The Effects of Genetic Mutations and Drugs on the Activity of the Thiamine Transporter, SLC19A2

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Abstract. A rare cause of megaloblastic anemia (MA) is thiamine-responsive megaloblastic anemia (TRMA), a genetic disorder caused by mutations in SLC19A2 (encoding THTR1), a thiamine transporter. The study objectives were to (1) functionally characterize selected TRMA-associated SLC19A2 variants and (2) determine whether current prescription drugs associated with drug-induced MA (DIMA) may act via inhibition of SLC19A2. Functional characterization of selected SLC19A2 variants was performed by confocal microscopy and isotopic uptake studies of [3H]-thiamine in HEK293 cells. Sixty-three drugs associated with DIMA were screened for SLC19A2 inhibition in isotopic uptake studies. Three previously uncharacterized SLC19A2 variants identified in TRMA patients exhibited disrupted localization to the plasma membrane along with near-complete loss-of-function. Ten of 63 drugs inhibited SLC19A2-mediated thiamine transport ≥50% at screening concentrations; however, with the exception of erythromycin, none was predicted to inhibit SLC19A2 at clinically relevant unbound plasma concentrations. Data from electronic health records revealed reduced levels of thiamine pyrophosphate (TPP) in patients prescribed erythromycin, consistent with inhibition of SLC19A2-mediated thiamine transport. Here, we confirmed the role of three SLC19A2 variants in TRMA pathology. Additionally, we report that inhibition of SLC19A2 is a potential, but uncommon mechanism for DIMA.

KEY WORDS: drug nutrient interactions; drug-induced megaloblastic anemia; thiamine diphosphate; THTR1; vitamin b1.

INTRODUCTION

Megaloblastic anemia (MA) describes a heterogeneous set of anemias characterized by the presence of megaloblasts (immature red blood cells) in the bone marrow. MA develops as a result of ineffective DNA synthesis in rapidly dividing hematopoietic cells (1). Major causes of MA include vitamin deficiencies and exposure to certain drugs (2), termed drug-induced megaloblastic anemia (DIMA). As nutritional deficiencies have decreased in Western populations, the percentage of MA caused by drugs (i.e., DIMA) has increased (2, 3). DIMA occurs through various mechanisms, some of which remain elusive (Fig. 1).

Thiamine deficiency can be caused by poor dietary intake, chronic alcoholism, poor absorption (e.g., gastric bypass surgery or diarrhea), increased utilization (e.g., pregnancy), genetic mutations, and use of particular medications (e.g., diuretics) (4). Thiamine deficiency usually occurs systemically but it can occur locally in specific tissue/organs. Systemic thiamine deficiency can cause disorders such as beriberi and Wernicke-Korsakoff syndrome, whereas tissue/organ-specific thiamine deficiency due to genetic inactivation of thiamine transporters can lead to thiamine metabolism dysfunction syndrome-2 (THMD2, OMIM# 607483) or thiamine-responsive megaloblastic anemia (TRMA, OMIM# 249270) (4).

TRMA is characterized by three hallmark signs: MA, non-autoimmune type 1 diabetes, and sensorineural hearing loss (5). TRMA is caused by loss-of-function genetic mutations in SLC19A2, encoding the thiamine human transporter 1.
(THTR1, SLC19A2) (6). SLC19A2 is the main thiamine transporter in pancreatic islet tissue and hematopoietic cells (7, 8). Approximately 60 SLC19A2 variants have been associated with TRMA (Supplemental Table 1) (5, 9), yet few have been functionally characterized to date. A mouse model of dietary thiamine deficiency (TD) has been used extensively to evaluate the effects of systemic thiamine depletion on neurological functions (10). However, there are no studies employing the TD model (or any in vivo system) to evaluate the impact of systemic thiamine depletion on erythropoiesis and megaloblastic anemia. Further, no studies have explored the idea that pharmacologic inhibitors of thiamine transporters may be causal for megaloblastic anemia (4). To our knowledge, SLC19A2 inhibition, which may phenocopy loss-of-function SLC19A2 variants, is not a known mechanism for DIMA.

In this study, we tested the hypothesis that drugs associated with DIMA may act (at least in part) as inhibitors of SLC19A2, and phenocopy genetic variants in the

Fig. 1. Common mechanisms underlying megaloblastic anemia and drug-induced megaloblastic anemia. Major causes of megaloblastic anemia (MA) include deficiencies of vitamin B₁₂ and folic acid due to inadequate dietary intake or malabsorption. MA due to drug exposure is also known as drug-induced megaloblastic anemia (DIMA). DIMA can be caused by a variety of drugs that inhibit DNA synthesis at different points of the DNA synthesis pathway. a Drugs can cause MA by reducing cellular availability of vitamin B₁₂ or folic acid through reduced absorption, plasma transport, or delivery of folate or vitamin B₁₂ or physical destruction of the vitamins (mechanisms #1 and #2). b Additional mechanisms of DIMA include inhibition of key enzymes such as dihydrofolate reductase resulting in tetrahydrofolate deficiency (mechanism #3) and drugs acting as purine and pyrimidine antagonists or analogs, including chemotherapies (5-fluorouracil), immune antagonists (e.g., leflunomide), and antiviral agents (mechanism #4). Interestingly, some drugs cause MA through an unknown mechanism. We propose an unknown mechanism of DIMA may involve inhibition of SLC19A2 in hematopoietic cells (mechanism #5).
transporter. The objectives of our study were to (1) functionally characterize selected \( SLC19A2 \) variants associated with TRMA and (2) identify inhibitors of \( SLC19A2 \) among drugs associated with DIMA. The current study extends our work on thiamine transport mechanisms with a focus on THTR1 (\( SLC19A2 \)). In particular, we functionally characterize several genetic mutations in \( SLC19A2 \) and identify clinically relevant \( SLC19A2 \) inhibitors. Collectively, our studies expand our understanding of drug-induced thiamine disorders.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

[^H]-Thiamine hydrochloride (catalog #ART 0710) was purchased from American Radiolabeled Chemicals Incorporation (St. Louis, MO, USA). Non-radiolabeled compounds were purchased from Sigma-Aldrich, Inc. (USA), Selleck Chemicals (Houston, TX), VWR International, Inc., and Thermo Fisher Scientific (USA). Cell culture supplies were purchased from Thermo Fisher Scientific (USA).

**Functional Studies**

Plasmids encoding wild-type \( SLC19A2 \) and three TRMA-associated variants were generated by Genscript Inc. (Piscataway, USA). Methods for generation of stably overexpressing \( SLC19A2 \) cell lines and uptake studies have been previously described (11, 12). Inhibition and kinetic studies were performed in \( SLC19A2 \)-overexpressing stable HEK293 cells transiently transfected with an additional 500 ng of \( SLC19A2 \) wild-type plasmid.

**Confocal Microscopy of \( SLC19A2 \) GFP-Tagged Variants**

The subcellular localization of wild-type and variant \( SLC19A2 \) was determined with confocal microscopy on a Nikon CSU-22 Spinning Disk. Samples were prepared as previously described (12).

**Inhibitor Screening Studies**

Prescription drugs associated with MA (2) in addition to others belonging to listed drug classes (e.g., proton-pump inhibitors; 63 compounds total) were screened in triplicate at 1 mM to evaluate inhibitory activity against \( SLC19A2 \). The screen was performed using \[^{3}H\]-thiamine (25 nM). Each plate contained control wells exposed for 10 min (within linear range, Supplemental Figure 1) to \[^{3}H\]-thiamine (positive control), \[^{3}H\]-thiamine plus 1 mM of amprolium (negative control), and \[^{3}H\]-thiamine plus 1 mM of screened drug (3 replicates per drug). Methods have been previously described (11). \( SLC19A2 \) inhibitors were defined as compounds that decreased \( SLC19A2 \)-mediated thiamine uptake by 50% or more at 1 mM. For compounds that met this definition, inhibition assays were conducted and \( IC_{50} \) values were calculated by nonlinear fitting using GraphPad Prism 7 (La Jolla, CA) (11).

**Calculation of \( SLC19A2 \) Inhibitor Systemic Blood Concentrations**

Clinical pharmacokinetic data from human studies (13-18) and drug monograph databases (e.g., Micromedex, FDA) were used to obtain \( C_{\text{max}} \) (maximum plasma concentration) and \( f_u \) (fraction of drug unbound in the plasma). Comparisons between unbound \( C_{\text{max}} \left( f_u C_{\text{max}} \right) \) and \( IC_{50} \) obtained \textit{in vitro} \( (f_u C_{\text{max}})/IC_{50} > 0.1 \) were used to estimate the likelihood that a drug may inhibit \( SLC19A2 \) clinically (Table I) (19, 20).

**EHR Data Analyses**

Methods detailing electronic health record (EHR) data extraction, filtering, and analysis are listed in Supplemental Table 2 and Supplemental Table 3.

**TPK1 Enzyme Assay**

Methods detailing thiamine pyrophosphokinase 1 (TPK1) enzyme assay development and execution are listed in Supplemental Figure 2 legend.

**RESULTS**

**\( SLC19A2 \) Variants Causal for TRMA Exhibit Complete Loss-of-Function**

HEK293 cells transiently transfected with three previously uncharacterized TRMA-associated \( SLC19A2 \) variants (p.T170P, p.G172R, and p.G334D) had significantly reduced thiamine transport activity compared to cells transfected with wild-type \( SLC19A2 \) (one-way ANOVA \( p \)-value < 0.0001, Fig. 2). The activities of variants p.T170P and p.G172R were not significantly different from cells transfected with empty vector (Fig. 2a; Student’s \( t \)-test \( p = 0.052 \) and 0.49, respectively), while variant p.G334D retained minimal but significant thiamine transport \( (p = 0.0009) \). Similarly, inhibition of \( SLC19A2 \) with 200 \( \mu \)M amprolium or trimethoprim (a commonly used antibiotic and \( SLC19A2 \) inhibitor) abolished wild-type \( SLC19A2 \) activity to levels comparable to the TRMA-associated variants (Fig. 2a).

**\( SLC19A2 \) Variants Exhibit Disrupted Plasma Membrane Localization**

Confocal microscopy of HEK293 cells expressing GFP-tagged wild-type and variant \( SLC19A2 \) revealed that the wild-type \( SLC19A2 \) localized primarily to the plasma membrane of the cell (Fig. 2b). In contrast, all three \( SLC19A2 \) variants exhibited partially disrupted membrane localization, and displayed concentrated puncta consistent with retention of the transporter variants in membrane-bound organelles.

**Inhibitor Screen Identifies Ten Drugs That Substantially Inhibit \( SLC19A2 \)**

Among the 63 drugs screened for \( SLC19A2 \) inhibition (Fig. 3, Fig. 4, Supplemental Table 4), ten were designated as inhibitors, that is, these 10 substantially inhibited \( SLC19A2 \) (≥ 50% reduction in thiamine transport) at 1 mM. Of the ten
inhibitors, four (fedratinib, amiloride, trimethoprim, amprolium) were previously identified as SLC19A2 inhibitors (11, 20), whereas six were novel SLC19A2 inhibitors, including erythromycin, mycophenolate mofetil, omeprazole, pantoprazole, lansoprazole, and chloroquine.

**IC₅₀ Assays Reveal Erythromycin Potently Inhibits SLC19A2**

Out of all the newly identified SLC19A2 inhibitors, erythromycin was the most potent with an estimated IC₅₀ of 20 μM. Inhibition potency was followed by amiloride (69 μM), mycophenolate mofetil (145 μM), omeprazole (207 μM), chloroquine (301 μM), and pantoprazole (429 μM) (Table I).

**Erythromycin Reaches Systemic Concentrations Relevant for SLC19A2 Inhibition**

Calculation of unbound $C_{\text{max}}/IC₅₀$ values for the SLC19A2 inhibitors listed in Table I revealed that erythromycin was the only drug that met the FDA’s criteria for clinically relevant transporter inhibition.

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**Table I. Predicted Risk of Causing an SLC19A2-Mediated Clinical Drug-Drug Interaction Using the FDA Criteria (19, 20)**

| Drug               | $f_u$  | $C_{\text{max}}, \mu M$ | IC₅₀, μM | *(f_u - C_{\text{max}}) ÷ (IC₅₀)* | Reference        |
|--------------------|--------|--------------------------|----------|----------------------------------|------------------|
| Erythromycin       | 0.1-0.3| 2.3-9.6                  | 20       | 0.144                            | PMID: 11294369   |
|                    |        |                          |          |                                  | PMID: 3606934    |
|                    |        |                          |          |                                  | PMID: 2656049    |
| Mycophenolate mofetil | 0.03 (label) | Below LLQ (0.9 μM) | 145      | NA                               | Package insert   |
| Omeprazole         | 0.05 (label) | 1-10                   | 207      | 0.002                            | PMID: 1458764    |
|                    |        |                          |          |                                  | PMID: 8675169    |
| Chloroquine        | 0.36-0.39 | 0.0034                 | 301      | $4 \times 10^{-6}$              | PMID: 23701202   |
|                    |        |                          |          |                                  | PMID: 6849768    |
| Pantoprazole       | 0.02 (label) | 6.5                    | 429      | 0.0003                           | Package insert   |
| Amiloride          | 0.77   | 0.04                     | 69       | 0.0004                           | Package insert   |
| Trimethoprim       | 0.56 (label) | 3.44                  | 6.84     | 0.28                             | PMID: 27803021   |

*FDA criteria* (19, 20) for predicting the potential of a drug to cause a transporter-mediated drug-vitamin interaction suggest that if the value in this column is > 0.1, the drug has the potential to cause a clinical drug-vitamin interaction. NA not applicable, LLQ lower limit of quantification.
Erythromycin Is a Substrate of TPK1

Luminescent signals from each enzyme reaction were measured and compared for omeprazole, erythromycin, thiamine (positive control or PC), and no substrate (negative control or NC). Luminescent signal in the erythromycin reaction was significantly higher than the negative control signal (Supplemental Figure 2, Student’s t-test, p-value < 0.0001), but still lower than the signal from the positive control (thiamine 1 mM). For omeprazole, the signal was statistically similar to the negative control (Supplemental Figure 2, Student’s t-test, p-value = 0.72).

DISCUSSION

In our previous research, we discovered that thiamine is the major endogenous substrate of OCT1 transporters (21), identified OCT1 as a key determinant of certain cardiometabolic (22), and hepatic (23) traits and identified several prescription drugs that are clinically relevant drug inhibitors of the intestinal thiamine transporter, SLC19A3 (24). In this study, we expand on our previous research by evaluating SLC19A2 expression and function and the physiological effects of SLC19A2 inhibition.

Approximately half of the 60 SLC19A2 mutations associated with TRMA are missense variants (Supplemental Table 1) (5, 25), yet few have been functionally characterized. In this study, we determined that three previously uncharacterized TRMA-associated SLC19A2 missense variants exhibited near-complete loss-of-function (Fig. 2). Consistent with loss-of-function, confocal microscopy revealed that the SLC19A2 variants largely failed to traffic to the plasma membrane. Instead, intracellular puncta were observed for all three variants, suggestive of impaired transporter trafficking or recycling, a pattern that has been observed previously for mutations in SLC19A2 (26, 27).

In the inhibitor screen, three prescription drugs associated with DIMA had IC50 values of < 200 μM. However, only the macrolide antibiotic, erythromycin, was predicted to inhibit SLC19A2 at clinically relevant concentrations when applying the FDA criteria for transporter-mediated drug-drug interactions. Erythromycin-induced MA is thought to occur through inhibition of folic acid absorption (2). However, our data suggest an additional mechanism via SLC19A2 inhibition (Fig. 4b), and potentially from inhibition of TPK1 (the enzyme that converts thiamine to TPP, the bioactive form of thiamine) (Supplemental Figure 2). Our finding that erythromycin is a substrate of TPK1 (Supplemental Figure 2) together with the fact that the drug is known to accumulate intracellularly to high levels (28) suggests that inhibition of TPK1 as well as of SLC19A2 may contribute to low TPP levels and ultimately to erythromycin-induced MA. EHR data were consistent with reduced levels of TPP in patients treated with erythromycin (Fig. 5, Supplemental Table 2, Supplemental Table 3). This mechanism may be particularly important in patients who may have higher systemic levels of erythromycin (e.g., patients with liver disease, the elderly, CYP3A4 poor metabolizers). Today, erythromycin use is rare compared to other macrolides (e.g., azithromycin).

CONCLUSION

Three TRMA-associated SLC19A2 variants were characterized, revealing complete loss of thiamine transport at least partially attributed to disrupted membrane localization. Our data suggest that SLC19A2 inhibition is not the mechanism...
Drug screen and potency studies against SLC19A2-mediated thiamine uptake. In the screen, SLC19A2 inhibitors were defined as compounds that inhibit at least 50% of thiamine uptake compared to the positive control. The positive control was thiamine uptake in the absence of inhibitor. Out of the 63 drugs screened, ten reached that threshold. Of the ten drugs, three (fedratinib, amprolium, and trimethoprim) have IC₅₀ values published in literature (a). IC₅₀ experiments were conducted using inhibitor concentrations from 0 to 1 mM. IC₅₀ curves for erythromycin, omeprazole, amiloride, mycophenolate mofetil, chloroquine, and pantoprazole were generated by fitting data to a nonlinear model (b). Figures representative of 3 independent experiments except for pantoprazole.
behind DIMA for most associated prescription drugs, although it may contribute to erythromycin-induced MA.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1208/s12248-021-00562-4.

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Fig. 5. Methods used to extract information from electronic health records (EHR) and results of thiamine pyrophosphate levels in patients prescribed erythromycin. To explore whether drugs predicted to cause a clinical drug-drug interaction in vitro studies inhibit SLC19A2 in vivo, we extracted and analyzed data from the UCSF EHR. Data was filtered by presence (“on”) or absence (“off”) of erythromycin prescription, medication order start date, and thiamine pyrophosphate laboratory data. Before analysis, individuals in the “on” and “off” group were age and sex-matched (a). The thiamine pyrophosphate levels of patients prescribed erythromycin (+, N = 4) versus not prescribed erythromycin (−, N = 20) were compared. The average TPP levels for patients prescribed erythromycin were lower (137 nM) than that of patients not prescribed erythromycin (186 nM) (Welch’s two sample t-test, p-value = 0.0016)
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