Drug repositioning for FGF23-mediated hypophosphatemia: Disulfiram lowers FGF23 production

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Introduction

Hypophosphatemic rickets is categorized as vitamin D-resistant rickets, exhibiting a common finding of renal phosphate wasting, hypophosphatemia, and inappropriate level of activated vitamin D.

X-linked hypophosphatemia (XLH) is the most common form of heritable rickets that constitutes more than 80% of all familial hypophosphatemia. XLH is caused by loss of function mutations in the phosphate-regulating endopeptidase gene on chromosome Xp22 (*PHEX*).

Excess levels of FGF23 are associated with XLH and other hypophosphatemic disorders. FGF23 suppresses transcription of the renal sodium/phosphate cotransporters (NaPi-IIa and NaPi-IIc), leading to decreased tubular phosphate reabsorption in the kidney. FGF23 inhibits expressions of the synthetic enzyme Cyp27b1 and stimulates the catabolic enzyme Cyp24a1, thereby reducing circulating levels of 1,25-dihydroxyvitamin D_3 (1,25$[OH]_2D_3$).

Current treatment for hypophosphatemic rickets is composed of medical therapy, high doses of 1,25-dihydroxycholecalciferol and phosphate supplementation, and orthopedic surgery (Fig. 1).

A drug repositioning strategy, in which a drug currently used for patients with a specific disease is applied to another disease, is gaining increasing attention.

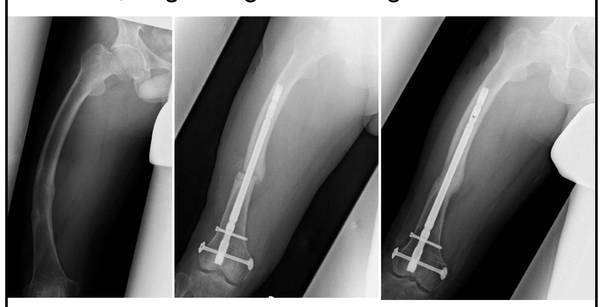

Fig. 1. A 22-year old adolescent with hypophosphatemic rickets had demonstrated remarkable anterolateral bowing of bilateral femur with progressive gait disturbance. Note pseudofracture at the distal diaphysis of the right femur (upper left). Corrective osteotomy and intramedullary fixation was performed with the T2 retrograde femoral nailing system® (upper middle). Complete union at the osteotomy site was achieved 2 years after the corrective surgery (upper right).

Objectives

In the present study, we screened FDA-approved compounds to identify clinically applicable drugs that inhibit expressions of FGF23 in osteogenic cells and examined the *in vivo* efficacy of hit compounds by using Hyp mice, a syngeneic murine model of XLH.

Disulfiram decreased *FGF23* mRNA and protein levels *in vitro*

Flow chart of our screening procedure

The Prestwick Chemical Library®
(FDA-approved drugs)
Total: 1,186 compounds
↓
1st screening at 10 μM
(*n*=6)
↓
0.2< Relative luciferase activity <0.8
↓
93 candidate compounds
↓
2nd screening at 10 μM
(*n*=6)
↓
Relative luciferase activity <0.7
80% < Relative cell viability
↓
16 candidate compounds
↓
Gene expression analysis at 10 μM
(Triplicate)
↓
Relative levels of endogenous *FGF23* mRNA <0.5
↓
2 candidate compounds
Disulfiram: Alcohol deterrent
Methiotepin: Serotonin antagonist
↓
Dose-response study at 1, 2, 5, and 10 μM
(Triplicate)
↓
We selected disulfiram as a hit compound based on lack of major adverse effects and a long track record in clinical settings

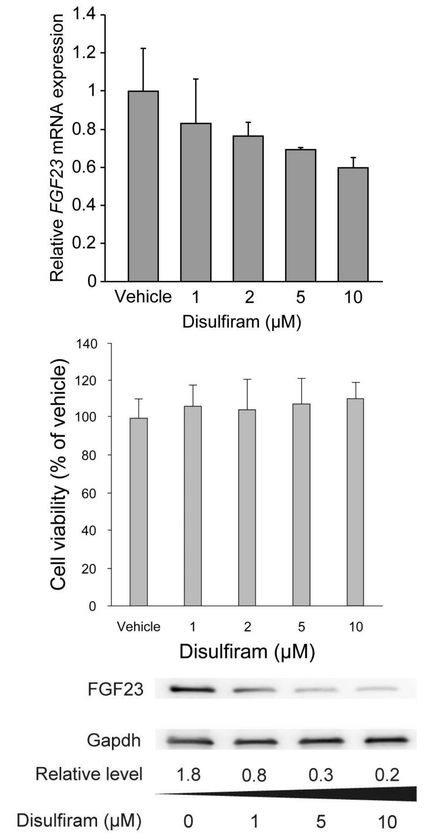

Fig. 2. After a multistep screening, we chose two best compounds that consistently exhibited beneficial effects such as averaged relative activity of human *FGF23* promoter < 0.5, averaged relative cell viability > 0.8, and averaged relative level of *FGF23* gene expression < 0.5. Disulfiram, commonly prescribed for alcohol abuse and dependence, dose-dependently decreased endogenous levels of *FGF23* mRNA without significant cell toxicity in MC3T3-E1 cells (upper and middle). We also confirmed that disulfiram suppressed expressions of endogenous FGF23 protein in a dose-dependent manner in MC3T3-E1 cells (lower). Date are shown as means + SD.

Disulfiram treatment decreased circulating FGF23 levels and ameliorated tubular phosphate reabsorption in Hyp mice.

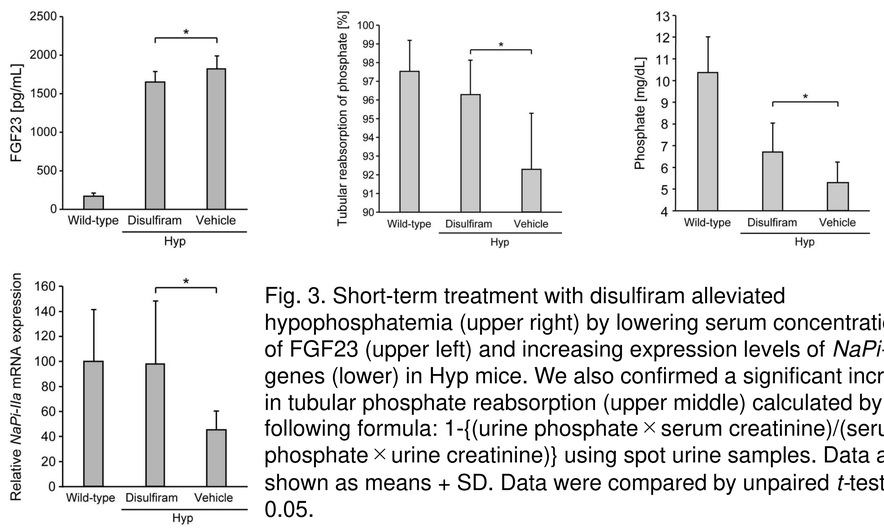

Fig. 3. Short-term treatment with disulfiram alleviated hypophosphatemia (upper right) by lowering serum concentrations of FGF23 (upper left) and increasing expression levels of *NaPi-IIa* genes (lower) in Hyp mice. We also confirmed a significant increase in tubular phosphate reabsorption (upper middle) calculated by the following formula: 1-{(urine phosphate × serum creatinine)/(serum phosphate × urine creatinine)} using spot urine samples. Data are shown as means + SD. Data were compared by unpaired *t*-test; *p < 0.05.

Disulfiram treatment induced 1,25$(OH)_2D_3$ production and alleviated hypocalcemia in Hyp mice.

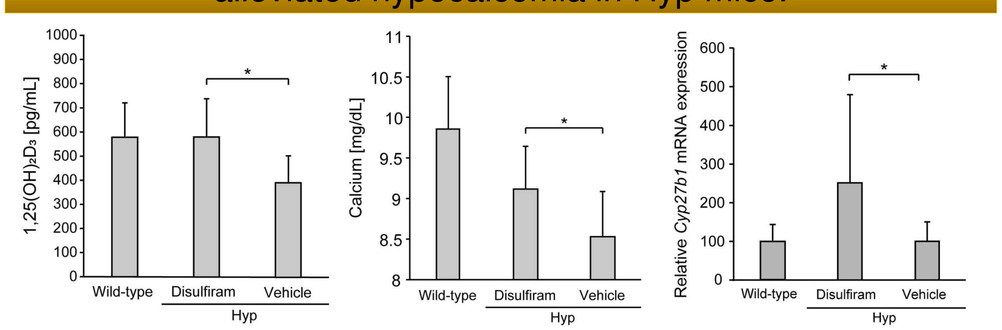

Fig. 4. Short-term treatment with disulfiram ameliorated hypocalcemia (upper middle) by upregulating serum concentrations of 1,25-dihydroxyvitamin D_3 (1,25$[OH]_2D_3$) (upper left) and gene expressions of the vitamin D anabolizing enzyme *Cyp27b1* in Hyp mice. Data are shown as means + SD. Data were compared by unpaired *t*-test; *p < 0.05.

Materials & Methods

Screening of off-label effects of 1,186 FDA-approved compounds:

We constructed a luciferase reporter vector (pGL4.10[luc2]) carrying a 5'-flanking region of human *FGF23* spanning about 1,500 bp upstream from the transcription start site. The MC3T3-E1 cells were transfected with this reporter vector and pcDNA3.1+ empty vector [Invitrogen] to establish stable cell lines. We next confirmed that the luciferase activity emanating from the stable clone cells was dose-dependently increased by adding Forskolin, a PKA activator, to osteogenic medium [1]. After overnight incubation of the stable clone cells in growth medium, cells were cultured in osteogenic medium containing 1 μM of Forskolin for 24 hours. We subsequently applied each of 1,186 bioactive chemical compounds (New Prestwick Chemical Library) at a final concentration of 10 μM and incubated for an additional 24 hours. We screened an effect of each compound on lowering *FGF23* promoter activity by quantifying the luciferase activity. We also examined the viability of cells using MTS assay.

Disulfiram decreased *FGF23* mRNA and protein levels *in vitro*:

Total RNA was isolated from MC3T3-E1 that were treated with 1, 2, 5, and 10 μM of Disulfiram for 24 hours and reverse-transcribed using oligo dT primers. We quantified endogenous *FGF23* mRNA using specific PCR primers. Total proteins were extracted from MC3T3-E1 cells that were harvested 24 hours after addition of disulfiram in RIPA buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membrane, and western blotting was performed using anti-FGF23 antibody. Results were expressed as a ratio of the positive bands of immunoblots against Gapdh.

Short-term treatment with disulfiram lowered FGF23 production and ameliorated renal FGF23 signaling in Hyp mice:

Hyp (B6.Cg-PhexHyp/J) male mice at 6 to 7 weeks of age were administered with 100 mg/kg of disulfiram or vehicle twice a day for 7 days by oral gavage. Mice were anesthetized by ether inhalation and blood/urine were collected. Mice were then euthanized by exsanguination and kidney was obtained. FGF23 or 1,25 [OH]2D3 serum level was analyzed by an ELISA kit detecting intact FGF23 or purified 1,25$[OH]_2D_3$, respectively. Serum/urine concentrations of phosphate and calcium were determined using an automated chemistry analyzer. After vigorous homogenization of approximately 30 mg of kidney tissues, renal RNA was isolated and reverse-transcribed using oligo-dT primers. Expression levels of *NaPi-IIa* (*Slc34a1*), *NaPi-IIc* (*Slc34a3*), *Cyp24a1,* and *Cyp27b1* genes were determined using a quantitative real-time PCR system. Results are expressed as means + SD. Statistical analyses were carried out using unpaired *t*-test with significance set at $p < 0.05$.

[1] Lavi-Moshayoff V, Wasserman G, Meir T et al. PTH increases FGF23 gene expression and mediates the high-FGF23 levels of experimental kidney failure: a bone parathyroid feedback loop. Am J Physiol Renal Physiol. 299 (2010) F882-9.

Conclusions

Disulfiram is a promising agent that can be applied to FGF23-mediated hypophosphatemia in clinical settings.