Interaction of 2,4-Diaminopyrimidine–Containing Drugs Including Fedratinib and Trimethoprim with Thiamine Transporters

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ABSTRACT

Inhibition of thiamine transporters has been proposed as a putative mechanism for the observation of Wernicke’s encephalopathy and subsequent termination of clinical development of fedratinib, a Janus kinase inhibitor (JAKi). This study aimed to determine the potential for other JAKi to inhibit thiamine transport using human epithelial colorectal adenocarcinoma (Caco-2) and thiamine transporter (THTR) overexpressing cells and to better elucidate the structural basis for interacting with THTR. Only JAKi containing a 2,4-diaminopyrimidine were observed to inhibit thiamine transporters. Fedratinib inhibited thiamine uptake into Caco-2 cells (IC₅₀ = 0.940 μM) and THTR-2 (IC₅₀ = 1.36 μM) and, to a lesser extent, THTR-1 (IC₅₀ = 7.10 μM) overexpressing cells. Two other JAKi containing this moiety, AZD1480 and cerdulatinib, were weaker inhibitors of the thiamine transporters. Other JAKi—including monoaminoypyrimidines, such as momelotinib, and nonaminoypyrimidines, such as filgotinib—did not have any inhibitory effects on thiamine transport. A pharmacophore model derived from the minimized structure of thiamine suggests that 2,4-diaminopyrimidine–containing compounds can adopt a conformation matching several key features of thiamine. Further studies with drugs containing a 2,4-diaminopyrimidine resulted in the discovery that the antibiotic trimethoprim also potently inhibits thiamine uptake mediated by THTR-1 (IC₅₀ = 6.84 μM) and THTR-2 (IC₅₀ = 5.56 μM). Fedratinib and trimethoprim were also found to be substrates for THTR, a finding with important implications for their disposition in the body. In summary, our results show that not all JAKi have the potential to inhibit thiamine transport and further establish the interaction of these transporters with xenobiotics.

Introduction

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is important in regulating development and homeostasis. This pleiotropic pathway is also the principal signaling mechanism for a wide array of cytokines and growth factors; thus, mutations that disrupt JAK/STAT signaling have been linked to the pathogenesis of various immune disorders, inflammatory conditions, and cancer types. Notably, in myeloproliferative neoplasms, the gain-of-function JAK2V617F mutation is present in more than 95% of cases and in as many as 57% of patients with essential thrombocythemia or primary myelofibrosis (Jones et al., 2005). Due to the widespread involvement of this pathway in diseases, there has been great effort to develop pharmacologic agents that target JAK/STAT. Ruxolitinib (Incyte, Wilmington, DE) was the first JAK1 and JAK2 inhibitor approved by the U.S. Food and Drug Administration for the treatment of myelofibrosis, and tofacitinib (Pfizer, New York, NY) is approved by the Food and Drug Administration for rheumatoid arthritis.

In November 2013, late-stage clinical development of fedratinib (Sanofi, Paris, France), a selective JAK2 inhibitor for the treatment of myelofibrosis, was terminated due to the observation of Wernicke’s encephalopathy, a severe neurologic disease associated with thiamine deficiency, in a small number of patients during clinical trials (Sechi and Serra, 2007; Rodriguez-Pardo et al., 2015). These adverse events have been attributed to the inhibition of thiamine transporter 2 (THTR-2)–mediated thiamine transport by fedratinib (Zhang et al., 2014b). Thiamine is an essential nutrient that cannot be synthesized in humans and must be obtained from the diet. In the body, thiamine is activated to thiamine pyrophosphate, which is an essential cofactor for several enzymes, including pyruvate dehydrogenase and α-keto dehydrogenase, essential enzymes in glycolytic energy production. Two known human thiamine transporters actively transport the nutrient across cell membranes: THTR-1 (SLC19A1) and THTR-2 (SLC19A3). These high-affinity thiamine transporters have distinct and overlapping expression levels in a wide variety of tissues, including the blood-brain barrier, liver, kidneys, placenta, muscle, and small intestine (Dutta et al., 1999; Eudy et al., 2000; Reidling et al., 2002; Larkin et al., 2003).

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ABBREVIATIONS: AZD1480, 5-chloro-2-N-[[S]-1-(5-fluoropyrimidin-2-yl)ethyl]-4-N-(5-methyl-1H-pyrazol-3-yl)pyrimidine-2,4-diamine; BBB, blood-brain barrier; Caco-2, human epithelial colorectal adenocarcinoma; CNS, central nervous system; DDI, drug-drug interaction; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; JAKi, Janus kinase inhibitor; JAK/STAT, Janus kinase/signal transducer and activator of transcription; MATE, multidrug and toxin extrusion protein; MSR, macrophage scavenger receptor; OCT2, organic cation transporter 2; P/S, penicillin/streptomycin; THTR, thiamine transporter; XL019, (2S)-N-[4-[2-(4-morpholin-4-yl)pyrimidin-4-yl]phenyl]pyrrolidine-2-carboxamide.
Fig. 1. Chemical structures of compounds tested. (A) 2,4-Diaminopyrimidine JAKi. (B) 2- or 4-monoaminopyrimidine JAKi. (C) Nonaminopyrimidine JAKi. (D) Non-JAKi. Aminopyrimidine core (bold). AT9283, (1-cyclopropyl-3-[(3Z)-3-[5-(morpholin-4-ylmethyl)benzimidazol-2-ylidene]-1,2-dihydropyrazol-4-yl]urea); WHI-P154, (2-bromo-4-[[6,7-dimethoxyquinazolin-4-yl]amino]phenol).
For example, THTR-2 is most highly expressed in the intestine, followed by kidney, liver, and adipose tissue (Rajgopal et al., 2001; Nabokina et al., 2013; Zhao and Goldman, 2013; Manzetti et al., 2014; GTEx Consortium, 2015; Mele et al., 2015). In most tissues, THTR-1 is also expressed, providing a redundant transporter for thiamine. However, in intestine, THTR-1 appears to be on the basolateral membrane and does not provide a redundant transport mechanism for thiamine uptake (Boulware et al., 2003). THTR-1 is the predominant transporter expressed in islet cells of the pancreas and in various blood cell types. Genetic defects in SLC19A2 result in the development of thiamine-responsive megaloblastic anemia and type 1 diabetes. THTR-2 is the predominant thiamine transporter in the intestine and in the blood-brain barrier. Mutations in SLC19A3 have been linked to biotin-responsive basal ganglia disease, an autosomal recessive disorder characterized by encephalopathy, which is reminiscent of Wernicke’s encephalopathy (Neufeld et al., 2001; Zeng et al., 2005). Although THTR-1 and THTR-2 play a role in thiamine absorption, only THTR-2 knockout mice exhibit reduced intestinal thiamine uptake and blood thiamine levels compared with wild-type littermates (Reidling et al., 2010). In addition, lower systemic levels of thiamine have been observed in biotin-responsive basal ganglia disease patients, thus establishing THTR-2 as the major absorptive transporter for thiamine (Neufeld et al., 2001; Zeng et al., 2005). Zhang et al. (2014b) showed that fedratinib was a potent inhibitor of THTR-2 and attributed the interaction between fedratinib and THTR-2 to the presence of an aminopyrimidine group present in the chemical structure of the Janus kinase inhibitor (JAKi). The main objectives of this study were to 1) determine the potential of JAKi to affect thiamine disposition, and 2) understand the molecular mechanism and structural basis of inhibition. Specifically, we wanted to examine the significance of the aminopyrimidine core present in thiamine and several of the JAKi. Our results provide potential explanations for the fedratinib-associated encephalopathy, and importantly, we show that this is not a class effect but rather is related to a specific structural element present in only a few JAKi. Finally, we report the novel finding that trimethoprim, a commonly used antibiotic that contains a 2,4-diaminopyrimidine structural moiety, is both an inhibitor and a substrate of THTR-1 and THTR-2. Our discoveries in this study not only demonstrate the importance of nutrient transporters in drug disposition, resulting in the potential for drug-vitamin interactions, but also highlight the emerging additional role of THTR-2 as a xenobiotic transporter.

Materials and Methods

Cell Lines, Compounds, and Reagents. Human epithelial colorectal adenocarcinoma (Caco-2) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Cells (passages 60–70) were plated at a density

![Fig. 2. The effect of JAK inhibitors on thiamine uptake in Caco-2 cells. (A) JAKi tested. [\(^3\)H]Thiamine (250 nM) uptake was conducted for 3 minutes in the presence of JAKi (30 μM), pyrithiamine (100 μM), and thiamine (1 mM). (B) IC50 determination. [\(^3\)H]Thiamine uptake was conducted in the presence of fedratinib (solid circles) or momelotinib (solid squares). Results shown represent the mean ± S.D. of triplicate determinations in a representative experiment of three independent experiments, except AT9283, baricitinib, gandolitinib, and WHI-P154, in which the compounds were tested only once. ***P < 0.001; ****P < 0.0001.](image-url)
of 2.5 x 10^5 cells/well in 12-well plates (Corning Inc., Corning, NY) in custom-formulated thiamine-deficient medium (Life Technologies, Carlsbad, CA), 10% FBS, and 1% P/S 4 days prior to experiments to stimulate transporter expression (Ashokkumar et al., 2006). HEK293 cells stably overexpressing THTR-1 and THTR-2 were provided by Dr. Kathy Giacomini (University of California, San Francisco, CA) and were cultured in DMEM/high-glucose medium containing 10% FBS, 1% P/S, and 10 μg/ml puromycin. These cells were plated at a density of 1.5 x 10^4 cells/well in 24-well poly-t-lysine–coated plates in normal growth medium 48 hours prior to experiments. HEK293 MSR THTR–2-overexpressing cells were generated by transiently expressing a plasmid containing the SLC19A3 open reading frame (Dharmacon, Lafayette, CO) in GripTite HEK293 MSR cells (Life Technologies). Cells were seeded in 24-well plates in regular growth medium at a density of 1.5 x 10^3 cells/well, transfections were performed according to the TransIT-293 transfection reagent protocol (Mirus Bio, LLC, Madison, WI), and experiments were conducted 48 hours post-transfection. All cells were maintained in DMEM/high-glucose medium containing 10% FBS, 1% P/S, 0.1 mM nonessential amino acids, and G418 (600 μg/ml) at 37°C, and 5% CO2. Compounds used were [3H]thiamine hydrochloride (American Radiolabeled Chemicals, St. Louis, MO), thiamine, amprolium, oxythiamine, and trimethoprim (Sigma-Aldrich, St. Louis, MO). All JAKi were purchased from Selleckchem (Houston, TX), except TG02 (MedKoo Biosciences, Chapel Hill, NC), Momelotinib and XL019 ([2S]-N-[4-[2-(4-amino-4-yl)-pyrimidin-4-yl]phenyl]pyrrolidine-2-carboxamide) were synthesized in house.

Real Time Polymerase Chain Reaction Analysis of mRNA Levels in Cell Lines and Human Tissue Samples. Total RNA was isolated from Caco-2, cell lines stably and transiently overexpressing THTR, and human tissue samples using the RNaseasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Total RNA (2 μg) from each sample was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. Quantitative real-time polymerase chain reaction was carried out in 96-well FAST reaction plates using 2 x TaqMan Fast Universal Master Mix (Applied Biosystems, Foster City, CA), 20 x TaqMan-specific gene expression probes, and 100 ng of the cDNA template. The reactions were performed on a QuantStudio 6 Flex Real-Time Polymerase Chain Reaction System (Applied Biosystems). The relative expression level of each mRNA transcript was calculated by the comparative C_T (ΔΔC_T) method and normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase.

Uptake Assays. Cells were preincubated at 37°C for 10–15 minutes in Hanks’ medium with the following composition (in mM): 137 NaCl, 5 KCl, 0.8 MgSO4, 1 MgCl2, 0.33 Na2HPO4, 0.44 KH2PO4, 0.25 CaCl2, 0.15 Tris-HCl, and 1 sodium butyrate, pH 7.4 (Lemos et al., 2012). [3H]Thiamine hydrochloride (250 nM) uptake assays were performed by adding unlabeled thiamine (225 nM) and radiolabeled thiamine (25 nM) to the cells. Reactions were terminated using ice-cold phosphate-buffered saline. Uptake assays with fedratinib (0.1 μM) and trimethoprim (0.1 μM) were conducted in the same Hanks’ medium. Cells were solubilized with 500 μl of lysis buffer (0.1% SDS, 0.1 N NaOH). Radioactivity in the cells was determined by liquid scintillation counting and normalized to protein using a bichinchoninic acid assay (Life Technologies). Samples treated with fedratinib or trimethoprim were subjected to liquid chromatography with tandem mass spectrometry analysis.

Liquid Chromatography with Tandem Mass Spectrometry. Cells were lysed with 400 μl of 70% methanol spiked with internal standard (150 μl of 100 nM labeled thiamine). Samples were centrifuged (3600 rpm, 20 minutes) and dried with a stream of nitrogen before reconstitution with 200 μl of water and acetonitrile [80:20 (v/v)]. After centrifugation again, 150 μl was transferred to a deep-well 96-well plate and then 5 μl of each sample was injected and analyzed using a Xevo TQ-S (Waters Corporation, Milford, MA).

Pharmacophore Model Generation. A qualitative, ligand-based pharmacophore model was generated by determining the global minimum conformation of thiamine using the Merck Molecular Force Field method. The spatial location and directionality of three pharmacophore features, two hydrogen bond donors and one hydrogen bond acceptor, were chosen as key recognition elements for thiamine uptake inhibition. All low-energy conformers of the JAKi compounds were calculated and aligned to the thiamine global minimum structure with the dianinopyrimidine group superimposed. The aligned structures were used to assess the extent to which the potential binding partners are in the same spatial location within the JAKi.

**Statistical and Data Analysis.** Data are expressed as mean ± S.D. Thiamine uptake was determined in the cells overexpressing THTR-1 and THTR-2 by subtracting the amount of uptake observed in the empty vector containing cells of three independent experiments.

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**Table 1**

Inhibition of thiamine uptake by JAKi and other xenobiotics

<table>
<thead>
<tr>
<th>Compound</th>
<th>Caco-2</th>
<th>THTR-1 IC50 Value</th>
<th>THTR-2 IC50 Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>Fedratinib</td>
<td>0.940</td>
<td>7.10 ± 1.26</td>
<td>1.36 ± 0.59</td>
</tr>
<tr>
<td>AZD1480</td>
<td>183 ± 68.9</td>
<td>22.2 ± 6.20</td>
<td>15.3 ± 2.90</td>
</tr>
<tr>
<td>Cerdulatinib</td>
<td>&gt;300</td>
<td>276 ± 117</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Momelotinib</td>
<td>&gt;30.0</td>
<td>0 ± 30.0</td>
<td>&gt;30.0</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>154 ± 22.4</td>
<td>6.84 ± 1.68</td>
<td>5.56 ± 0.65</td>
</tr>
<tr>
<td>Oxythiamine</td>
<td>198 ± 16.0</td>
<td>67.3 ± 7.50</td>
<td>66.4 ± 21.4</td>
</tr>
<tr>
<td>Amprolium</td>
<td>9.40 ± 2.80</td>
<td>2.60 ± 0.93</td>
<td>0.620 ± 0.270</td>
</tr>
</tbody>
</table>

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Fig. 3. The interaction of fedratinib with thiamine transporters. (A) HEK293 THTR-1–overexpressing cells. (B) HEK293 MSR THTR-2–overexpressing cells. [3H]Thiamine (250 nM) uptake was conducted in the presence of fedratinib (solid circles) or momelotinib (solid squares). Results represent the mean ± S.D. of triplicate determinations in a representative experiment of three independent experiments.
from the overexpressing cells. \[^{[3]}H\]Thiamine uptake was expressed as “percentage control” by normalizing the amount of uptake observed in compound-treated cells to dimethylsulfoxide vehicle–treated cells. Data were analyzed using GraphPad Prism software (version 6.01; GraphPad Software, La Jolla, CA). The data were analyzed using a one- or two-way analysis of variance with a post hoc Tukey’s or Dunnett’s multiple comparisons test. Values of \( P < 0.05 \) were considered statistically significant. All experiments were performed on at least three separate occasions, except where indicated, and data presented are from representative experiments.

Results

The Interaction of JAKi in Caco-2 Cells. To determine the potential of momelotinib and other JAKi on disrupting intestinal uptake of thiamine, we examined the effect of a number of JAKi on thiamine transport using Caco-2 cells as an in vitro model (Fig. 1) (Said et al., 1999). We observed that fedratinib potently inhibited thiamine transport (IC\(_{50} = 0.940 \pm 0.080 \) \( \mu M \)). We also observed weak inhibition of thiamine uptake by AZD1480 [(5-chloro-2-N-[(1S)-1-(5-fluoropyrimidin-2-yl)ethyl]-4-N-(5-methyl-1H-pyrazol-3-yl)pyrimidine-2,4-diamine)] (AstraZeneca, London, UK). Specificity of thiamine uptake was confirmed by treating cells with unlabeled thiamine or pyrithiamine, which inhibited thiamine uptake by 73 and 45%, respectively. The other JAKi examined, including momelotinib and its metabolite, did not significantly affect thiamine uptake (Fig. 2, A and B; Supplemental Fig. 1A; Table 1). Amprolium and oxythiamine, two thiamine analogs, also demonstrated inhibitory effects on thiamine transport (Supplemental Fig. 1, B and C; Table 1).

The Interaction of Fedratinib with Human Thiamine Transporters. To elucidate a potential mechanistic basis of this inhibitory effect on thiamine uptake observed in the Caco-2 cells, we examined the expression of the thiamine transporters in our cells. The mRNA levels of the transporters in Caco-2 were similar to those of the colon based on analysis of human intestinal tissue (Supplemental Fig. 1D).

To further examine the interaction of fedratinib with the individual thiamine transporters, we tested the compound in HEK293 cells stably overexpressing THTR-1 or HEK293 MSR macrophage scavenger receptor transiently overexpressing THTR-2. Thiamine uptake kinetics for both cell lines was similar to previous reports (Said et al., 2004; Liang et al., 2015). Thiamine had a \( K_m = 6.98 \pm 2.23 \) \( \mu M \) and \( V_{\text{max}} = 6.44 \pm 4.29 \) pmol/mg protein/min in HEK293 THTR-1 cells, and \( K_m = 3.85 \pm 1.14 \) \( \mu M \) and \( V_{\text{max}} = 82.1 \pm 20.9 \) pmol/mg protein/min in HEK293 MSR THTR-2 cells (Supplemental Fig. 2). Consistent with a previous study, we observed fedratinib to most potently inhibit THTR-2–mediated thiamine transport (IC\(_{50} = 1.36 \pm 0.59 \) \( \mu M \)) (Zhang et al., 2014b). Interestingly, we found that fedratinib also inhibited THTR-1 thiamine transport (IC\(_{50} = 7.10 \pm 1.26 \) \( \mu M \)) in our system. Perhaps the discrepancy between the studies is due to differences in the composition of the uptake buffers or experimental conditions. Momelotinib did not inhibit thiamine uptake in Caco-2 cells. To confirm the compound does not interact with the thiamine transporters, momelotinib was tested in the HEK293 THTR-1 and HEK293 MSR THTR-2 overexpressing cell lines, and no inhibitory effects on thiamine uptake were observed (Fig. 3, A and B; Table 1).

To determine if fedratinib could also be a substrate of THTR-1 and THTR-2, we examined fedratinib uptake in the presence or absence of pyrithiamine as a control in the HEK293 stably overexpressing THTR-2 cell line were similar to the HEK293 MSR THTR-2 cell line were similar to the HEK293 MSR THTR-2 cell line.

Fig. 4. Interaction and kinetic characterization of fedratinib with thiamine transporters. (A) HEK293 cells overexpressing THTR-1. (B) HEK293 cells stably overexpressing THTR-2. Uptake with fedratinib (0.1 \( \mu M \)) was performed for 3 minutes in the presence or absence of pyrithiamine (100 \( \mu M \)) in empty vector cells (black bar) and in transporter-overexpressing cells (gray bar). (C) HEK293 cells stably overexpressing THTR-2. Uptake with fedratinib (0.1 \( \mu M \)) was performed over time (0–30 minutes). (D) HEK293 cells stably overexpressing THTR-2. Uptake was performed with increasing concentrations of fedratinib (0–1 \( \mu M \)) over 3 minutes. Results represent the mean ± S.D. of triplicate determinations in a representative experiment of three independent experiments. ns, not statistically significant. **** \( P < 0.0001 \).
The interaction of other 2,4-diaminopyrimidine compounds with the thiamine transporters. To further examine the interaction of the 2,4-diaminopyrimidine structural moiety with the thiamine transporters, we tested drugs from various therapeutic classes that contained the structural group. We discovered trimethoprim, a commonly used antibiotic in the prevention and treatment of urinary tract infections and other bacterial infections, inhibited thiamine uptake in Caco-2, and in cells overexpressing THTR-1 (IC$_{50}$ = 6.84 ± 1.68 μM) and those overexpressing THTR-2 (IC$_{50}$ = 11.5 ± 4.73 μM) (Fig. 5, A–C; Table 1). In addition, we determined that trimethoprim is a substrate of both THTR-1 (K$_{m}$ = 22.1 ± 13.2 μM, V$_{max}$ = 65.7 ± 58.1 pmol/mg protein/min) and THTR-2 (K$_{m}$ = 1.01 ± 0.10 μM, V$_{max}$ = 22.1 ± 5.10 pmol/mg protein/min) (Fig. 6, A–F).

Role of 2,4-Diaminopyrimidine Structure in Affecting Thiamine Transport. To further explore the structural basis of thiamine uptake inhibition, we generated a ligand-based pharmacophore model of the JAKi that were tested in the in vitro thiamine uptake assays. Our model indicated fedratinib can adopt a conformation similar to thiamine with three key pharmacophore features in the same spatial location, whereas momelotinib cannot. We also determined that AZD1480, cerdulatinib (Portola Pharmaceuticals, San Francisco, CA), and trimethoprim can adopt a conformation similar to thiamine; however, the reduced overlap of the pharmacophore features in the aligned structures suggests a weaker interaction with the thiamine transporters (Fig. 7). The difference in inhibitory potency between amprolium (aminopyrimidine donor and acceptor but lacks alcohol) and oxythiamine (alcohol donor but lacks aminopyrimidine contacts) illustrates the relative importance of the proper alignment of the aminopyrimidine for high-affinity binding.

Discussion

The major findings in this study are that fedratinib and the widely used antibiotic trimethoprim are inhibitors of THTR-1 and THTR-2. In addition, we discovered that fedratinib is a substrate of only THTR-2, whereas trimethoprim is a substrate of both thiamine transporters. Finally, the interaction of fedratinib and trimethoprim with the thiamine transporters may be attributed to the 2,4-diaminopyrimidine pharmacophore present in both compounds. Our findings provide further evidence that the adverse effects observed with fedratinib administration are related to thiamine transport and may explain the observation of high brain penetration relative to other molecules in their respective classes observed with fedratinib and trimethoprim.

Thiamine deficiency is particularly important in vulnerable populations, such as patients who suffer from alcoholism or surgical patients with gastrointestinal bypass. In individuals diagnosed with myeloproliferative neoplasms, a higher incidence of Wernicke's encephalopathy has been reported (1.09/1000 person-years) compared with non-myeloproliferative neoplasm patients (0.39/1000 person-years), suggesting that patients who are already susceptible to vitamin deficiency may be more prone to developing Wernicke's than other disease populations upon administration of compounds that affect thiamine disposition (Wu et al., 2015). Although both thiamine transporters are involved in absorption of dietary thiamine, only THTR-2 knockout mice exhibit reduced intestinal thiamine uptake and blood thiamine levels compared with wild-type littermates (Reidling et al., 2010). Recently, Zhang et al. (2014b) showed that fedratinib was a potent inhibitor of THTR-2, providing a potential mechanism for the observation of Wernicke's encephalopathy in the fedratinib clinical trial. Our finding that fedratinib inhibits both THTR-1- and THTR-2-mediated thiamine uptake adds to the growing body of evidence that the drug is a potent...
inhibitor of thiamine transporters, and that fedratinib-associated encephalopathy could be due to the compound affecting absorption of dietary thiamine. To determine the likelihood of an intestinal drug-drug interaction (DDI), we estimated the $I_2/IC_{50}$ of fedratinib to be 2803, which greatly exceeds the threshold for recommended DDI studies suggested by the International Transporter Consortium for other transporters (any compound with an $I_2/IC_{50}$ ratio greater than 10) (Giacomini et al., 2010). Thus, fedratinib would be expected to be a potent inhibitor of thiamine absorption in the intestinal tract. Interestingly, when we calculated the $C_{\text{max,unbound}}/IC_{50}$ for fedratinib for THTR-2 (1.69), the value also exceeded the threshold for a clinical DDI study ($I_1/IC_{50}$ ratio greater than 0.1), suggesting that the previously observed Wernicke’s encephalopathy following fedratinib administration may have been a result of both intestinal inhibition of thiamine absorption and inhibition of thiamine uptake across the blood-brain barrier, as THTR-2 is the major thiamine transporter in the blood-brain barrier. The $I_1/IC_{50}$ and $I_2/IC_{50}$ values (0.32 and 540, respectively) for fedratinib inhibition of THTR-1 also exceed thresholds proposed for other transporters, suggesting the potential for more global changes in thiamine handling.

Momelotinib was a JAK1 and JAK2 inhibitor in phase 3 trials for myelofibrosis (Pardanani et al., 2013). Although peripheral neuropathy, almost exclusively grade 1 or 2, has been reported with momelotinib treatment, no central neurotoxicity has been reported (Verstovsek et al., 2014; Abdelrahman et al., 2015). The clinical experience with momelotinib is unlike that of other JAKi, such as fedratinib, AZD1480, and XL019, where effects on the central nervous system were observed (Pimack et al., 2013; Verstovsek et al., 2014; Zhang et al., 2014a; Verstovsek et al., 2015). Similar to momelotinib, administration of a number of JAKi has not been associated with central nervous system effects. Based on structural features common between fedratinib and AZD1480, momelotinib was predicted to affect thiamine disposition, suggesting patients on the drug would be at risk for developing Wernicke’s encephalopathy (Ratner, 2014). Specifically, interaction with the thiamine transporters has been attributed to the
aminopyrimidine group (Greenwood and Pratt, 1985). As evident from our in vitro studies, fedratinib, which contains a 2,4-diaminopyrimidine group in its chemical structure, interacts with THTR-1 and THTR-2. AZD1480 and cerdulatinib, both containing a 2,4-diaminopyrimidine group, also affect thiamine transport, whereas all of the non-2,4-diaminopyrimidine-containing JAKi tested have no effect. Our findings suggest that momelotinib and most JAKi do not have the potential to affect thiamine transport, a potential contributing factor to the

Fig. 7. Pharmacophore model of JAKi. (A) 2,4-Diaminopyrimidine JAKi. (B) 2- or 4-monoaminopyrimidine JAKi. (C) Nonaminopyrimidine JAKi. (D) Non-JAKi. The global minimum conformation for each compound was generated. The potential binding partners were assessed to determine if they were in the same spatial location as thiamine. Blue, H-bond donor; red, H-bond acceptor. Aminopyrimidines that do not share the minimal pharmacophore of thiamine are marked with an X.
observation of central nervous system effects observed with some JAKi, fedratinib and AZD1480 in particular.

Consistent with our in vitro results, the pharmacophore model of the compounds demonstrates that the 2,4-diaminopyrimidine structural group in JAKi is necessary for potent thiamine inhibition, but not sufficient to mimic key contacts of thiamine with its transporters. To further explore the effect of compounds in this structural group on their interaction with the thiamine transporters, we tested drugs from various therapeutic classes that contained the 2,4-diaminopyrimidine structural core on thiamine uptake. Trimethoprim is a commonly used antibiotic on the World Health Organizations Essential Medicines list that also contains a 2,4-diaminopyrimidine. Consistent with the structure-activity relationship developed for JAKi, we found that trimethoprim is also a potent inhibitor of THTR.

Although the mechanism is unknown, thiamine deficiency has been infrequently reported with trimethoprim use (Thorne Research, 2003). We estimated trimethoprim would achieve high concentrations relative to its inhibition constant for THTR-2 (IIC50 = 248 for THTR-2). In addition, the Cmax,unbound/IC50 for tissues (in particular brain) via THTR-2. Similar to fedratinib, the I1/IC50 suggests the compound may inhibit uptake of thiamine into other tissues (in particular brain) via THTR-2. Similar to fedratinib, the I1/IC50 and I2/IC50 values (0.33 and 200, respectively) for trimethoprim inhibition of THTR-1 also exceed thresholds proposed for other transporters, suggesting the potential for trimethoprim to inhibit both thiamine transporters at clinically relevant concentrations.

In addition, although rare, several case reports involving elderly or immunocompromised patients associate trimethoprim treatment with neurotoxic effects, such as encephalopathy, transient psychosis, acute delirium with agitation, visual and auditory hallucinations, and transient tremor (Cooper et al., 1994; Patey et al., 1998; Patterson and Coughenour, 1999; Saidinejad et al., 2005). Similar to fedratinib, it is possible that these effects may only manifest and be relevant in sensitive populations who are already thiamine deficient.

THTRs are expressed in many tissues, including the intestine, blood-brain barrier, and kidney, where they play an important physiologic role in the absorption, brain penetration, and reabsorption, respectively, of this important nutrient. Notably, SLC19A3 mRNA expression was found to be especially enriched on brain microvessels, establishing that the transporter may play a role in delivering its substrates across the blood-brain barrier to the central nervous system (CNS) (Giefer et al., 2013). Both thiamine transporters are highly homologous and were previously thought to share similar substrate specificity that did not include transporting antifolate compounds (Dutta et al., 1999; Rajgopal et al., 2001; Zhao and Goldman, 2013). One aspect that makes trimethoprim a valuable antibiotic is its CNS penetration. High-dose trimethoprim is used to treat CNS infections (Dudley et al., 1984; Nau et al., 2010).

Potentially related to the observation of trimethoprim being a substrate for THTR-1 and -2, trimethoprim ratios of cerebral spinal fluid to plasma in human subjects have been observed to be about 0.2, which is greater than the brain permeation for other antibiotic classes, including penicillins (0.02) and cephalosporins (range 0.007-0.1) (Nau et al., 2010). In addition, our finding that fedratinib is a substrate of THTR-2 may help to provide a mechanistic basis for the observation that fedratinib had a free brain to plasma ratio (Kp,brain) 7- to 10-fold higher than ruxolitinib or tofacitinib in rats (Zhang et al., 2014b). Also, recent data demonstrate that THTR-2—but not THTR-1—transports metformin, famotidine, and 1-methyl-4-phenylpyridinium (Liang et al., 2015). Our data showing that THTR-2 transports fedratinib and trimethoprim support the emerging additional role of THTR-2 as a xenobiotic transporter. In this study, only a select set of compounds were tested for their interaction with thiamine transporters. Coupled with our prior work showing metformin to be a substrate and inhibitor of THTR-2 (Liang et al., 2015), these results support the more broadly screening of drugs for their interaction with thiamine transporters.

Thiamine disposition in the kidney is complex. In the proximal tubule, the nutrient interacts with several secretory transporters, such as organic cation transporter 2 (OCT2; SLC22A2) and multidrug and toxin extrusion protein (MATE; SLC47), with much higher Kmax values than the THTRs, both of which are also expressed in the proximal tubule (Ashokkumar et al., 2006; Reidling et al., 2006). In healthy individuals who do not suffer from thiamine insufficiency, thiamine is substantially secreted in the urine, presumably by OCT2, MATE1, and MATE2 (Kato et al., 2014). However, in the case of thiamine deficiency, net reabsorption is likely. That is, reabsorptive flux may predominate. It is possible that both fedratinib and trimethoprim may reduce thiamine reabsorption, which would be particularly relevant in patients with thiamine deficiency. For example, trimethoprim levels in urine have been measured to be in the hundreds of micromolars over 24 hours after a 100-mg dose. The inhibition constants for THTR-1 and THTR-2 determined for trimethoprim in this study are also similar to transporters known to be inhibited by trimethoprim in the kidney (e.g., OCT2, MATE1, and MATE2-K) (Lepist et al., 2014). Inhibition of nutrient reabsorption has also been reported for antibiotics. Carnitine deficiency observed with cephaloridine and etoposide has been attributed to inhibition of carnitine reabsorption mediated by organic cation/ carnitine transporter (SLC22A5) at the apical membrane of the renal proximal tubule (Tune and Hsu, 1994; Ganapathy et al., 2000; Yang et al., 2012). Combined, these results illustrate that some drugs have the potential to inhibit reabsorptions of nutrients in the kidney.

In conclusion, a majority of JAKi show no potential to affect thiamine transport. The results with fedratinib, AZD1480, and trimethoprim on affecting THTR-1 and THTR-2-mediated thiamine disposition suggest that drug compounds able to adopt a similar confirmation to thiamine due to the presence of a 2,4-diaminopyrimidine group can be involved in drug-vitamin interactions. Finally, our results further establish THTR-1 and THTR-2 as a potential source for drug-nutrient interactions and suggest they may have a broader role in the disposition of some drugs, and that monitoring of vitamin deficiency and appropriate supplementation may be warranted in some populations being chronically treated with drugs affecting nutrient pathways.

**Authorship Contributions**
- **Participated in research design:** Giacomini, Hao, Liang, Chandrasekhar, Whitney, Lepist, Ray.
- **Conducted experiments:** Giacomini, Hao, Liang, Chandrasekhar, Twelves.
- **Contributed new reagents or analytic tools:** Liang, Whitney.
- **Performed data analysis:** Giacomini, Hao, Liang, Chandrasekhar, Lepist, Ray.
- **Wrote or contributed to the writing of the manuscript:** Giacomini, Chandrasekhar, Lepist, Ray.

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