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

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**ABBREVIATIONS**

BBGD, biotin-responsive basal ganglia disease

Caco-2, Human epithelial colorectal adenocarcinoma

DMEM, Dulbecco's Modified Eagle's Medium

FBS, fetal bovine serum

FDA, US Food and Drug Administration

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

JAK/STAT, Janus Kinase/Signal Transducer and Activator of Transcription

JAKi, Janus Kinase inhibitor

LS-MS/MS, liquid chromatography–mass spectrometry

P/S, penicillin/streptomycin

PCR, polymerase chain reaction

THTR, thiamine transporter

DDI, drug-drug interaction

BBB, blood-brain barrier

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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 Caco-2 and thiamine transporter (THTR) over-expressing 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 ($IC_{50}$ = 0.940 μM) and THTR-2 ($IC_{50}$ = 1.36 μM) and, to a lesser extent, THTR-1 ($IC_{50}$ = 7.10 μM) over-expressing cells. Two other JAKi containing this moiety, AZD1480 and cerdulatinib, were weaker inhibitors of the thiamine transporters. Other JAKi—including monoaminopyrimidines, such as momelotinib, and non-aminopyrimidines, 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 THTR-1 ($IC_{50}$ = 6.84 μM) and THTR-2 ($IC_{50}$ = 5.56 μM)-mediated thiamine uptake. 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 establishes the interaction of these transporters with xenobiotics.

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## 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 $JAK2^{V617F}$ mutation is present in greater than 95% of polycythemia vera 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) was the first JAK1 and JAK2 inhibitor approved by the US Food and Drug Administration (FDA) for the treatment of myelofibrosis, and tofacitinib (Pfizer) is FDA approved for rheumatoid arthritis.

In November 2013, late-stage clinical development of fedratinib (Sanofi), a selective JAK2 inhibitor for the treatment of myelofibrosis, was terminated due to the observation of Wernicke's encephalopathy, a severe neurological disease associated with thiamine deficiency, in a small number of patients during clinical trials (Sechi and Serra, 2007; 2013; Rodriguez-Pardo et al., 2015). These adverse events have been attributed to the inhibition of 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 co-factor for several enzymes including pyruvate dehydrogenase and alpha-keto dehydrogenase, essential enzymes in glycolytic energy production.

Two known human thiamine transporters actively transport the nutrient across cell membranes: THTR-1 (*SLC19A2*) 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., 2012). 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; 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 (BBGD), an autosomal recessive disorder characterized by encephalopathy, which is reminiscent of Wernicke's encephalopathy (Neufeld et al., 2001; Zeng et al., 2005). While 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 BBGD patients; thus, establishing THTR-2 as the major absorptive transporter for thiamine (Neufeld et al., 2001; Zeng et al., 2005). Zhang et al., 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 JAKi (Zhang et al., 2014b).

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 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 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 they also highlight the emerging additional role of THTR-2 as a xenobiotic transporter.

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## 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 of 2.5 x $10^5$ cells/well in 12-well plates (Corning Inc., Corning, NY) in custom-formulated thiamine-deficient media (Life Technologies, Carlsbad, CA), 10% FBS, and 1% P/S four days prior to experiments to stimulate transporter expression (Ashokkumar et al., 2006). THTR-1 and THTR-2 stably over-expressing HEK293 cells were provided by Dr. Kathy Giacomini (University of California, San Francisco, CA) and were cultured in DMEM/High Glucose media containing 10% FBS, 1% P/S, and puromycin (10 µg/mL). These cells were plated at a density of 1.5 x $10^5$ cells/well in 24-well poly-D-lysine–coated plates in normal growth media 48 hours prior to experiments. HEK293 MSR THTR-2 over-expressing cells were generated by transiently expressing a plasmid containing the *SLC19A3* open reading frame (Dharmacon, Lafayette, CO) in GripTite™ HEK293 MSR cells (Life Technologies, Carlsbad, CA). Cells were seeded in 24-well plates in regular growth media at a density of 1.5 x $10^5$ cells/well, transfections performed according to the *Trans*IT®-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 media containing 10% FBS, 1% P/S, 0.1 mM Non-Essential Amino Acids, and G418 (600 µg/mL) and at 37°C, 5% $CO_2$. Compounds used were [$^3$H]-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 were synthesized in-house.

*Real Time Polymerase Chain Reaction (RT-PCR) Analysis of mRNA Levels in Cell Lines and Human Tissue Samples.*

Total RNA was isolated from Caco-2, THTR stably and transiently over-expressing cell lines, and human tissue samples using the RNeasy 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 PCR was carried out in 96-well FAST reaction plates using 2X Taqman Fast Universal Master Mix (Applied Biosystems, Foster City, CA), 20X Taqman specific gene expression probes, and 100 ng of the cDNA template. The reactions were performed on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). The relative expression level of each mRNA transcript was calculated by the Comparative $C_T$ ($\Delta\Delta C_T$) Method and normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

*Uptake Assays.*

Cells were pre-incubated at 37$^{o}$C for 10 to 15 minutes in Hanks' medium with the following composition (in mM): 137 NaCl; 5 KCl; 0.8 $MgSO_4$; 1 $MgCl_2$; 0.33 $Na_2HPO_4$; 0.44 $KH_2PO_4$; 0.25 $CaCl_2$; 0.15 Tris-HCl; and 1 sodium butyrate, pH 7.4 (Lemos et al., 2012). [$^3$H]-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 PBS. 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 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 bicinchoninic acid assay (BCA) (Life Technologies). Samples treated with fedratinib or trimethoprim were subjected to liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

*LC-MS/MS.*

Cells were lysed with 400 μL 70% methanol spiked with internal standard (150 μL of 100 nM labetalol in acetonitrile). Samples were centrifuged (3600 rpm, 20 min) and dried down with a stream of nitrogen before reconstitution with 200 μL of water and acetonitrile (80:20, v/v). After centrifugation again, 150 μL were 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).

*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 diaminopyrimidine 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 ± standard deviation (SD). Thiamine uptake was determined in the THTR-1 and THTR-2 over-expressing cells by subtracting the amount of uptake observed in the empty vector containing cells from the over-expressing cells. [$^3$H]-thiamine uptake was expressed as "% control" by normalizing the amount of uptake observed in compound-treated cells to dimethyl sulfoxide vehicle treated cells. Data were analyzed using GraphPad Prism software (version 6.01). The data were analyzed using a one or two-way analysis of variance (ANOVA) 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 (Figure 1) (Said et al., 1999). We observed that fedratinib potently inhibited thiamine transport ($IC_{50}$ = 0.940 ± 0.080 µM). We also observed weak inhibition of thiamine uptake by AZD1480 (AstraZeneca). 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; Table 1; Supplemental Figure 1A). Amprolium and oxythiamine, two thiamine analogs, also demonstrated inhibitory effects on thiamine transport (Table 1; Supplemental Figure 1, B and C).

*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 the colon based on analysis of human intestinal tissue (Supplemental Figure 1D).

To further examine the interaction of fedratinib with the individual thiamine transporters, we tested the compound in HEK293 cells stably over-expressing THTR-1 or HEK293 MSR transiently over-expressing 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 ± 2.23 µM and $V_{max}$ of 6.44 ± 4.29 pmol/mg protein/min in HEK293 THTR-1 cells and $K_m$ = 3.85 ± 1.14 µM,

$V_{max}$ = 82.1 ± 20.9 pmol/mg protein/min in HEK293 MSR THTR-2 cells (Supplemental Figure 2). Consistent with a previous study, we observed fedratinib to most potently inhibit THTR-2–mediated thiamine transport ($IC_{50}$ = 1.36 ± 0.59 μM) (Zhang et al., 2014b). Interestingly, we found that fedratinib also inhibited THTR-1 thiamine transport ($IC_{50}$ = 7.10 ± 1.26 μM) 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 over-expressing cell lines, and no inhibitory effects on thiamine uptake were observed (Figure 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 over-expressing THTR-1 and THTR-2 cell lines. Thiamine kinetics in the HEK293 THTR-2 cell line were similar to the HEK293 MSR THTR-2 transiently over-expressing cell line (Liang et al., 2015). We discovered that fedratinib is a substrate of THTR-2 ($K_m$ = 0.44 ± 0.32 μM and $V_{max}$ of 33.07 ± 11.44 pmol/mg protein/min), with more than four-fold uptake in THTR-2 over-expressing cells compared with empty vector cells. Interestingly, fedratinib is not a substrate of THTR-1, as no significant uptake over empty vector was observed in over-expressing cells (Fig. 4, A and B).

*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 both THTR-1 ($IC_{50}$ = 6.84 ± 1.68 µM) and THTR-2 ($IC_{50}$ = 11.5 ± 4.73 µM) over-expressing cells (Fig. 5, A to 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) (Figure 6, A to 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, while momelotinib cannot. We also determined that AZD1480, cerdulatinib (Portola Pharmaceuticals), and trimethoprim can adopt a similar conformation to thiamine; however, the reduced overlap of the pharmacophore features in the aligned structures suggest 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, while 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 also 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 (MPN), a higher incidence of Wernicke's encephalopathy has been reported (1.09/1000 person-year) compared to non-MPN patients (0.39/1000 person-year), 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). While 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., 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 2,803, 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) (International Transporter 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 $C_{max,unbound}/IC_{50}$ 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, as well as inhibition of thiamine uptake across the blood brain barrier (BBB), as THTR-2 is the major thiamine transporter in the BBB. 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 is a JAK1 and JAK2 inhibitor in phase 3 trials for myelofibrosis (Pardanani et al., 2013). While 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 (Plimack 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 affects thiamine transport, while 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 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 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 with 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. While the mechanism is unknown, thiamine deficiency has been infrequently reported with trimethoprim use (2003). We estimated trimethoprim would achieve high concentrations relative to its inhibition constant for THTR-2 ($I_2/IC_{50}$ =248 for THTR-2). In addition, the $C_{max,unbound}/IC_{50}$ for trimethoprim for THTR-2 was approximately 0.41, again, exceeding the threshold for a clinical DDI study with other transporters and suggests the compound may inhibit uptake into other tissues, in particular brain,

of thiamine via THTR-2. Similar to fedratinib, the $I_1/IC_{50}$ and $I_2/IC_{50}$ 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 Couchenour, 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.

THTR 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) (Geier 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, cerebral spinal fluid (CSF) to plasma ratios of trimethoprim 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 to 0.1) (Nau et al., 2010). In addition, our finding that fedratinib is a substrate of THTR-2 may help to provide mechanistic basis for the observation that fedratinib had a free brain to plasma ratio ($K_{p,uu}$) 7- to 10-fold higher than ruxolitinib or tofacitinib in rats (Zhang et al., 2014b). There is also recent data that demonstrate THTR-2—but not THTR-1—transports metformin, famotidine, and MPP+ (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 more broadly screening 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 $K_m$ values than the THTRs, which are also both 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 micromolar over 24 hours post a 100 mg dose. The inhibition constants for THTR-1 and THTR-2 determined for trimethoprim in this study are also similar to transporters that it is known to inhibit 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 (OCTN2; *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.

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## AUTHORSHIP CONTRIBUTIONS

*Participated in research design:* Giacomini, Hao, Liang, Chandrasekhar, Whitney, Lepist, and Ray

*Conducted experiments:* Giacomini, Hao, Liang, Chandrasekhar, and Twelves

*Contributed new reagents or analytic tools:* Liang and Whitney

*Performed data analysis:* Giacomini, Hao, Liang, Chandrasekhar, Lepist, and Ray

*Wrote or contributed to the writing of the manuscript:* Giacomini, Chandrasekhar, Lepist, and Ray

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**FOOTNOTES**

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## FIGURE LEGENDS

**Fig. 1.** Chemical structures of compounds tested. (A) 2,4-diaminopyrimidine JAKi. (B) 2 or 4-monoaminopyrimidine JAKi. (C) Non-aminopyrimidine JAKi. (D) Non-JAKi. Aminopyrimidine core (bold).

**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) $IC_{50}$ determination. [$^3$H]-Thiamine uptake was conducted in the presence of fedratinib (solid circles) or momelotinib (solid squares). Results shown represent the mean ± SD 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. ***$P < 0.001$, ****$P < 0.0001$.

**Fig. 3.** The interaction of fedratinib with thiamine transporters. (A) HEK293 THTR-1 overexpressing cells. (B) HEK293 MSR THTR-2 overexpressing cells. [$^3$H]-Thiamine (250 nM) uptake was conducted in the presence of fedratinib (solid circles) or momelotinib (solid squares). Results shown represent the mean ± SD of triplicate determinations in a representative experiment of three independent experiments.

**Fig. 4.** Interaction and kinetic characterization of fedratinib with thiamine transporters. (A) HEK293 THTR-1 overexpressing cells. (B) HEK293 THTR-2 stably overexpressing cells. Uptake with fedratinib (0.1 μM) was performed for 3 minutes in the presence or absence of pyrithiamine (100 μM) in empty vector cells (black bar) and in transporter over-expressing cells

(gray bar). (C) HEK293 THTR-2 stably overexpressing cells. Uptake with fedratinib (0.1 μM) was performed over time (0 – 30 minutes). (D) HEK293 THTR-2 stably overexpressing cells. Uptake was performed with increasing concentrations of fedratinib (0 – 1 μM) over 3 minutes. Results shown represent the mean ± SD of triplicate determinations in a representative experiment of three independent experiments. Ns = not statistically significant, ****$P < 0.0001$.

**Fig. 5.** The interaction of trimethoprim with Caco-2 and thiamine transporters. (A) Caco-2 cells. [$^3$H]-Thiamine uptake (250 nM) assay was conducted for 3 minutes in the presence of trimethoprim (30 μM), pyrithiamine (100 μM), and thiamine (1 mM). (B) HEK293 THTR-1 overexpressing cells. (C) HEK293 THTR-2 stably overexpressing cells. [$^3$H]-Thiamine uptake (250 nM) assays were conducted in the THTR overexpressing cells for 3 minutes in the presence of increasing concentrations of trimethoprim (0 – 300 μM). Inhibitory effects are reported as a percentage of vehicle-treated controls. Results shown represent the mean ± SD of triplicate determinations in a representative experiment of three independent experiments. ****$P <$ 0.0001.

**Fig. 6.** Interaction and kinetic characterization of trimethoprim with thiamine transporters. (A-C) HEK293 THTR-1 overexpressing cells. (D-F) HEK293 THTR-2 stably overexpressing cells. Uptake with trimethoprim (0.1 μM) was performed for 3 minutes in the presence or absence of pyrithiamine (100 μM), over various time points (0 – 30 minutes), and with increasing concentrations of trimethoprim (0 – 30 μM) in empty vector cells (black bar) and in transporter over-expressing cells (gray bar). Results shown represent the mean ± SD of triplicate

determinations in a representative experiment of three independent experiments. ****$P <$ 0.0001.

**Fig. 7.** Pharmacophore model of JAKi. (A) 2,4-diaminopyrimidine JAKi. (B) 2 or 4-monoaminopyrimidine JAKi. (C) Non-aminopyrimidine 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. H-bond donor (blue), H-bond acceptor (red). Aminopyrimidines that do not share the minimal pharmacophore of thiamine are marked with an "X."

**Table 1. Inhibition of thiamine uptake by JAKi and other xenobiotics.**

| | $IC_{50}$ Value (μM) for Thiamine Uptake | | |
|---|---|---|---|
| **Compound** | **Caco-2** | **THTR-1** | **THTR-2** |
| Fedratinib | 0.940 ± 0.080 | 7.10 ± 1.26 | 1.36 ± 0.59 |
| AZD1480 | 183 ± 68.9 | 22.2 ± 6.20 | 15.3 ± 2.90 |
| Cerdulatinib | >300 | 276 ± 117 | >300 |
| Momelotinib | >30.0 | >30.0 | >30.0 |
| Trimethoprim | 154 ± 22.4 | 6.84 ±1.68 | 5.56 ± 0.65 |
| Oxythiamine | 198 ± 16.0 | 67.3 ± 7.50 | 66.4 ± 21.4 |
| Amprolium | 9.40 ± 2.80 | 2.60 ± 0.93 | 0.620 ± 0.270 |

Values represent the mean ± SD of triplicate determinations in a representative experiment of three independent experiments.

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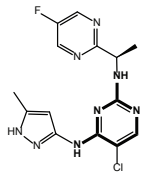
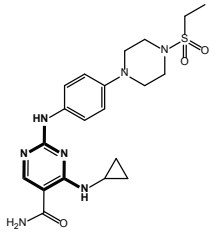
**Figure 1**

**A.**

AZD1480



Cerdulatinib



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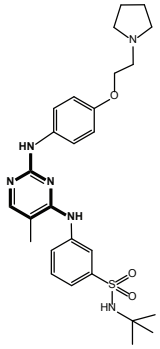
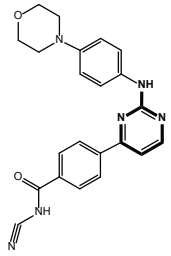


**B.**

Momelotinib



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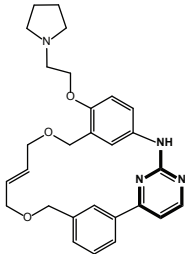

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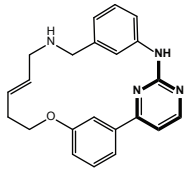

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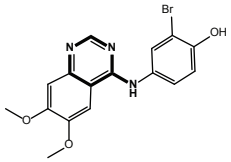

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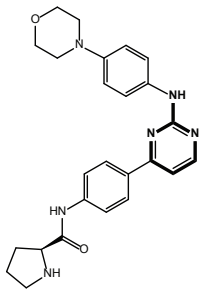
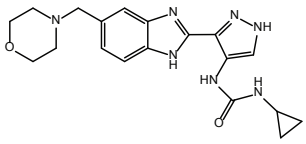


**C.**

AT9283



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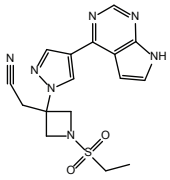

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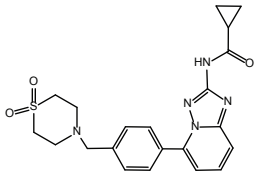

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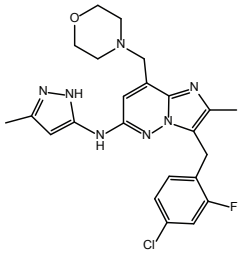

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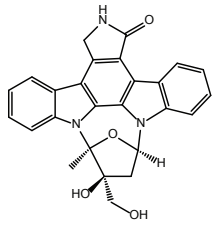

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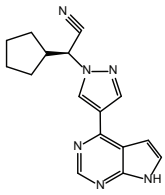

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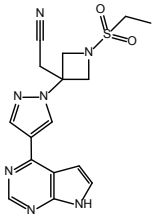
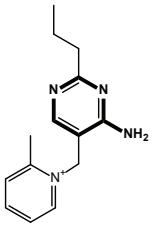
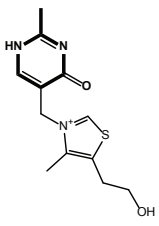


**D.**

Amprolium



Oxythiamine



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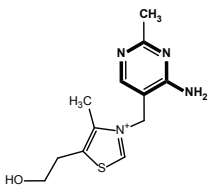

Trimethoprim

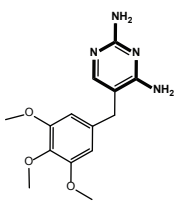

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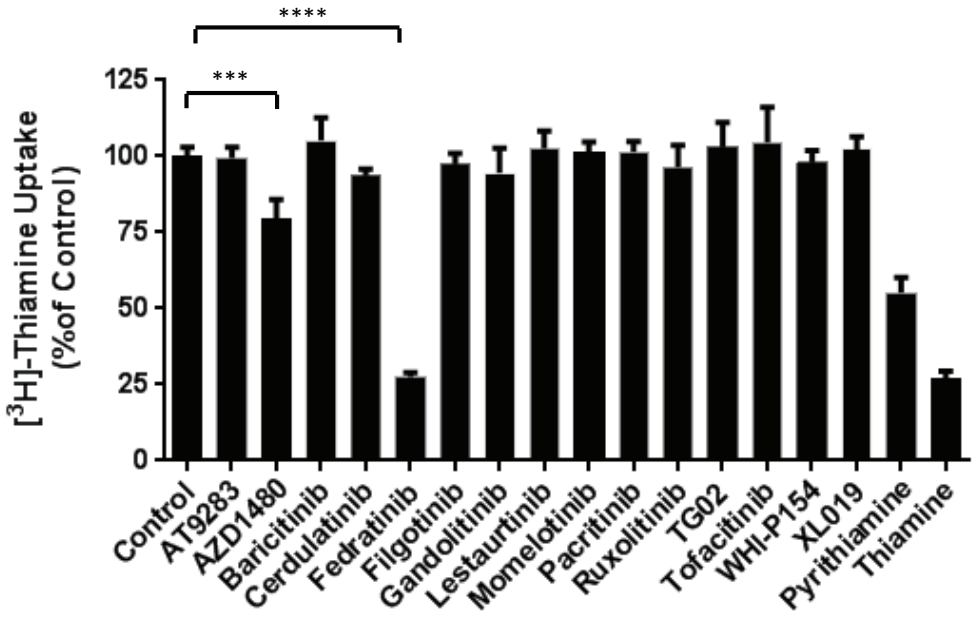
**Figure 2**

**A.**

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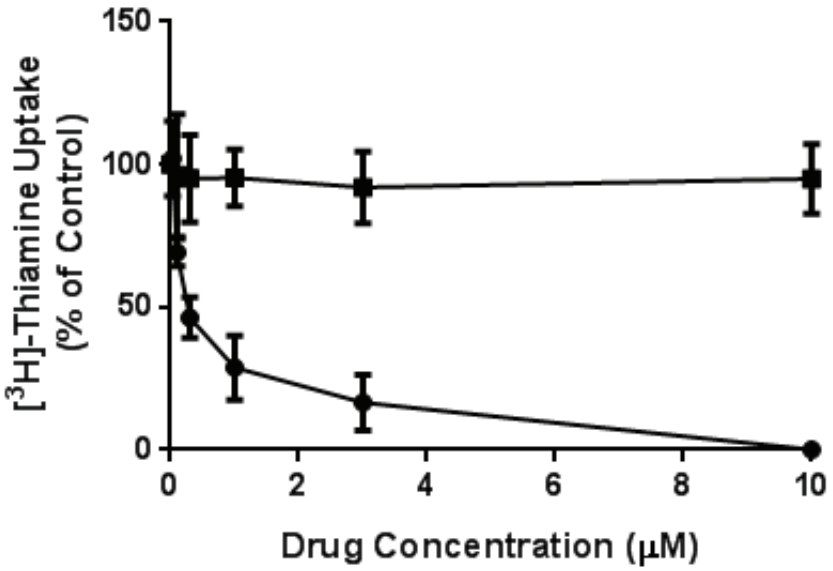

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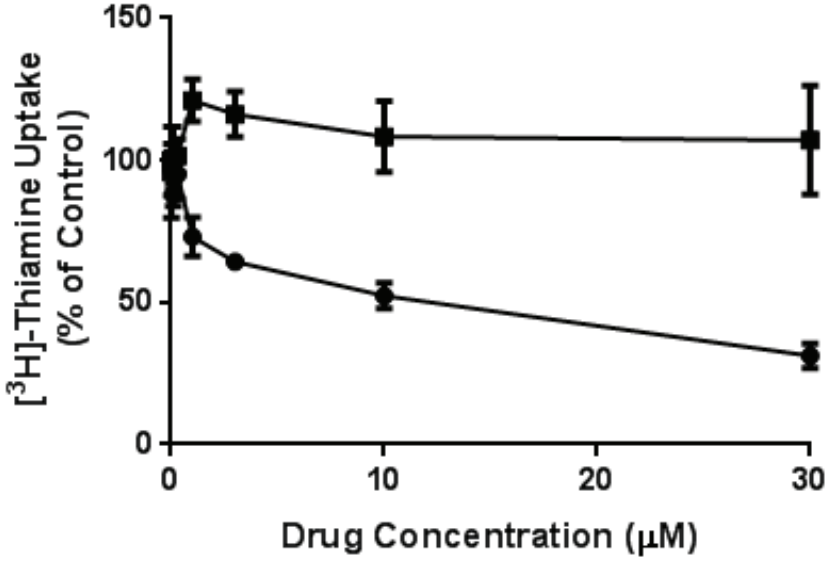
**Figure 3**

**A.**




**B.**

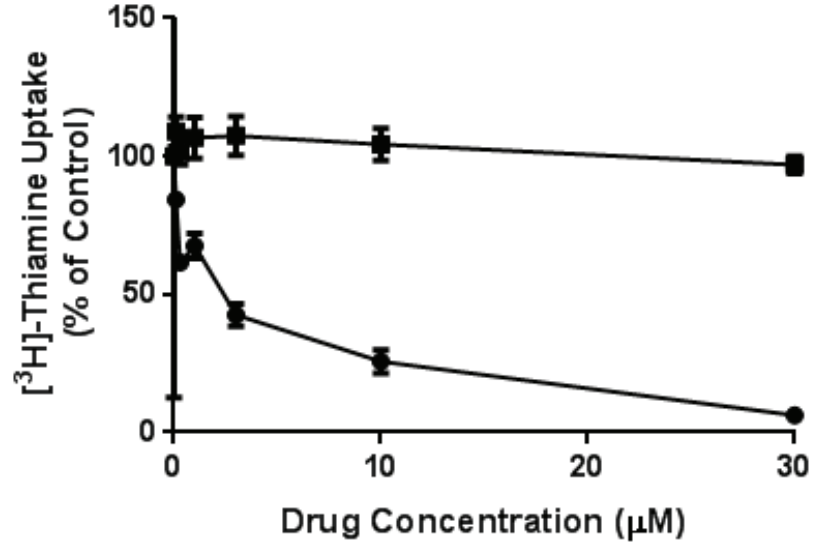


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**Figure 4**

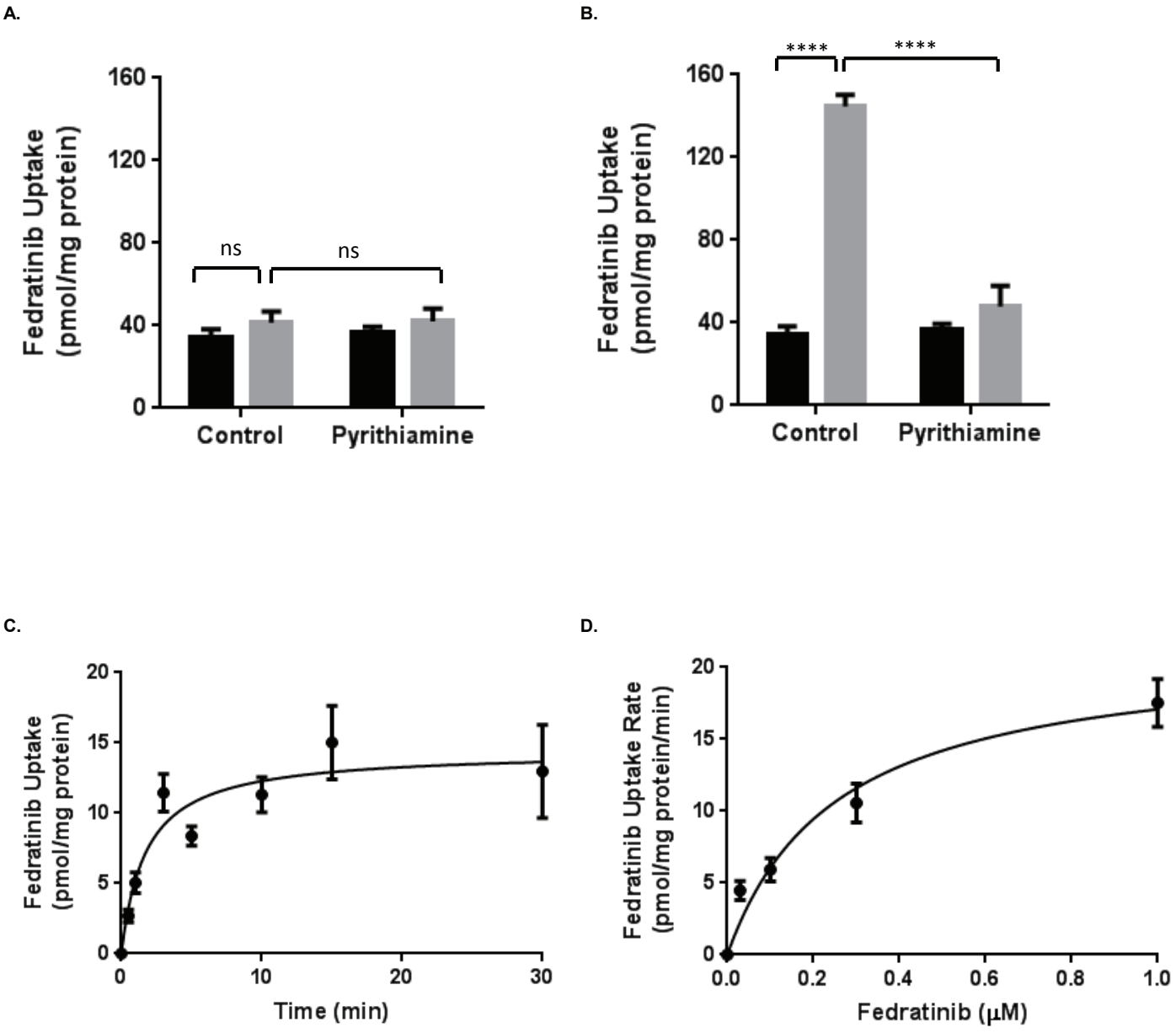

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Figure 5

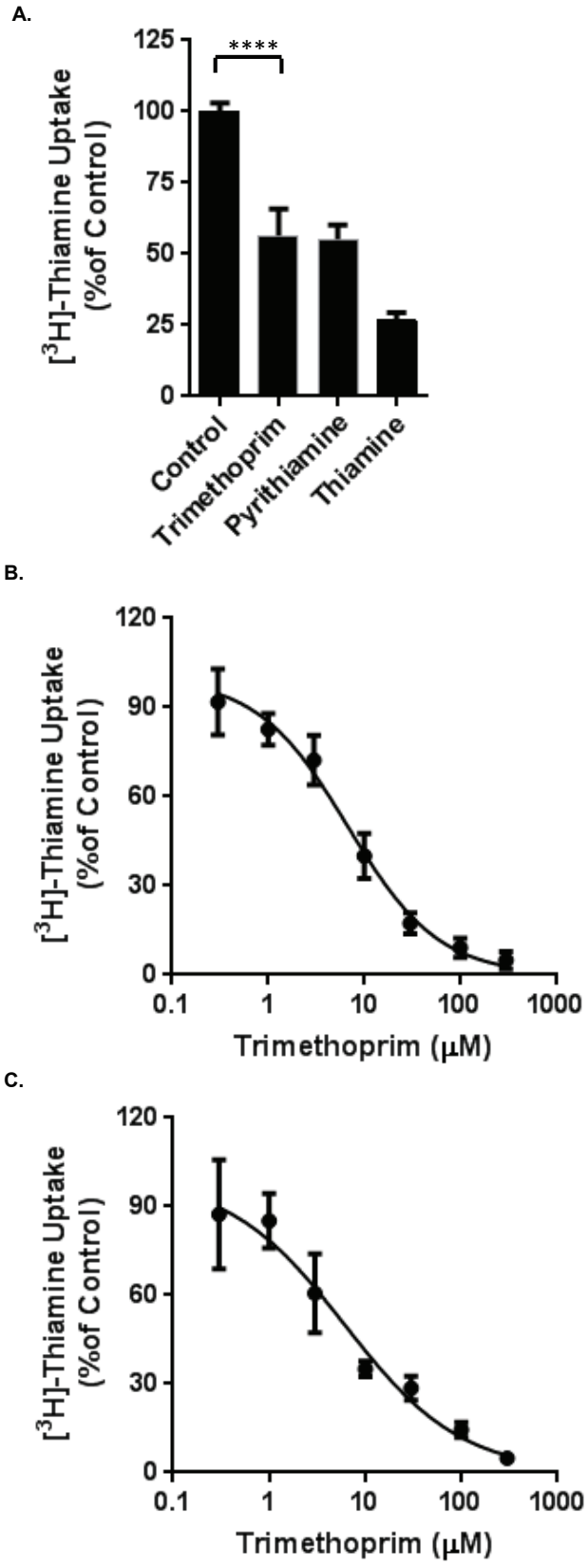

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Figure 6

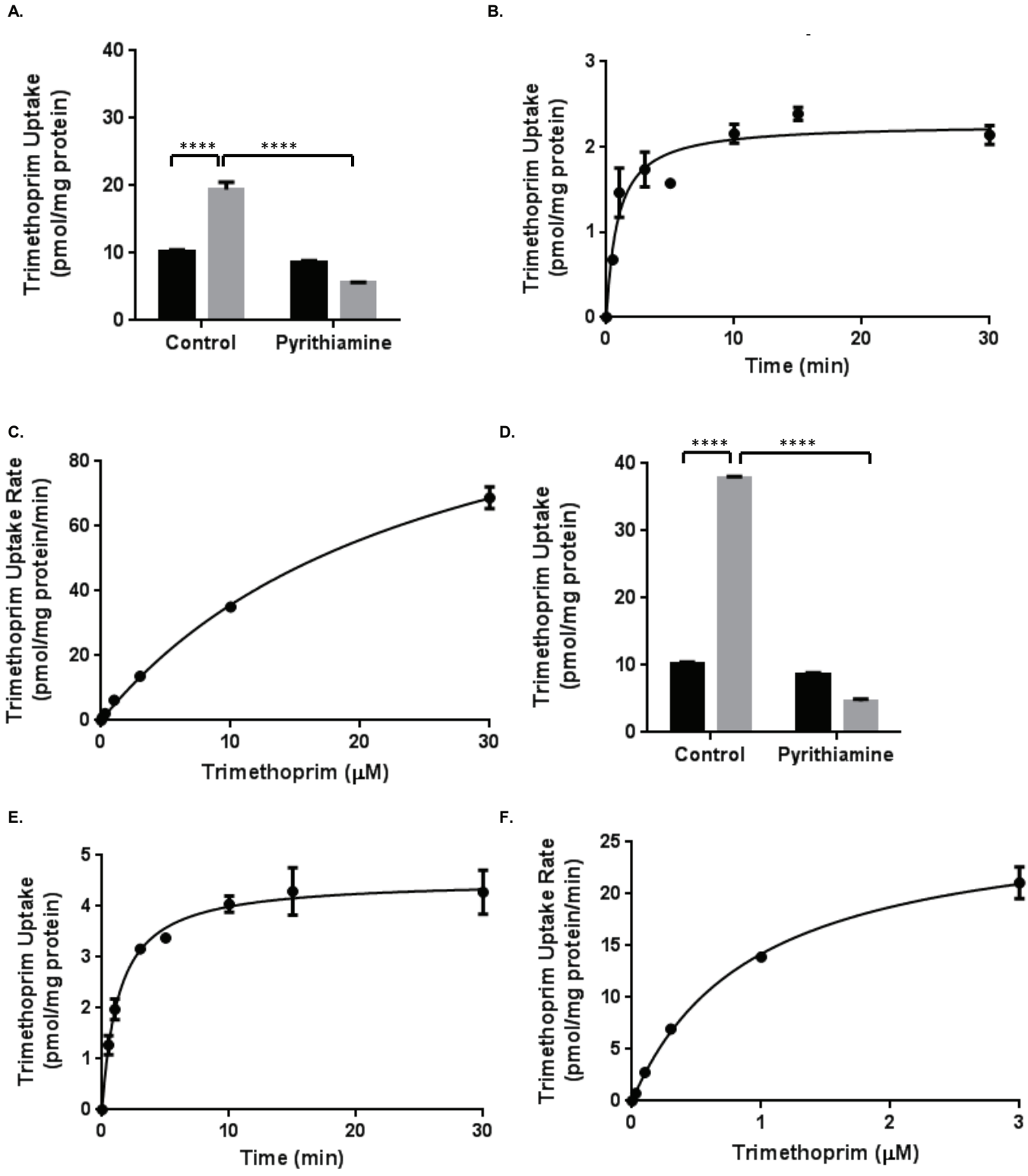

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**Figure 7**