Tim44, Tim23, and Tim17, based on mislocalization of the Su9-Ura3 fusion protein from mitochondria to the cytosol (15). The targeting sequence, abbreviated Su9, is derived from subunit 9 of the *Neurospora crassa* ATPase and confers robust import. If Su9-Ura3 is targeted to the mitochondrial matrix, yeast fails to grow in media lacking uracil because Ura3 must be localized to the cytosol to participate in the uracil biosynthetic pathway. When protein translocation is attenuated, the Su9-Ura3 protein remains in the cytosol, and growth in media lacking uracil is restored to the yeast cells (15). A plasmid encoding Su9-Ura3 with a C-terminal myc tag was integrated into wild type (WT) and the *tim23-2* mutant at the *LEU2* locus (16). In addition, the multidrug resistance ABC transporters *SNQ2* and *PDR5* were deleted to increase the concentration of small molecules in the cells (17).

A small-molecule screen was conducted with an integrated robotic system. Briefly, the Prestwick library of FDA-approved compounds (~2,000) at a concentration of ~10 μM was screened against the yeast strain WT[Su9-URA3]. The strain was aliquotted into 384-well pates consisting of 24 columns followed by compound addition with robotic pinning into the assay wells (column 3–22) in minimal glucose media lacking uracil. For a negative control of cell growth, column 2 contained the WT[Su9-URA3] strain with 1% DMSO. For a positive control of cell growth, column 23 contained the *tim23-2*[Su9-URA3] strain with 1% DMSO, which has compromised protein import. After incubation at 30 °C for 24 h, cultures in each well were measured for optical density (OD_{600}) as a measure of growth. Wells that had a growth increase of greater than 30% compared with the negative control were selected as potential candidates. Of two candidates that were reconfirmed by repeating the assay, one compound reproducibly increased the growth of the WT[Su9-URA3] strain. Here, we characterize this FDA-approved compound, termed MitoBloCK-12 (MB-12), which was identified as dequalinium chloride (DECA) (Fig. 1*A*). DECA has been approved as an antibacterial treatment for oral and vaginal applications (18). Moreover, DECA is used as a "nano-particle" to deliver cargo to the mitochondria (19, 20). DECA can form a micelle-like structure with an encapsulated target that is driven to the mitochondrial matrix because the matrix has a net negative charge (19, 20); these studies suggest that DECA is well-tolerated by cells, but little is understood about the specific mechanism of action.

DECA was identified based on the ability to inhibit Su9-Ura3 import into mitochondria and confer growth in media lacking uracil. At an optimal concentration of 0.7–4 μM, a yeast strain expressing integrated plasmid pSu9-URA3 showed increased growth in media lacking uracil, likely because protein import into mitochondria was impaired (Fig. S1*A*). At higher concentrations, the strain failed to grow because protein import may have been limiting. In contrast, treatment with carbonyl cyanide m-chlorophenyl hydrazone (CCCP) caused only a slight increase in growth from 0.1 μM to 0.6 μM. The import of radiolabeled Su9-Ura3 was tested into isolated yeast mitochondria in the presence of DECA (Fig. 1*B*). Whereas 2 μM DECA did not inhibit import of Su9-Ura3, 4 μM and 10 μM DECA strongly inhibited Su9-Ura3 import. Thus, DECA treatment attenuated protein translocation of Su9-Ura3 in yeast mitochondria in vitro and in vivo.

We investigated the minimal inhibitory concentration required to inhibit growth of 50% of the yeast (MIC_{50}) for DECA and

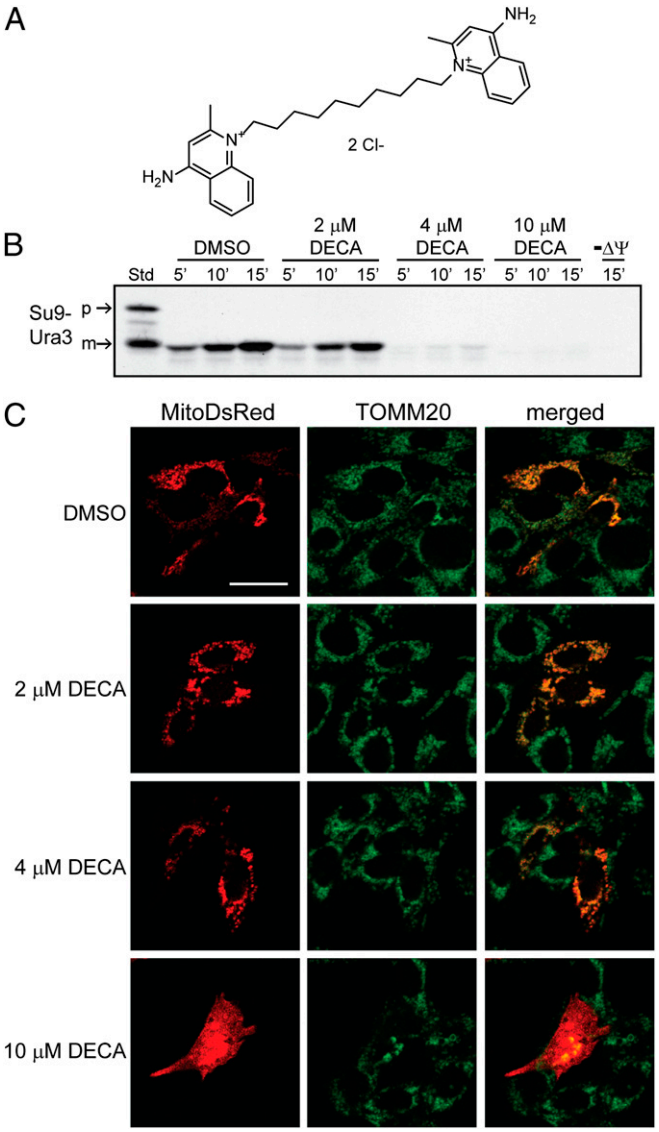

Fig. 1. DECA (10 μM) attenuates protein translocation into mitochondria. (*A*) Structure of MitoBloCK-12, the chemical name of which is dequalinium chloride (DECA). (*B*) Isolated WT yeast mitochondria were preincubated with vehicle (1% DMSO) or DECA (2 μM, 4 μM, 10 μM) for 15 min, followed by the import of radiolabeled Su9-Ura3. Aliquots were removed after 5 min, 10 min, and 15 min, and trypsin was added to remove nonimported precursor. Samples were subsequently treated with trypsin inhibitor and were separated by SDS/PAGE and developed by autoradiography. As a negative control for import, the membrane potential was inhibited by CCCP treatment. A 10% standard is included. p, precursor; m, mature. (*C*) MitoDsRed (DsRed targeted to the mitochondrial matrix) was transiently expressed in CHO-K1 cells that were treated with 1% DMSO or DECA as indicated. At 24 h posttransfection, cells were fixed and stained with anti-TOMM20 antibody, and images were taken. (Scale bar: 20 μm.)

CCCP in WT and the *tim23-2* mutant strain in rich glucose media (Fig. S1 *B* and *C*); in both strains, the drug pumps Snq2 and Pdr5 were deleted to increase the concentration of the small molecule in the cell (17). For CCCP, the MIC_{50} was ~0.8 μM for both the *tim23-2* mutant and WT strains. The MIC_{50} for DECA was 0.38 μM for WT and 0.17 μM for the *tim23-2* mutant. Thus, this analysis suggests that the *tim23-2* mutant has increased sensitivity to DECA.

A potential mechanism by which DECA may alter protein translocation is that DECA may nonspecifically permeabilize mitochondrial membranes, and proteins may be released from the mitochondrion to the supernatant (17). We incubated mitochondria with DECA for 30 min followed by centrifugation at 14,000 × *g* to separate the intact mitochondria (P) from proteins that may have been released into the supernatant (S) (Fig. S1 *D* and *E*). Proteins were separated by SDS/PAGE and visualized with Coomassie staining for the collective release of proteins (Fig. S1*D*) or detected by immunoblot analysis for the release of specific proteins (Fig. S1*E*). Candidate proteins that were tested by immunoblotting included α-ketoglutarate dehydrogenase (KDH), Tim44, Tim23, and cytochrome *c* (cyt c). Compared with the vehicle control DMSO, proteins were not extensively released to the supernatant in the presence of DECA. Thus, mitochondrial membrane integrity was not compromised by DECA addition.

Dequalinium Chloride Inhibits Protein Import into Mitochondria, but Does Not Uncouple Mitochondria. We tested the ability of DECA to inhibit import of Su9-DHFR into mitochondria isolated from CHO-K1 cells (Fig. S1*F*). Radiolabeled Su9-DHFR precursor was imported into isolated mitochondria in the presence of DECA or CCCP. The 2 μM DECA inhibited Su-DHFR import by 40% whereas treatment of mitochondria with 4 μM and 10 μM DECA inhibited import similarly to treatment with 2 μM CCCP. Because DECA is effective at inhibiting protein import into isolated mammalian mitochondria, these experiments were extended into CHO-K1 cells to determine whether DECA treatment altered the import of DsRed targeted to the mitochondrial matrix (MitoDsRed) in vivo (Fig. 1*C*). Treatment of cells with 2 μM or 4 μM DECA did not alter mitochondrial targeting of MitoDsRed. However, when cells were treated with 10 μM DECA, MitoDsRed import into mitochondria was impaired, and the precursor showed diffuse staining in the cytosol. A similar set of experiments was done with CCCP in CHO-K1 cells (Fig. S2). A concentration of 2 μM or 4 μM did not impair MitoDsRed import, but 10 μM CCCP inhibited import of MitoDsRed, and the precursor accumulated in punctate spots in the cytosol.

We tested whether DECA uncoupled mitochondria in CHO-K1 cells. We assessed the membrane potential by staining cells with the membrane potential-sensitive dye, MitoTracker Red (Fig. 2*A*). At concentrations up to 10 μM DECA, the mitochondria still maintained a membrane potential as shown by intense staining with MitoTracker Red; in contrast, cells treated with as little as 2 μM CCCP displayed a markedly decreased membrane potential because mitochondrial staining could not be detected with MitoTracker Red treatment (Fig. S3*A*). As the cells became uncoupled by CCCP treatment, the ATP levels dropped (Fig. S3*B*); in contrast, 10 μM DECA treatment did not alter the ATP abundance of the cells. Finally, we also investigated the toxicity of DECA in zebrafish embryos (Fig. S4). When asynchronous cell division started at 3 h postfertilization (hpf) (21, 22), embryos were treated with CCCP or DECA in the vehicle DMSO. At 150 nM CCCP, the zebrafish embryos failed to develop, indicating that CCCP is very toxic to embryos (Fig. S4*C*). In contrast, addition of 20 μM DECA in DMSO vehicle did not alter zebrafish development (Fig. S4*A*). Because concentrations greater than 20 μM DECA were not soluble in DMSO, a slightly aqueous buffer [10% H_2O/90% DMSO (vol/vol)], in which 100 μM DECA could be reached, was incubated with the zebrafish embryos. At 50 μM DECA, embryos became malformed, and embryos did not survive in solution with 100 μM DECA (Fig. S4*B*). In summary, DECA inhibits mitochondrial protein translocation in vivo at a concentration greater than 4 μM

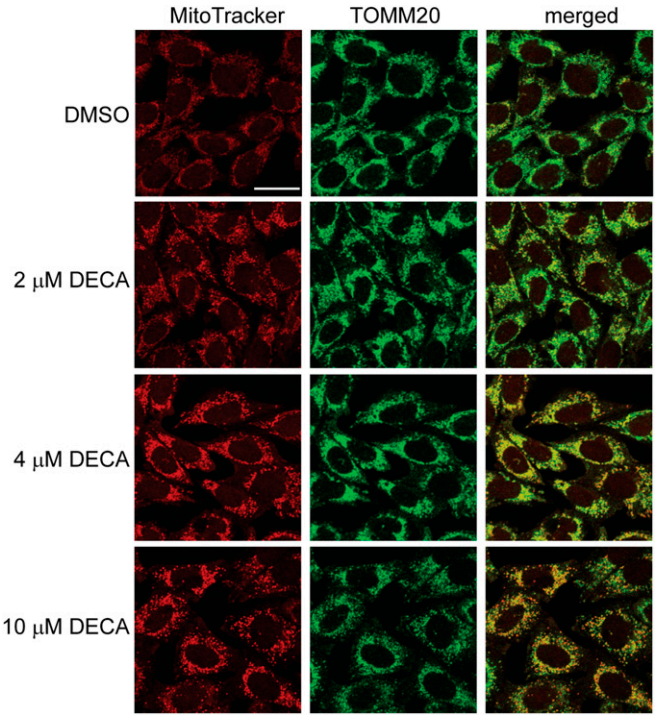

Fig. 2. DECA (10 μM) does not decrease the membrane potential. CHO-K1 cells were treated with the indicated concentrations of DECA for 4 h. Cells were stained with the membrane potential-dependent dye MitoTracker CMXRos, fixed, and incubated with anti-TOMM20 antibody. (Scale bar: 20 μm.)

(Fig. 1*C*) but does not uncouple cells or embryos (Fig. 2 and Fig. S4). DECA therefore does not act like CCCP, and DECA does not interfere with embryogenesis at the working concentrations below 10 μM.

$AGT^{P11LG170R}$ Localizes to the Mitochondrial Matrix. DECA seemed like an ideal candidate to determine whether attenuated mitochondrial import of $AGT^{P11LG170R}$ could restore trafficking back to the peroxisome. We tested the import of WT AGT and the $AGT^{P11LG170R}$ mutant into isolated yeast mitochondria (Fig. S5*A*). The WT AGT with the weak mitochondrial targeting sequence did not import into mitochondria; in contrast, $AGT^{P11LG170R}$ import into mitochondria was robust and dependent on the presence of a membrane potential. To confirm a mitochondrial localization in the matrix, we performed osmotic shock followed by centrifugation to separate the soluble intermembrane space fraction (S) from mitoplasts (intact inner membrane/matrix fraction that is recovered in the pellet fraction, P) (Fig. S5*B*). $AGT^{P11LG170R}$ was recovered in the mitoplast fraction, and subsequent treatment with proteinase K resulted in degradation of intermembrane space protein cytochrome b_2 (cyt b2), but not matrix-localized α-ketoglutarate dehydrogenase (KDH) or $AGT^{P11LG170R}$. Treatment with detergent resulted in degradation of $AGT^{P11LG170R}$, confirming localization to the matrix. Note that KDH was recovered in the supernatant as a fragment of decreased molecular mass (indicated by an asterisk in Fig. S5*B*), indicating that detergent treatment solubilized the membranes. The resistance of KDH to the added protease in the presence of detergent is likely caused by a tightly folded domain that is protease-resistant (23). Analysis by carbonate extraction showed that $AGT^{P11LG170R}$ was a soluble protein (S) like matrix-localized metallopeptidase 112 (Mop112) rather than a membrane protein (P) like ADP/ATP carrier (Fig. S5*C*). The import of $AGT^{P11LG170R}$ into WT yeast mitochondria in the presence of DECA was also investigated as described in Fig. 1*B* (Fig. S5*D*). DECA inhibited

the import of AGT^{P11LG170R} at 4 μM and 10 μM, which mirrors the import inhibition of Su9-DHFR and Su9-Ura3. Thus, AGT^{P11LG170R} is a resident matrix protein (3) with import that is abrogated by the addition of DECA.

DECA Blocks Protein Import of AGT^{P11LG170R} into Mitochondria, Restoring Peroxisomal Import. To determine whether DECA attenuated mitochondrial import of AGT^{P11LG170R} in cultured cells, we transiently transfected CHO-K1 cells with the WT AGT or AGT^{P11LG170R} construct followed by fixing and immunostaining for AGT and mtHsp70 to mark mitochondria (Fig. 3*A*). In the presence of DMSO, WT AGT translocated to peroxisomes, but AGT^{P11LG170R} localized to mitochondria. Treatment with 1 μM DECA resulted in AGT^{P11LG170R} localization to mitochondria and small punctate loci that resembled peroxisomes. An increase to 2 μM DECA altered AGT^{P11LG170R} targeting from mitochondria to these small punctate loci. In a subsequent set of experiments, peroxisomes were marked with GFP-SKL, which has a C-terminal peroxisomal targeting sequence to efficiently target GFP to peroxisomes (Fig. 3*B*). Indeed, AGT^{P11LG170R} colocalized with peroxisomal GFP, supporting that DECA treatment attenuates AGT^{P11LG170R} import into mitochondria in a manner that allows the peroxisomal targeting sequence the ability to restore translocation to the peroxisome. Because it has been suggested that uncouplers such as CCCP may function similarly (24), we replaced DECA with CCCP and examined AGT^{P11LG170R} import (Fig. 4). In the presence of 2 μM CCCP, AGT^{P11LG170R} imported into mitochondria whereas 4 μM CCCP blocked AGT^{P11LG170R} import into mitochondria, with the precursor accumulating in the cytosol. Thus, CCCP is not effective at redirecting AGT^{P11LG170R} import to peroxisomes, and DECA works through a mechanism independent of uncoupling mitochondria.

DECA Treatment Reduces Oxalate Levels in a PH1 Cell Model. To confirm that DECA treatment restored AGT function to cells, we took advantage of a CHO-K1 cell model in which glycolate oxidase (GO) with AGT or AGT^{P11LG170R} was stably expressed (25). This cell line was chosen because this glyoxylate pathway is not active in this cell type. Peroxisomal GO converts glycolate to glyoxylate. In the aminotransferase reaction, AGT then converts glyoxylate to glycine, which reduces glyoxylate levels. If functional AGT is absent from the peroxisome, glyoxylate is transported from the peroxisome to the cytosol and lactate dehydrogenase, which is active in CHO-K1 cells, converts glyoxylate to oxalate that is directly measured in this assay.

Cells stably expressing GO and WT AGT were fed 200 μM glycolate; the oxalate levels were measured and normalized to 1 (Fig. 5). The presence of DECA did not alter the oxalate levels in the cells expressing GO and AGT, indicating that DECA alone had no influence on the pathway and that oxalate levels did not increase when AGT was targeted normally to peroxisomes. Oxalate levels were approximately threefold higher in cells expressing GO and AGT^{P11LG170R} in the presence of the vehicle control DMSO, indicating that AGT^{P11LG170R} was not functional as it localized to mitochondria. Pyridoxal HCl treatment (converted to pyridoxine in the cell) has been shown to partially rescue the metabolic defect in a subset of patients with AGT^{P11LG170R}. Studies in cell models indicate that pyridoxine addition may stabilize AGT^{P11LG170R} in an active form in the cytosol (10); alternatively, pyridoxine may act as a chaperone to restore a low level of AGT^{P11LG170R} import back to peroxisomes, but localization studies were not convincing (10). The amount of pyridoxal HCl in the culture media is 0.3 μM. Because the addition of pyridoxine to 50–250 μM was shown to be inhibitory for AGT activity (10), pyridoxal HCl was added at 10 μM in this assay. Treatment with pyridoxal HCl reduced the oxalate level to twofold compared with cells expressing AGT^{P11LG170R} in the presence of DMSO. Treatment with 2 μM DECA had a similar

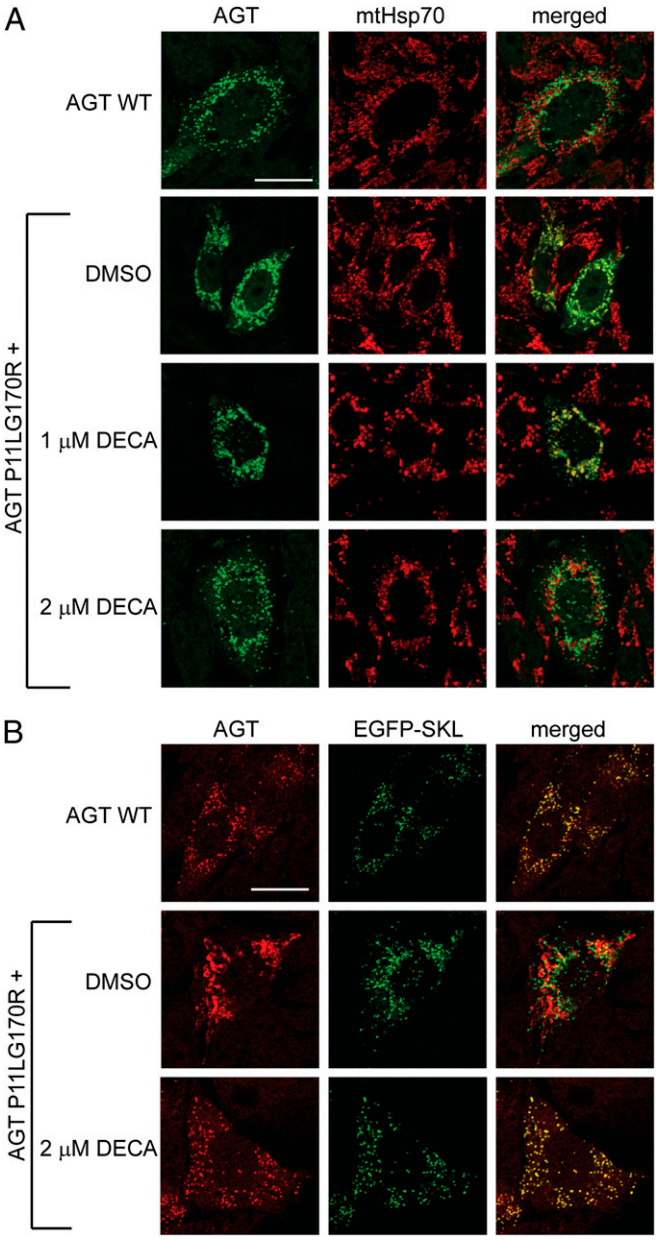

Fig. 3. DECA treatment stops mitochondrial mislocalization of mutant AGT^{P11LG170R} and restores peroxisomal targeting. (*A*) CHO-K1 cells that were transiently transfected with AGT WT or AGT^{P11LG170R} constructs and then were treated with DECA or DMSO as indicated. At 48 h posttransfection, cells were fixed and stained with antibodies against AGT and mtHsp70. (*B*) CHO-K1 cells were transiently cotransfected with AGT WT or AGT^{P11LG170R} mutant and EGFP-SKL to mark peroxisomes. At 48 h posttransfection, cells were fixed and stained with anti-AGT antibody. (Scale bar: 20 μM.)

effect; and the addition of both DECA and pyridoxal HCl was additive in reducing the oxalate levels to 1.5-fold compared with cells expressing AGT^{P11LG170R} in the presence of DMSO. Additional combinations of DECA and pyridoxal HCl were not successful in reducing oxalate levels to that of cells expressing GO and WT AGT, but this lack of reduction may be a result of parameters that are difficult to control, such as the expression level of the enzymes, efficiency in retargeting AGT^{P11LG170R}, and transport of glyoxylate from the peroxisome to the cytosol. Because CCCP also inhibited AGT^{P11LG170R} import into mitochondria, we tested CCCP in this system, but the cells died.

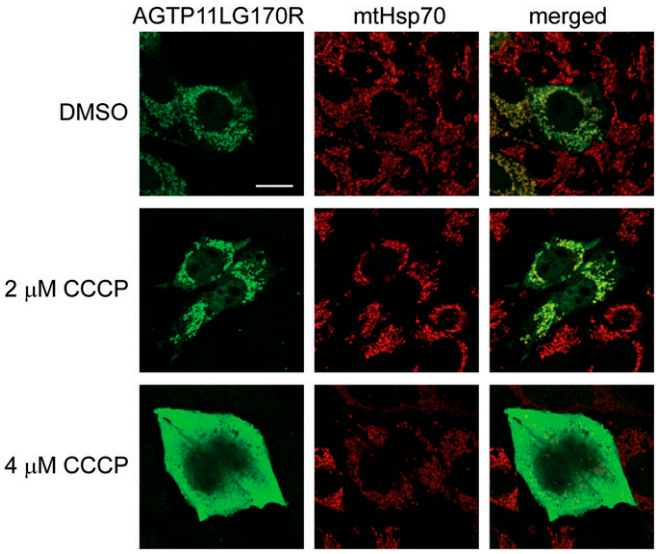

Fig. 4. CCCP treatment blocks AGT^{P11LG170R} translocation into mitochondria but does not restore peroxisomal targeting. As in Fig. 3*A*, but cells were treated with the indicated concentration of CCCP. (Scale bar: 20 μM.)

This line of investigation shows that DECA can block AGT^{P11LG170R} import into mitochondria, restore import into peroxisomes, and partially rescue the oxalate accumulation defect in a cell model. Thus, repurposing FDA-approved DECA as a pharmacologic treatment for PH1 may be a platform for therapeutic strategies in a subset of patients.

Discussion

The molecular basis of PH1 is complicated because, in a subset of patients, AGT^{P11LG170R} is functional but solely traffics to the wrong compartment (1). AGT normally localizes to peroxisomes in human and is mistargeted to mitochondria, where the active enzyme cannot reach substrate. Thus, AGT has a complex trafficking pathway. Although it is a rare, autosomal recessive disease, it is the most common type of hyperoxaluria, with an estimated incidence rate of ~1:100,000 live births per year in Europe (26). PH1 accounts for ~1% of pediatric end-stage renal disease. In contrast, PH1 is more prevalent in countries where consanguineous marriages are common (10% and 13% of children in Kuwait and Tunisia, respectively) (27, 28). The unfortunate endpoint with PH1 is renal failure, and the age at which it presents and the severity vary greatly as is typical with many mitochondrial and peroxisomal diseases (26). Current treatments include pyridoxine supplementation and, later, liver or liver and kidney transplantation (10). These treatments, however, have limited success (7).

We therefore considered whether a small molecule that inhibits mitochondrial protein translocation might be promising to retarget AGT^{P11LG170R} back to peroxisomes. From a small-molecule screen in yeast, based on blocking import of Su9-Ura3, we have identified several potential small molecules that abrogated import. Indeed, treatment of cells expressing AGT^{P11LG170R} with DECA blocked import of AGT^{P11LG170R} into mitochondria and restored import to peroxisomes. DECA does not act like the uncoupler CCCP, which resulted in AGT^{P11LG170R} accumulation in the cytosol. Thus, a suggested strategy of an uncoupler will not likely be successful in retargeting AGT^{P11LG170R} to mitochondria, especially because uncoupler treatment impaired all mitochondrial import functions and induced death in zebrafish embryos and the PH1 cell model. DECA likely works differently because AGT^{P11LG170R} may arrest in the mitochondrial TOM and TIM translocons with the C terminus facing the cytosol; this conformation of AGT^{P11LG170R} may maintain import-competency for peroxisomes, and chaperones such as Pex5 may subsequently mediate peroxisomal import. DECA treatment also reduced the oxalate load in a cell model, suggesting that DECA has potential in a therapeutic platform.

DECA is an FDA-approved compound and has been used as a delivery system to mitochondria (19, 20). DECA is quite potent at blocking import of AGT^{P11LG170R} into mitochondria in cell models. Studies with purified mitochondria and yeast indicate that a low concentration (1-2 μM) of DECA is inhibitory. In contrast, the import of Hsp70 and MitoDsRed was not markedly decreased by DECA. This selective difference in import inhibition may be imparted on a weaker mitochondrial targeting sequence in AGT^{P11LG170R} vs. bona fide mitochondrial proteins.

Mammalian cells and zebrafish embryos also seem to tolerate DECA at low concentrations well; zebrafish development was affected only when the concentration of DECA reached 50 μM and CHO-K1 cells were not uncoupled in the presence of 10 μM DECA. Thus, DECA seems to be promising for further studies in animal models. Recently, an additional 75 missense mutations in AGT have been identified, and almost half are uncharacterized (7, 29). DECA or similar small molecules may be beneficial for rescuing PH1 caused by these new AGT mutants. Given that DECA is FDA-approved, repurposing this old drug may be an attractive strategy in developing therapeutics for a subset of patients with PH1 (30) because pyridoxine treatment has not resulted in biochemical remission in a recent clinical trial (7). In the short term, DECA and similar mitochondrial protein import blockers should be useful for studying PH1 in new model systems that are being developed and for mechanistic studies to determine the complex AGT-trafficking pathway.

Experimental Procedures

High-Throughput Screening. The screen was performed using the WT[Su9-URA3] strain that was grown in SD media supplemented with uracil and without leucine. Yeast were washed with sterile H_2O twice to remove uracil and diluted in SD media lacking uracil to an OD_{600} of 0.05. A Titertek multidrop instrument was used to dispense 50 μL of cell suspension into each clear 384-well plate (Greiner Bio-One). The Biomek FX (Beckman Coulter) was used to pin transfer 0.5 μL of compounds from the 1 mM stock or DMSO to respective wells. The approximate screening concentration of the small molecules was 10 μM. As controls, column 2 and 23 consisted of the

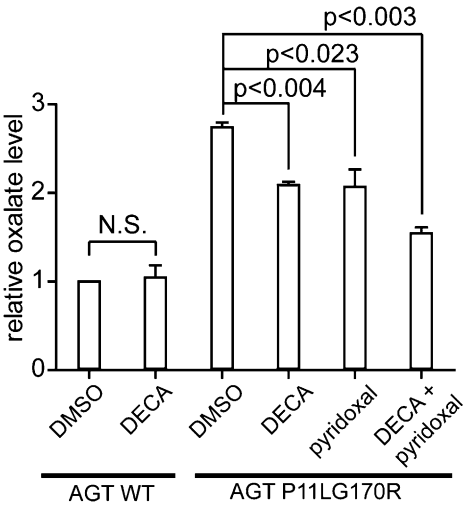

Fig. 5. DECA ameliorates oxalate accumulation in a PH1 model in CHO-K1 cells. CHO-K1 cells stably expressing glycolate oxidase with AGT WT (WT) or AGT^{P11LG170R} were treated with 2 μM DECA and 10 μM pyridoxal HCl for 4 d. After drug treatment, cells were incubated with 200 μM glycolate for 4 h, followed by oxalate measurement. The number 1 was set as the oxalate level of WT cells treated with DMSO. Average oxalate level ± SD of $n = 3$ trials. N.S., not significant. A paired *t* test was used to assess significance.

WT[Su9-URA3] and *tim23-2*[Su9-URA3] strains, respectively, supplemented with the vehicle 1% DMSO. After completing compound transfer, all plates were incubated at 30 °C in a humidified incubator for 24 h. Each plate was shaken in a Beckman orbital shaker to resuspend the settled cells, and the OD_{600} was read by a Wallac Victor plate reader (Perkin-Elmer). The compounds that augmented growth of the WT[Su9-URA3] strain by more than 30% of that of the *tim23-2*[Su9-URA3] strain were identified and cherry-picked into one plate for rescreening. Hit compounds that conferred growth in the repeated assay were reordered from the Prestwick library for follow-up analysis.

Cell Culture and DNA Transfection. CHO-K1 cells, including CHO-K1 cells stably expressing GO with WT AGT or $AGT^{P11L\ R170R}$ (25), were maintained in Ham's F-12 medium supplemented with 10% (vol/vol) FBS at 37 °C under 5% (vol/vol) CO_2. DNA transfections were performed using FuGENE HD (Promega).

Plasmids and Antibodies. cDNAs encoding WT AGT and $AGT^{P11L\ R170R}$ cloned in pcDNA3.1 were provided by C.J.D. (25). pMitoDsRed (Clontech Laboratories) and EGFP fused to SKL (peroxisomal targeting signal type 1) (EGFP-SKL) in pEGFP-C1 were used to visualize mitochondria and peroxisomes, respectively. A polyclonal rabbit antibody to TOMM20 (Santa Cruz) and a monoclonal mouse antibody to mitochondrial Hsp70 (University of California, Davis/National Institutes of Health NeuroMab Facility) were purchased from commercial vendors. The polyclonal rabbit antibody to AGT was provided by C.J.D. (25).

Zebrafish Manipulations. Zebrafish lines derived from the characterized TL background were maintained in a 14-h light/10-h dark cycle and mated for 1 h to obtain synchronized embryonic development. Embryos were grown for 3 hpf in E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM Mg_2SO_4) and then incubated with E3 buffer supplemented with 1% DMSO, DECA in 1% DMSO, or DECA in 10% H_2O/90% DMSO (vol/vol) for 3 d at 28.5 °C. Following treatment, embryos were imaged with a stereomicroscope under white light using a Leica S8AP0 at 1.575× magnification. Images were resized to 300 dpi without resampling using Photoshop software (Adobe).

Oxalate Measurements. CHO-K1 cells stably expressing GO with WT AGT or $AGT^{P11L\ R170R}$ were treated with 0.2% DMSO, 2 μM DECA, 10 μM pyridoxal HCl or both of 2 μM DECA and 10 μM pyridoxal HCl for 3 d. Cells (5×10^5) were transferred into a six-well dish and incubated in the presence of respective drugs for an additional 24 h. Cells were then treated with 200 μM glycolate for 4 h, followed by collection of the culture supernatant. The supernatants were treated with charcoal, and oxalate levels were measured with a commercial kit according to the manufacturer's protocols (Trinity Biotech). Three independent measurements were taken, and a student's paired *t* test was used to assess significance.

ATP Measurements. To measure cellular ATP level, cells from a six-well dish were trypsinized, pelleted, and lysed by 200 μL of 1% trichloroacetic acid. After neutralization with 1 M Tris·HCl (pH 7.4), ATP levels in the extracts were measured with a luciferase-based ATP measurement kit according to the manufacturer's protocol (Promega).

Mitochondrial Manipulations. Import assays, carbonate extraction analysis, and osmotic shock experiments were performed as described previously (17, 21).

Immunofluorescence Microscopy. Cells on coverslips were fixed with 3.7% (vol/vol) formaldehyde in PBS, permeabilized with ice-cold methanol, and blocked with 1% BSA in PBS (PBS-BSA). Subsequently, cells were incubated for 2 h at room temperature with appropriate primary antibodies diluted in PBS-BSA, and then cells were washed extensively with PBS and incubated for 1 h at room temperature with Alexa Fluor 488- or Alexa Fluor 568-conjugated secondary antibody diluted in PBS-BSA. For staining with MitoTracker Red CMXRos (Invitrogen), cells were incubated with 100 nM MitoTracker Red CMXRos for 30 min before fixation. Images were obtained using a confocal laser microscope (Carl Zeiss).

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