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Results of a Pilot Study of Isoniazid in Patients with Erythropoietic Protoporphyrria

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Abstract

Erythropoietic protoporphyria (EPP), the most common porphyria of childhood and the third most common porphyria of adulthood, is characterized clinically by painful, non-blistering cutaneous photosensitivity. Two distinct inheritance patterns involving mutations affecting genes that encode enzymes of the heme biosynthetic pathway underlie the clinical phenotype. Aminolevulinic acid synthase 2 (ALAS2), the rate limiting enzyme of the heme pathway in the erythron, is a therapeutic target in EPP because inhibiting enzyme function would reduce downstream production of protoporphyrin IX (PPIX), preventing accumulation of the toxic molecule and thereby ameliorating symptoms. Isoniazid (INH) is widely used for treatment of latent and active *M. tuberculosis* (TB). Sideroblastic anemia is observed in some patients taking INH, and studies have shown that this process is a consequence of inhibition of ALAS2 by INH. Based on these observations, we postulated that INH might have therapeutic activity in patients with EPP. We challenged this hypothesis in a murine model of EPP and showed that, after 4 weeks of treatment with INH, both plasma PPIX and hepatic PPIX were significantly reduced. Next, we tested the effect of INH on patients with EPP. After eight weeks, no significant difference in plasma or red cell PPIX was observed among the 15 patients enrolled in the study. These results demonstrate that while INH can lower PPIX in an animal model of EPP, the standard dose used to treat TB is insufficient to affect levels in humans.

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Introduction:

All porphyrias are a consequence of disturbances in the formation of heme, an essential component of both hemoglobin and cellular cytochromes. Each type of porphyria is due to a hereditary or acquired defect in one of the enzymes of the heme synthetic pathway. With one exception (x-linked erythropoietic protoporphyria), the enzyme defect restricts conversion of porphyrin precursors to the enzyme's product with subsequent accumulation of the substrate or its metabolic intermediates. The porphyrias are grouped into acute neurovisceral and chronic cutaneous forms. Erythropoietic protoporphyria (EPP) is a member of the family of chronic cutaneous porphyrias. EPP is inherited as an autosomal recessive disease. (1, 2) In most cases, an inactivating mutation of ferrochelatase (FECH), the enzyme that catalyzes incorporation of ferrous iron into PPIX, is present on one allele with the other allele being under-expressed (hypomorphic) due to a common polymorphism that creates a cryptic splice site. In a small minority of cases, EPP is inherited as an X-linked disease due to a gain-of-function mutation of ALAS2, the erythroid-specific form of the enzyme that catalyzes formation of ALA from glycine and succinyl-CoA. (3) This form of the disease, termed X-linked EPP (XLP), is typically more severe in males than in females, however females can be affected to varying degrees depending upon the extent of mosaicism resulting from random X-chromosome inactivation. The gain-of-function mutation, increases production of PPIX sufficiently to exceed the capacity of FECH to generate heme. Consequently, PPIX accumulates in erythroid cells, phenocopying the autosomal form of EPP.

Clinically, EPP is characterized by painful, non-blistering cutaneous photosensitivity with onset in early childhood. (4) Reports of prevalence varies between 5 and 15 cases per million population. (4, 5) Protoporphyrinic hepatopathy is a potentially fatal complication of the disease that is estimated to occur in less than 5% of patients. Avoidance of exposure to sunlight to prevent the debilitating symptoms of the disease requires major changes in lifestyle and work environment. Topical sunscreens that absorb ultraviolet A and sun blocks containing zinc oxide or titanium dioxide may lessen symptoms. β -carotene, which may act by quenching oxygen radicals, (6) is the most studied treatment for EPP. (7) It may afford some protection after 1 to 3 months of therapy, but results vary. Oral cysteine may quench excited oxygen species and thereby increase tolerance to sunlight in patients with EPP. Other treatments that aim either to increase skin pigmentation or to scavenge activated oxygen species include dihydroxyacetone/Lawsone, vitamin C, and narrow wave ultraviolet B phototherapy. (8) Afamelanotide, an α -melanocyte-stimulating hormone analogue that increases skin melanin, has now been approved as treatment for EPP in many countries in Europe and an application is under review at the Food and Drug Administration (FDA). (9) Clearly more specific therapy is needed.

The functional activity of ALAS2 is inhibited by the pyridine derivative, isonicotinic acid hydrazide (isoniazid, INH), a drug used to treat tuberculosis that has been in use worldwide for nearly 60 years. (10) Pyridoxal phosphate (PLP) is an essential cofactor of ALAS2. (11) Studies have shown that INH restricts PLP function both by inhibiting pyridoxal kinase activity and by binding to PLP to form an inactive pyridoxal hydrazine. (10) In these cases, ALAS2 activity is reduced indirectly by limiting availability of its cofactor, PLP. (12) Recent in vitro studies suggest that INH may also directly inhibit ALAS2 activity by binding to the

protein and causing a structural rearrangement that disrupts the interaction between ALAS2 and PLP. (10) Herein we report the results of experiments using a murine model of XLP that show that INH reduces plasma and hepatic concentrations of PPIX, and we describe the findings of our study designed to determine if INH, given at a dose similar to that used to treat tuberculosis, reduces the concentration of ALA and PPIX in the erythrocytes and plasma of patients with EPP and XLP.

A mouse model of XLP has been described where deletion of the gene that encodes iron regulatory protein 2 (*Irp2*) *Irp2*^{-/-}, leads to overproduction of ALA. Without *Irp2*, translation of *Alas2* is unregulated because IRP2 is not available to bind to the 5' Iron Responsive Element (IRE) present in the *Alas2* mRNA, and thereby modulate translation. The unregulated production of ALAS protein leads to excess ALA that is converted to PPIX by subsequent steps in the pathway, the activity of FECH then becomes rate-limiting with respect to PPIX production and excess PPIX accumulates much the same as in EPP. (13)

Methods:

Vertebrate Animal Studies

Mouse experiments were approved by the University of Utah Institutional Animal Care and Use Committee. Wild-type and *Irp2*^{-/-} mice were a kind gift of Dr. Elizabeth Leibold, University of Utah School of Medicine. (13) In the current study, *Irp2*^{-/-} mice and wild-type (WT) littermates were dosed by gavage with INH (100 mg/kg in normal saline) (12) weekdays (5 of 7 days) for 4 weeks, and plasma concentrations of PPIX were monitored. Approximately 50 μ L of blood was removed by venous jugular bleed on a weekly basis for analysis of PPIX levels. After 4 weeks of treatment, cohorts were sacrificed, exsanguinated, and subjected to hepatectomy. Analysis of free PPIX and ZnPPIX in the red blood cells, plasma and liver was performed using standard procedures. (13) Briefly, serum was diluted with water to about 10 mg/mL protein. While vortexing, 50 μ L of 6 M HCl was added to 150 μ L diluted serum to give a final acid content of 1.5 M. The mixture was incubated for 30 min at 37°C and then centrifuged at 13000 rpm for 10 min at room temperature. The supernatant fluid was injected into the UPLC as described in the Iron and Heme Core website: http://cihd.cores.utah.edu/wp-content/uploads/2016/06/UPLC_of_Porphyrins.pdf. When samples were visualized for hemolysis it was evident that several samples appeared to have more hemolysis than others. Based on the observed hemolysis in some of the blood samples the levels of plasma PPIX were considered to be unacceptable since the amount contributed from the lysed red blood cells was not consistent across samples.

Patient Studies

Studies in humans were approved by the local Ethics Committees in accordance with the ethical principles for medical research involving human subjects and its subsequent amendments of the Declaration of Helsinki (R162-16-7 and 145-15-4, French ethical agreement). All participants provided written informed consent. All research subjects were adults (18 years old), capable of understanding, and in agreement with, the purposes of the study that included genetic testing. The study was performed in patients with biochemically and genetically confirmed EPP or XLP. Laboratory values for CBC, urinalysis, liver

function, metabolic panel, iron measurements were determined at each participating center. Plasma and red blood cell PPIX and ZnPPIX were quantified in the laboratory of Dr. Karl Anderson, University of Texas, Galveston or in the Central Porphyria Laboratory, Paris, France. Patient were enrolled from five Porphyria Research Consortium Centers (University of Utah; University of Alabama; Mt. Sinai, New York City; University of California, San Francisco; University of Texas, Galveston) and a single site in Paris.

Patients with evidence of active liver injury as defined by serum transaminase concentrations greater than three times the upper limit of normal, those with a history of recent (within 3 months of enrollment) or ongoing alcohol abuse, those with diabetes mellitus requiring therapy, renal insufficiency (serum creatinine >2.0 mg/ml), or evidence of malnutrition (based on subnormal plasma concentration of transthyretin) were ineligible for participation in the study. Pregnant and/or lactating women were excluded from the study. The design of the study and the laboratory samples obtained at each of the scheduled patient visits are shown in Table 1.

Data and Statistical Analysis

Erythrocyte PPIX, plasma PPIX, urinary ALA, and plasma PLP, were compared between groups using Wilcoxon tests when 2 groups were compared and Kruskal-Wallis tests when 3 or more were compared. The longitudinal values for each patient were evaluated using ANOVA. All analyses were considered exploratory and were conducted using SAS, version 9.4 (SAS Institute Inc), with $P < .05$ considered significant. The Students T-test was used to compare results between groups of mice assuming unequal sample size. Values with $P < .05$ were considered significant.

Results:

Proof of Concept Studies in Mice

In XLP, the pathophysiologic process that underlies the clinical phenotype is due to overproduction of PPIX. One of the primary observations in the *Irp2*^{-/-} mouse is development of a protoporphyria phenotype (14) including excess hepatic porphyrins. We tested the hypothesis that INH has the capacity to limit PLP cofactor availability for Alas2 and thereby diminish enzymatic activity sufficiently to affect hepatic PPIX levels. To test this hypothesis, mice were treated with 100 mg/kg INH for four weeks. (12) Sampling of ~ 50 μ L of blood on a weekly basis provided sufficient plasma to measure PPIX without altering the rate of hematopoiesis due to lowering the hematocrit. There was insufficient sample to also measure plasma PLP levels directly and any lowering of ALA would be inferred to be due to decreased PLP levels.

Treatment with INH caused a decrease in the plasma concentration of PPIX in both the *Irp2*^{-/-} mice and WT animals with the effect being most apparent after 4 weeks of treatment ($p < 0.05$, Initial vs. week 4 each mouse) (Table 2). The plasma concentration of PPIX in the *Irp2*^{-/-} mice, at week 4, was similar to that of the initial concentration of PPIX in WT mice (Table 2). For the *Irp2*^{-/-} animals, the concentration of PPIX at week 4 of treatment was significantly lower when compared with the initial concentration ($p < 0.05$) (Table 2),

however while a decrease was seen in wild type mice the result was not significant and there were not significant changes in hepatic PPIX (see below). No significant changes in blood counts were observed, no ringed sideroblasts were noted in the bone marrow at week 4, and neither behavioral nor neurological changes were seen in mice from either group. Together, these results demonstrate that treatment with INH can lower the concentration of PPIX in an animal model of X-linked EPP.

The properties of PPIX [large (563 g/mole), planar, hydrophobic], lead to metabolic elimination challenges. PPIX enters the enterohepatic circulation where it is metabolized in hepatocytes and transported into the biliary system. When present in above normal amounts, complications can arise including biliary stones, hepatobiliary damage, and liver failure. After sacrificing the animal, the liver was removed and PPIX levels were evaluated by UPLC (Table 3). The levels of PPIX in the liver from WT mice pre- and post-treatment were not significantly different. Prior to treatment with INH, PPIX concentrations in the livers of *Irp2*^{-/-} mice were 18.0 fold greater than in WT mice (Table 3). PPIX levels in the *Irp2*^{-/-} group decreased 3.3-fold over the four-week treatment period. This approximately 60% reduction in PPIX in the *Irp2*^{-/-} mice, after four weeks of INH treatment, was statistically significant ($p < 0.05$).

Based on the results of the above preclinical experiments, a pilot study was initiated to analyze the effects of INH on patients with EPP and XLP to evaluate the potential use of INH to suppress ALAS2 activity and thereby ameliorate the symptoms of EPP and XLP.

Pilot Study in EPP and XLP Patients

Subjects with biochemically and genetically confirmed EPP or XLP were recruited into a single arm study to receive INH at a dose of 5 mg/kg up to 300 mg (the standard dose for treatment of *M. tuberculosis*).⁽¹⁵⁾ A total of 15 patients were enrolled, 9 with EPP and 6 with XLP (Table 3).

Each of the patients participated in the study for a total of 12 weeks. Patients 1–11 were seen at clinics of Porphyria Consortium members (www.rarediseasesnetwork.org/cms/porphyrias) and patients 12–15 were seen at the Porphyria Center, Paris, France. Subjects were treated with INH for 8 weeks consecutively, followed by a 4-week post-treatment observation period. The primary endpoint for the study was a decrease in plasma PPIX concentration compared to baseline values.

The hypothesis under investigation was that addition of INH would limit the availability of PLP to serve as a cofactor for ALAS, thereby reducing enzymatic activity. None of the subjects showed a decrease in the levels of PLP below the lower limit of normal (20 nM/L) (Data not shown). Urinary ALA was monitored as an indirect measure of ALAS activity. No significant change in urinary ALA was observed in any of the subjects over the 8-week treatment period (Data not shown). Conceivably the levels of ALA remained unchanged but accumulation of PPIX decreased. If so, a reduction in RBC and plasma PPIX should be observed. However, no significant reduction in the levels of RBC or plasma PPIX was noted in any of the test subjects (Data not shown). None of the parameters measured showed a trending pattern for any subset of patients even when segregated based and were quite

variable in some patients. The reasons for these variations were not apparent based on any of the clinical parameters monitored.

Discussion and Conclusions:

The results of our studies demonstrate that INH can reduce the production of erythrocyte PPIX in an animal model of EPP as evidenced by a reduction in plasma PPIX of the INH treated *Irp2*^{-/-} mice (Table 2, 3). Accumulation of hepatic PPIX is a consequence of excess plasma PPIX (16). In support of the effects of INH on erythrocyte PPIX production, treatment led to a reduction in hepatic PPIX in the *Irp2*^{-/-} mice at 4 weeks with no change in hepatic levels of PPIX in WT mice. These mice were treated with a higher dose of INH based on literature values. (17) When translated to humans, the standard dose of INH used to treat latent and active TB is insufficient to affect PPIX in patients with EPP or XLP that are consuming a normal Western diet replete with PLP and precursors. (15) The mechanisms involved in heme biosynthesis have been studied for more than 150 years, and generation of ALA by ALAS1 and ALAS2 as the rate limiting enzymes in the liver and the erythron, respectively, has been established. (2) The need for a regulated system that ties the availability of iron to the production of porphyrin precursors is important in the case of the erythron as this requirement ensures that neither excess porphyrin precursors nor free iron accumulates intercellularly, as both substances are potentially toxic. (18–21) In EPP and XLP the clinical phenotype is due to excess PPIX in circulation. Approximately 5% of EPP patients are at risk of accumulating large amounts of PPIX that are hepatotoxic, causing cholestatic liver disease that can be fatal. In EPP, excess PPIX is due to diminished enzymatic activity of FECH. In XLP, the excess PPIX is due to a 2–3 fold increase in ALAS2 activity. In the latter case, PPIX accumulates because FECH becomes rate limiting as does availability of iron. (2, 22) In XLP there is also a significant increase in zinc-PPIX as a result of insertion of zinc rather than iron into the PPIX macrocycle by FECH. (23) While a decrease in heme synthesis occurs in patients with EPP, the disease phenotype is a consequence of accumulation of PPIX rather than of subnormal heme production.

Patients with either EPP or XLP would benefit from a reduction in the activity of ALAS. By titrating the level of ALA produced, it should be possible to balance PPIX production with iron availability and FECH activity. Pharmacologically controlling the amount of available enzyme cofactor should be a way to achieve this objective. Our studies in the *Irp2*^{-/-} mice confirmed that reducing the activity of ALAS, by limiting the availability of PLP through treatment with INH, decreased PPIX accumulation as reflected by a significantly lower concentration of plasma PPIX in the plasma at week 4 (Table 2) and a significantly lower amount of PPIX in the liver after 4 weeks of treatment (Table 3). In this model, there were no apparent adverse effects in mice treated with a dose of INH that decreased ALAS2 activity.

In the pilot study of INH treatment of patients with EPP and XLP, the plasma concentration of PLP was not reduced to levels below the lower limit of normal (Data not shown). The concentration of PLP precursors in the average Western diet varies, and no dietary restrictions were imposed on patients as part of this study protocol. Therefore variations in PLP among the study patients may reflect lack of dietary consistency. Conceivably, the PLP

cofactor activity could be rendered limiting for ALAS2 in humans if higher doses of INH were used. In the mouse study, animals were treated with a standard dose of 100 mg/kg, whereas in the human study, the maximum dose of INH allowed was 5 mg/kg up to a maximum of 300 mg/d. Thus, mice received a much higher weight-based dose (~100 mg/kg in mice vs 4.6 mg/kg in humans) of INH compared to humans. Our study was constrained by regulatory guidelines that limited the dose of INH to a schedule that has been shown to be safe for treatment of patients with TB, however lowering of PLP has been shown in subjects treated with therapeutic INH who consume a vitamin deficient diet including deficiency of B6 (PLP). Early studies where doses were more than three times this limit showed significant toxicity due to depletion of PLP indicating that B vitamin supplementation was required at these doses. (24, 25) Pyridoxal-phosphate serves as a coenzyme in reaction involved in amino acid, glucose and lipid metabolism, and at higher doses of INH, peripheral neuropathy, transaminitis and sideroblastic anemia have been observed. (15, 26, 27) These adverse reactions are generally mitigated by supplementing the diet with vitamin B6. (25) A number of foods contain large quantities of vitamin B6, and some breakfast cereals are fortified with vitamin B6. We did not restrict the diet of patients with EPP or XLP, and differences in dietary intake of vitamin B6 likely accounts, at least in part, for PLP concentrations that are in the normal range in test subjects (Data not shown).

For maximum efficacy and safety, identification of drugs that can be targeted to the erythron and that specifically inhibit ALAS2 are needed for optimal treatment of X-linked EPP. (10) This strategy is being tested in clinical trials in humans where an siRNA that targets hepatic ALAS1 is being tested for treatment of patients with acute hepatic porphyrias (28), however, the erythropoietic porphyrias have not been targeted using this approach.

Our studies show that standard doses of INH are insufficient to reduce PLP coenzyme activity in patients with EPP and XLP such that it becomes limiting for ALAS2 activity. Higher doses of INH may be efficacious, and development of novel agents that specifically target ALAS2 are warranted to address the absence of effective treatments for the erythropoietic porphyrias. Although these are rare diseases that are uncommonly fatal, the debilitating impact that they inflict upon individual patients is life-altering and life-long.

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Table 1.

Study design and laboratory samples collected.

	Base Line	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
Informed Consent	X						
Complete systems review	X	X	X	X	X	X	X
Plasma PPIX	X	X	X	X	X	X	X
Plasma and Urine ALA, PBG	X	X	X	X	X	X	X
Plasma B6, PLP	X	X	X	X	X	X	X
CBC, metabolic panel, liver function tests, serum transthyretin, urinalysis	X	X	X	X	X	X	X
Serum ferritin, serum iron, TIBC, Tf-sat.	X				X		X

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Table 2.Weekly Plasma Protoporphyrin, μM on treatment with 100 mg/Kg INH

Week	*WT-1	WT-2					Median	Range
0	0.038	0.024					0.031	0.014
1		0.154					0.154	
2	0.068	0.042					0.055	0.026
3	0.020	0.016					0.018	0.004
4	0.008	0.004					0.006	0.004
Week	δ^{\S} KO-1	KO-2	KO-3	KO-4	KO-5			
0	0.281	0.328	0.454	0.221	0.233			0.233
1	0.237	0.444	0.320	0.410	0.380		0.380	0.207
2	0.177	0.197	0.290	0.252	0.504		0.252	0.327
3	0.127	0.166	0.216	0.404	0.202		0.202	0.277
4	0.076	0.051	0.035	0.028	0.046		0.046	0.048

* WT-wild type,

 δ^{\S} KO- *Irf2*^{-/-} mice.

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Table 3.

Liver Protoporphyrin nmol/g tissue.

	Mouse	1	2	3	4	5		Median	Range
Untreated	*WT	0.141	0.219	0.180	0.097	0.205		0.160	0.122
Untreated	§KO	2.093	4.272	3.658	4.089	1.793		2.875	2.479
†Treated	*WT	0.148	0.143	0.220	0.176	1.567		0.162	1.424
Treated	§KO	0.613	2.121	0.139	1.378	0.546		0.579	1.982

* WT-wild type,

§ KO- *Irf2*^{-/-} mice.

† Treated mice received INH, 100 mg/Kg 5 of 7 days/week for 4 weeks.

Table 4.

Subject demographics.

	EPP	Gender	XLP	Gender
Caucasian	8	3 F; 5M	3	2 F; 1M
Hispanic	1	1 F	0	
Arabic	0		3	1 F; 2M

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