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Influence of Tyrosine Kinase Inhibition on Organic Anion Transporting Polypeptide 1B3-Mediated Uptake[S]

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ABSTRACT

The organic anion transporting polypeptide family member (OATP) 1B3 is a hepatic uptake transporter that has a broad substrate recognition and plays a significant role in regulating elimination of endogenous biomolecules or xenobiotics. OATP1B3 works in tandem with OATP1B1, with which it shares approximately 80% sequence homology and a high degree of substrate overlap. Despite some substrates being recognized solely by OATP1B3, its ability to compensate for loss of OATP1B1-mediated elimination and recognition by regulatory agencies, little is known about OATP1B3 regulatory factors and how they are involved with drug-drug interaction. It was recently discovered that OATP1B1 function is mediated by the activity of a particular tyrosine kinase that is sensitive to a variety of tyrosine kinase inhibitors (TKIs). This study reports that OATP1B3 is similarly regulated, as at least 50% of its activity is reduced by 20 US Food and Drug Administration -approved TKIs. Nilotinib was assessed as the most potent OATP1B3 inhibitor among the investigated TKIs, which can occur at clinically relevant concentrations and acted predominantly through noncompetitive inhibition without impacting membrane expression. Finally, OATP1B3 function was determined to be sensitive to the knockdown of the Lck/Yes novel tyrosine kinase that is sensitive to nilotinib and has been previously implicated in mediating OATP1B1 activity. Collectively, our findings identify tyrosine kinase activity as a major regulator of OATP1B3 function which is sensitive to kinase inhibition. Given that OATP1B1 is similarly regulated, simultaneous disruption of these transporters can have drastic effects on systemic drug concentrations, which would promote adverse events.

SIGNIFICANCE STATEMENT

The organic anion transporting polypeptide family member (OATP) 1B3 is a facilitator of hepatic drug elimination, although much is unknown of how OATP1B3 activity is mediated, or how such regulators contribute to drug-drug interactions. This study reports that OATP1B3 activity is dependent on the Lck/Yes novel tyrosine kinase, which is sensitive to numerous tyrosine kinase inhibitors. These findings provide insight into the occurrence of many clinical drug-drug interactions, and a rationale for future study of tyrosine kinases regulating drug disposition.

Introduction

The organic anion transporting polypeptide transporters (OATPs) recognize a broad range of endogenous substrates, such as bile acids and thyroid hormones, as well as xenobiotics that include statins, anti-diabetic therapeutics, and anti-cancer drugs (Alam et al., 2018; McFeely et al., 2020). Among the OATP family are OATP1B1 and OATP1B3, which share ~80% homology and are highly expressed in hepatocytes, where they mediate the initial step of metabolism or biliary elimination of substrates (Konig et al., 2000; Kalliokoski and Niemi, 2009; McFeely et al., 2019). The pharmacological importance of these transporters is represented by increased systemic concentrations of drug substrates when transport function is lost, which can result in life threatening adverse effects.

The clinical relevance of these transporters, along with their potential as sites of drug-drug interactions (DDIs), has received recognition by regulatory agencies, such as the US Food and Drug Administration (FDA) (Konig et al., 2013;

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ABBREVIATIONS: CCK-8, cholecystokinin 8; DDI, drug-drug interaction; DMEM, Dulbecco's Modified Eagle's Medium; 8-FcA, 8-(2-[Fluoresceinyl]-aminoethylthio)-adenosine-3′, 5′-cyclic-monophosphate; FDA, US Food and Drug Administration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney cells; LC-MS/MS, liquid chromatography tandem mass spectrometry; LYN, Lck/Yes novel tyrosine kinase; OATP, organic anion transporting polypeptide family member; PKC, protein kinase C; TKI, tyrosine kinase inhibitor.

https://www.fda.gov/regulatory-information/search-fda-guidance-documents/vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions). As a result, great effort has been made to document various factors that mediate the transport activity of OATP1B1 and OATP1B3. In addition to DDIs, genetic polymorphisms have also been shown to regulate function of these transporters (Ieiri et al., 2009). Approximately 20% of patients carry reduced functional OATP1B1 and OATP1B3 genetic variants, while individuals with simultaneous genetic deficiency of both transporters are especially rare (van de Steeg et al., 2012; Dhumeaux and Erlinger, 2013; Gong and Kim, 2013; Nies et al., 2013). Although patients deficient of these transporters experience hyperbilirubinemia (van de Steeg et al., 2012), individuals typically lead healthy lives, with the exception of being at significantly high risk for DDIs when administered substrate drugs. For example, increased statin concentrations and high rates of rhabdomyolysis are common with standard dosing regimens in patients deficient of OATP1B activity (Abu Mellal et al., 2019). The same risks exist in patients that are administered different OATP1B1 and OATP1B3 substrates concomitantly with OATP1B inhibitors (Abu Mellal et al., 2019). While the newly published FDA guidelines have provided conditions to investigate OATP1B--dependent DDIs, they have only recently been finalized and are not yet mandatory practices. Therefore, influence of new drugs on OATP1B function is not always considered in drug development, and perhaps more important, there are no current tests performed to measure or predict drug-induced changes to post-translational events that may be necessary for transport function.

Recently, there have been reports of DDIs involving OATP1B substrates and tyrosine kinase inhibitors (TKIs) (Kendra et al., 2015; Martin et al., 2016; Logue et al., 2017; Strumberg et al., 2016; https://www.accessdata.fda.gov/drugsatfda_docs/label/2014/202806s002lbl.pdf), and some TKIs have been identified as OATP1B inhibitors in vitro (Pahwa et al., 2017; Leblanc et al., 2018; Kayesh et al., 2021) . TKIs are a class of drugs used to treat a variety of diseases including cancer, rheumatoid arthritis, and neurologic disorders (Gomez-Puerta and Mócsai, 2013; Gągało et al., 2015; Pottier et al., 2020). These DDIs were unexpected, as no interaction involving metabolism was anticipated. Furthermore, while some TKIs have been identified as weak OATP1B substrates, these interactions were not anticipated to be clinically relevant, especially when concomitantly used with strong recognized substrates, such as statins (Zimmerman et al., 2013). We recently discovered that particular TKIs inhibit OATP1B1-dependent transport noncompetitively through interference of Lck/Yes novel tyrosine kinase (LYN), a Src kinase that is expressed in hepatocytes (Hayden et al., 2021). Although this observation provides partial insight into the mechanism that contributes to TKI-dependent DDIs associated with OATP1B substrates, it remains unknown whether OATP1B3 activity is maintained, or if function is similarly sensitive to tyrosine kinase activity and TKIs. OATP1B3 has been previously reported as a phosphorylated protein (Powell et al., 2014), and given the structural similarities or high degree of functional and substrate overlap with OATP1B1, we hypothesized that OATP1B3 activity is also sensitive to TKIs noncompetitively and regulated by LYN kinase. To test this hypothesis, overexpressing cell lines and primary human hepatocytes were used to measure OATP1B3-mediated uptake in the presence or absence of FDA-approved TKIs. Furthermore, these cells were used for kinetic analysis and genetic knockdown approaches to collectively assess the impact of kinase activity or TKI exposure on OATP1B3 function.

Materials and Methods

Cell Culture and Reagents. Human embryonic kidney (HEK293) cells overexpressing OATP1B3 with a V5-tag (HEK293-OATP1B3) were generated with the PMIG II vector, engineered from a murine stem cell virus (MSCV)-internal ribosome entry site GFP and kindly provided by Dario Vignali (St. Jude Children's Research Hospital, Memphis, TN). These cells were used in a previous study to assess OATP1B3 activity in the presence of nilotinib (Leblanc et al., 2018). Cell culturing was carried out using Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2 and were not cultured beyond 30 passages. Cells were supplemented with 0.2 mg/ml of G418 to isolate cells possessing the PMIG II vector. Phosphate buffered saline and 0.25% trypsin-EDTA were used during cell culture procedures. Cells were seeded with a density of 100,000 cells per well or 400,000 cells per well and were grown until 80–90% confluent. Human plateable hepatocytes (Thermo Fisher Scientific) from a 19 year old female donor (See Supplemental Table 1 for additional donor information) were thawed and cultured at 37°C as previously described (Hayden et al., 2021), with a density of 0.8 $X10^6$ cells per well in 24-well collagen coated tissue culture plates (Thermo Fisher Scientific) and a volume of 0.5 ml per well for 6 hours.

TKIs were purchased from vendors that included Sigma-Aldrich, AstaTech, Inc., AChemBlock, MedChemExpress, LLC, Tocris Bioscience, Selleck Chemical LLC,ArkPharm Inc, and Apexbio Technology LLC (Supplemental Table 2) and dissolved at 10–1 mM in DMSO before further dilutions were prepared in DMEM. All other materials were obtained from Thermo Scientific unless otherwise stated. All solutions were visually assessed for the absence of precipitation before cell exposure. Solubility of nilotinib was confirmed by diluting the concentrated nilotinib solution in DMSO to 10 μM in serum/phenol red-free DMEM and incubation at 37°C for 15, 30, and 60 minutes. Solutions then underwent centrifugation at 13,000 rpm for 5 min at 4°C and the supernatant was analyzed by a validated method using reversed-phase liquid chromatography coupled to tandem mass-spectrometric detection (LC-MS/MS). A Vanquish ultra-high-performance liquid chromatography system coupled with a Quantiva triple quadrupole mass spectrometer from Thermo Fisher Scientific was used for LC-MS/MS analysis. An Accucore aQ column (150 × 2.1 mm, dp = 2.6 μm, Thermo Fisher Scientific) was protected by a C18 AQUASIL guard cartridge (2.1 mm × 10 mm, dp = 3 μm, Thermo Fisher Scientific). The injection volume of sample was 5.0 μl. The temperature of the autosampler was 4°C, and the temperature of the column was maintained at 50°C. Mobile phase A consisted of water with 0.1% (v/v) formic acid and mobile phase B consisted of acetonitrile: methanol (1:1) with 0.1% (v/v) formic acid. The total run time was 4.6 minutes. The gradient conditions were as follows: 0–0.5 minutes, 45% B; 0.5–3.5 minutes, 45%–90% B; 3.5–4.0 minutes, 90% B; 4.0–4.1 minutes, 90% to 45% B; 4.1–4.6 minutes, 45% B with a flow rate of 0.4 ml/min. The MS assay setting with the positive voltage applied to the ESI capillary was set at 4000 V, and the capillary temperatures was 375°C with a vaporizer temperature of 450°C. Argon was used as the collision gas at a pressure of 1.5 mTorr. Precursor molecular ions and product ions were recorded for confirmation and detection of nilotinib (530.162→289.025), using [$^2H_3$]-nilotinib (nilotinib-d3) as an internal standard (533.1→288.94). Results from assay validation studies revealed that the within-day precision and between-day precision ranged 0.61%–3.43%, and the

accuracy ranged 96.9%–103%. The lower limit of quantification was 5 ng/ml.

Transport Activity. OATP1B3 transport activity was measured in vitro using 8-(2-[Fluoresceinyl]-aminoethylthio)-adenosine-3′,5′-cyclic-monophosphate (8-FcA) (5 - 40 μM). OATP1B3-mediated transport in HEK293 cells was assessed in phenol-red free and serum free DMEM during an interval of 15 minutes preincubation at 37°C with various TKIs ranging from 0.1–10 μM, or DMSO as a vehicle control, followed by the aspiration of pretreatment and coincubation with TKIs and 8-FcA for 30 minutes. To assess recovery of OATP1B3 transport function, 8-FcA uptake was measured 15–1500 minutes following exposure to TKIs for 15 minutes. After a 30-minute incubation with 8-FcA, media was aspirated followed by three washes with ice-cold PBS. A plate reader was used to measure fluorescence at an excitation wavelength of 485 nm and emission detection at 535 nm. OATP1B3 activity was determined by normalizing 8-FcA uptake to vehicle treated cells and nonspecific accumulation was accounted for by normalizing fluorescence to nontransfected HEK-293 cells exposed to 8-FcA. All cellular accumulation experiments were conducted using phenol red and serum-free media. Substrate concentrations were then normalized to total protein content which was assessed using the Pierce protein assay (Fisher Scientific), following the supplier's instructions, and measuring absorption at 563 nm.

OATP1B3-mediated uptake was also assessed in human plateable hepatocytes using the cholecystokinin 8 (CCK-8) peptide substrate (Letschert et al., 2005). Uptake was measured using a pre-exposure to nilotinib (4 μM) or vehicle (DMSO) for 15 minutes followed by a coincubation of nilotinib and ^{3}H-CCK-8 (2 μM) for 10 minutes. Assessment of CCK-8 uptake was conducted in the presence or absence of 10% FBS (in both preincubation and coincubation) and at 4°C and 37°C. After the 10-minute exposure to CCK-8 in the above conditions, hepatocytes were washed with ice cold PBS and lysed with NaOH (1N) for 30 minutes. The lysate was neutralized with HCl (2M), after which protein quantification was conducted using the Pierce protein assay, and detection of radioactivity was measured by scintillation counting. OATP1B3 mediated activity was defined as CCK-8 uptake (pmol) at 37°C normalized to protein concentration (mg) subtracted by uptake measured at 4°C.

Protein Analysis and Localization of OATP1B3. HEK293-OATP1B3 cells were exposed to nilotinib (10 μM) for 15 minutes and whole cell lysates were collected using modified radioimmunoprecipitation assay buffer [20 mM of Tris-HCl (pH 7.5) 150 mM of NaCl, 1 mM of Na2 EDTA, 1 mM of EGTA 1% NP-40 1% sodium deoxycholate 2.5 mM of sodium pyrophosphate 1 mM of b-glycerophosphate 1 mM Na_3VO_4, 0.2% protease inhibitor cocktail (Sigma), and 0.1% SDS]. Human liver and hepatocytes lysates were purchased from Novus Biologicals and ScienCell, respectively. Protein levels were assessed using the Pierce protein assay to quantify cellular protein concentrations. Western blot analysis was conducted using Invitrogen Bis-tris gradient mini-gels (Invitrogen), followed by detection with chemiluminescence (Bio-Rad). Primary antibodies recognizing V5, LYN, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling and used at a 1:1,000 dilution. Horseradish peroxidase-conjugated goat anti-rabbit antibody (Cell Signaling) was used as a secondary antibody at a 1:10,000 dilution. Surface expression of biotinylated protein after exposure to nilotinib (10 μM) or vehicle was measured following the manufacturer's instructions (EZ-Link Sulfo-NHS-SS-Biotin; Thermo Fisher Scientific) and as previously outlined (Hayden et al., 2021).

siRNA LYN Knockout Studies. HEK293-OATP1B3 cells were transfected with 12.5 nM siRNA designed to specifically knockdown LYN expression (Life Technologies), using Lipofectamine RNAiMAX reagent (Life Technologies). After 48-hour exposure to siRNA or Lipofectamine alone, OATP1B3 activity was assessed by exposing cells to 8-FcA (25 μM) for 30 minutes as outlined by previous work from our group (Leblanc et al., 2018; Hayden et al., 2021). Whole cell lysates were also collected after the 48-hour exposure to siRNA or Lipofectamine alone to confirm LYN knockdown by western blot analysis.

Mass Spectrometry Analysis of OATP1B3. To assess the phosphorylated tyrosines located within OATP1B3, we isolated the protein by immunoprecipitation using an anti-V5 antibody (Cell Signaling) and measured phosphorylation status of peptides by liquid chromatography-mass spectrometry based analysis, as previously described (Hayden et al., 2021).

Data Analysis. Data are presented as mean ± S.D. from 3 independent experiments unless stated otherwise. Each independent experiment consisted of 3–6 replicates with the exception of experiments with human hepatocytes that were conducted with 2 replicates. Differences between 2 groups were determined using a paired *t* test as part of GraphPad Prism version 8.1.2. *P*-values of < 0.05 were considered significant. To assess the influence of FDA approved TKIs on OATP1B1 activity a mixed effects model was used to account for the correlation that may be present in the technical replicates. The mixed effects model used OATP1B1 activity as determined by 8-FcA uptake modeled as a fixed drug effect (drug or vehicle) and a random intercept effect for biologic replicates. Significance of the drug effect is evaluated by a maximum likelihood test and we use a Bonferroni correction to control the family wise error rate at level 0.05 to account for the multiple testing problem present in evaluating 46 distinct drugs. The lme4 package was used in addition to the R programming language for all computations.(Douglas Bates, 2015; Team, 2020) A multiple linear regression with maximum likelihood estimation was employed with CCK-8 uptake as the response and predictors being the binary indicators of presence status for serum and nilotinib. Significance of variables was assessed by a *t* test of the corresponding coefficients at level 0.05. Computations were conducted using the R programming environment.

Results

Inhibition of OATP1B3 by TKIs. To assess the impact of TKIs on OATP1B3-mediated transport, we measured cellular accumulation of 8-FcA in OATP1B3 overexpressing HEK293 cells, in the presence or absence of TKIs. Solutions prepared with TKIs were assessed as soluble by a lack of precipitate with visual inspection, while solubility of nilotinib at 10 μM after a 30-minute incubation in solution was confirmed by LC-MS/MS. The study revealed that 20 of the 46 FDA-approved TKIs assessed reduced OATP1B3 activity below 50% and eight (imatinib, dabrafenib, bosutinib, ibrutinib, gilteritinib, cabozantinib, lapatinib, and nilotinib) reduced function to 25% or below compared with vehicle, with nilotinib being identified as the most potent inhibitor (Fig. 1, A and B). Meanwhile, many TKIs such ruxolitinib had no effect on transport activity.

Influence of Nilotinib on OATP1B3-Mediated Uptake. After identification of nilotinib as the most potent OATP1B3 inhibitor we assessed the potency and clinical relevance of this interaction (Fig. 2). The concentration of nilotinib at which 50% of transport function was lost (IC_{50}) in our OATP1B3 overexpressing HEK293 cells was 0.68 μM (Fig. 2A). This concentration is below the maximum total nilotinib systemic concentration of ~4 μM reported in FDA and EMA prescribing information (https://www.accessdata.fda.gov/drugsatfda_docs/label/2010/022068s004s005lbl.pdf and https://www.ema.europa.eu/en/documents/scientific-discussion/tasigna-epar-scientific-discussion_en.pdf, respectively), but above the predicted 80 nM of unbound drug present in serum. Nonetheless, the above inhibitory data were confirmed in human hepatocytes using CCK-8, where 4 μM of nilotinib was capable of

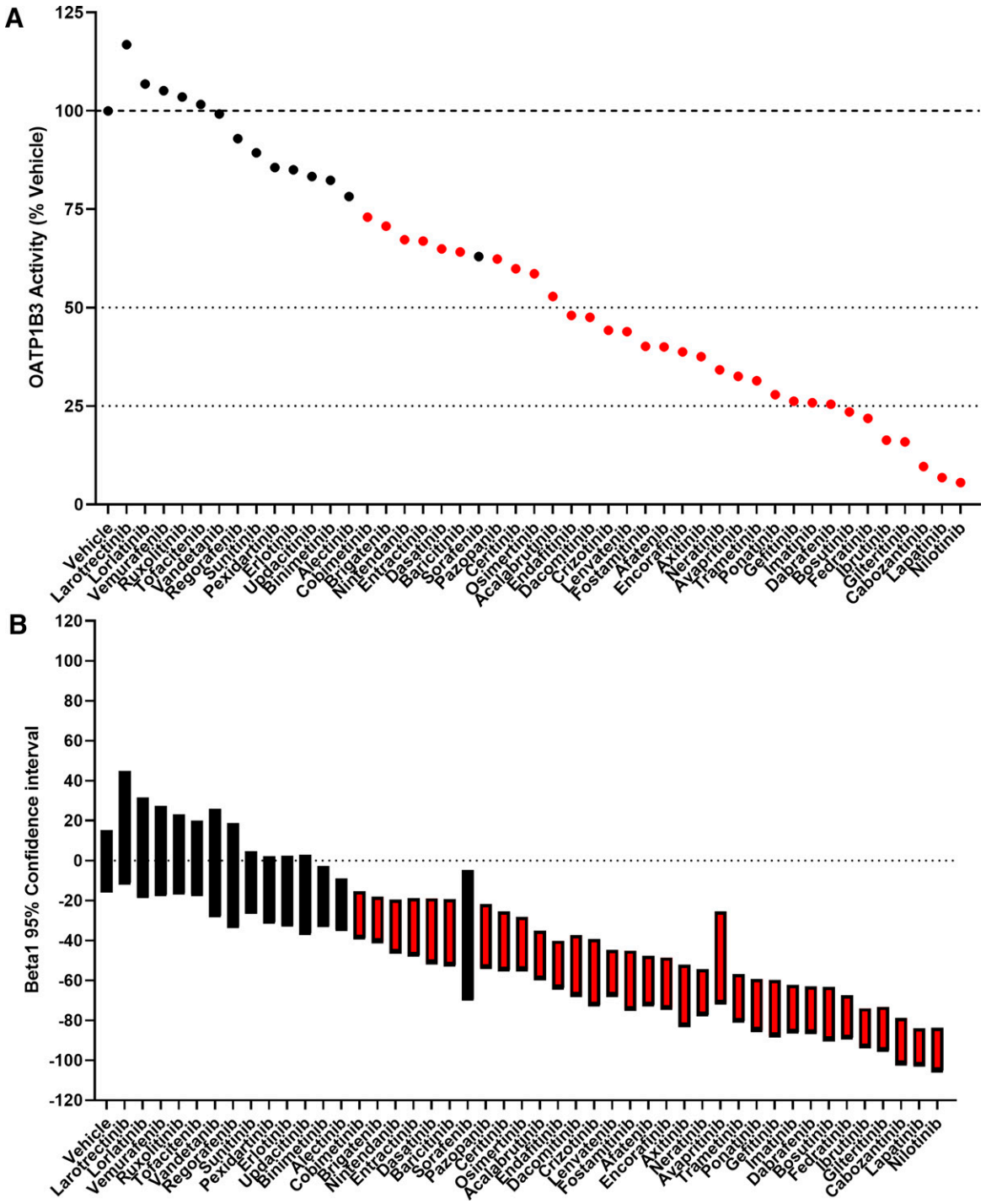

Fig. 1. Influence of TKIs on OATP1B3 activity. (A) Activity of OATP1B3 in overexpressing HEK293 cells as determined by uptake of 8-FcA (25 μM) following a 15-minute preincubation and 30-minute coincubation with various TKIs (10 μM) in serum and phenol red free DMEM. Data are represented by relative transport function in cells exposed to vehicle (DMSO) alone (black dotted line). TKIs that significantly reduced transport function are depicted in red. Values represent predicted mean values from three independent experiments. (B) Beta1 95% confidence intervals associated with the mixed effects model to measure random intercept effect for biologic replicates of TKI impact on OATP1B1 activity as determined by 8-FcA uptake. Significant difference is depicted by samples highlighted in red.

reducing OATP1B3-mediated uptake (Fig. 2B). Addition of serum also reduced CCK-8 uptake in human hepatocytes, which is consistent with cholecystokinin binding to bovine serum albumin (Huang et al., 1995); however, inhibition of CCK-8 uptake was further reduced in the presence of both nilotinib and serum, suggesting that nilotinib-mediated inhibition is likely retained despite protein binding.

A potential limitation to the above results involving uptake of 8-FcA at 30 minutes is that this condition is above the linear range of cellular accumulation (Supplemental Fig. 1), and therefore effects on efflux transporters could be a confounding complication. Further kinetic analysis was performed using varying concentrations of nilotinib and 8-FcA for 10 minutes, where activity remains in the linear range

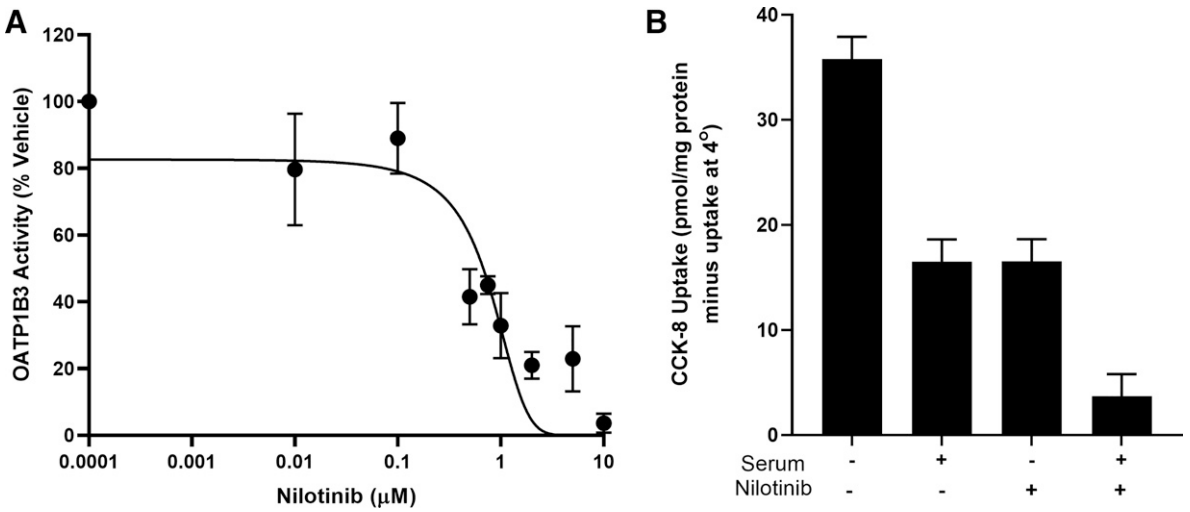

Fig. 2. Potency of OATP1B3 inhibition by nilotinib. (A) Inhibition of OATP1B3 dependent uptake of 8-FcA (25 μM) in overexpressing HEK293 cells by varying concentrations of nilotinib (IC_{50} of OATP1B3 = 0.68 μM). Data are represented by relative OATP1B3 activity compared with exposure of vehicle (DMSO) alone. Values represent mean ± S.D. from three independent experiments. (B) Assessment of CCK-8 (2 μM) uptake in human hepatocytes for 10 minutes in the presence or absence of 5% FBS (serum) and or nilotinib (4 μM) at 37°C normalized to uptake with the same conditions at 4°C. Values represent mean ± S.D. from two independent experiments. Inhibition was assessed using a 15-minute preincubation with nilotinib (+/− 5% FBS) followed by a coincubation of 8-FcA (30 minutes) or CCK-8 (10 minutes) in the presence or absence of nilotinib and or 5% FBS.

(Supplemental Fig. 1) in our OATP1B3 overexpressing HEK293 cells. A Dixon plot from this analysis revealed that nilotinib inhibits OATP1B3 predominantly noncompetitively with intersecting lines at the x-axis and an inhibition constant of 3.3 μM (Fig. 3A). A similar observation was seen when assessing uptake of 8-FcA for 30 minutes (Supplemental Fig. 2). Consistently, preincubation alone with nilotinib was equally effective in reducing OATP1B3-mediated transport as pre- and coincubation with the TKI (Fig. 3B). Activity of OATP1B3 is eventually restored following removal of nilotinib, although full activity is not observed again until 24 hours later (Fig. 3C). Meanwhile, nilotinib had no significant impact on OATP1B3 protein expression or membrane localization (Fig. 3D).

Regulation of OATP1B3 Activity by LYN Kinase. The observations made above are similar to those we previously reported with nilotinib and OATP1B1 (Hayden et al., 2021), where we found that the Src kinase LYN which is expressed in liver tissues can mediate OATP1B1 function. We have since confirmed that LYN expression is detectable in human hepatocytes (Fig. 4, A and B), that OATP1B3 is phosphorylated at multiple tyrosine amino acids (Fig. 5), and that there is a large overlap of inhibitors with OATP1B3. Therefore, we sought to determine whether OATP1B3-mediated transport can be altered when LYN expression is reduced. Using siRNA designed to specifically target LYN, we successfully reduced protein expression of LYN in OATP1B3 overexpressing HEK293 cells after 48 hours compared with mock transfected cells (Fig. 6A). Furthermore, we assessed OATP1B3 activity under these conditions, which revealed that reduced LYN expression led to significantly diminished OATP1B3-dependent transport compared with OATP1B3 overexpressing cells exposed to lipofectamine only (Fig. 6B).

Discussion

The current study provides insight into a potential mechanism associated with DDIs in patients receiving TKIs and expands on the knowledge that Src-kinase activity regulates OATP1B transport by confirming that OATP1B3 function is dependent on LYN expression (Fig. 6) and is inhibited by certain TKIs in vitro (Fig. 1). These findings imply that TKI-mediated DDIs involving OATP1B substrates may be more devastating than originally anticipated, as absence of both OATP1B1 and OATP1B3, which often compensate for one another, would significantly increase the systemic concentrations and risk of adverse events for a variety of drugs (Kendra et al., 2015; Martin et al., 2016; Strumberg et al., 2016; Logue et al., 2017; https://www.accessdata.fda.gov/drugsatfda_docs/label/2014/202806s002lbl.pdf).

Several investigations have shown that many TKIs are potent OATP1B1 inhibitors (Zimmerman et al., 2013; Hu et al., 2014; Khurana et al., 2014; Leblanc et al., 2018; Hayden et al., 2021). Meanwhile OATP1B3 has received less attention, with the exception of reports highlighting the inhibitory effect of dasatinib, nilotinib and vemurafenib on OATP1B3 (Pahwa et al., 2017; Leblanc et al., 2018; Kayesh et al., 2021). However, nilotinib was only assessed at a single high dose concentration,(Leblanc et al., 2018) and dasatinib, which is reported as a low risk contributor to OATP1B-mediated DDIs, was only investigated in overexpressing HEK293 cells and in the absence of serum (Pahwa et al., 2017). Vemurafenib's inhibitory potential was also characterized using overexpressing cells, which revealed no inhibition in the absence of serum but loss of transport activity at a high dose when serum was present (Kayesh et al., 2021). Despite these findings, the mechanism by which these TKIs inhibit OATP1B3 remained unexplored, and the effect of other TKIs on OATP1B3 activity was largely unknown. We observed that 20 FDA approved TKIs reduced OATP1B3 activity by more than 50%, while imatinib, dabrafenib, bosutinib, ibrutinib, gilteritinib, cabozantinib, lapatinib, and nilotinib were considered the most potent inhibitors capable of reducing function by 75% or more (Fig. 1). These findings are highly consistent with previous reports of TKIs impacting

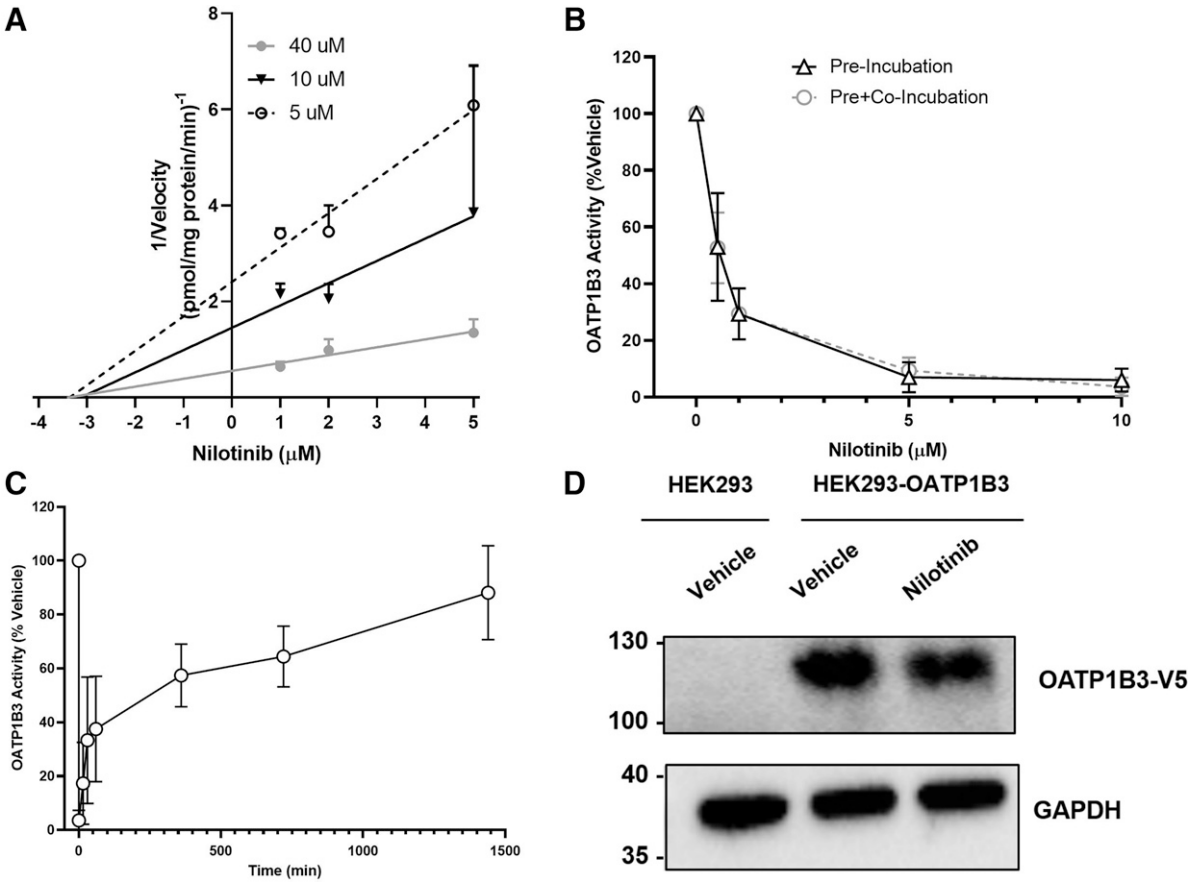

Fig. 3. Kinetic analysis of nilotinib-mediated inhibition of OATP1B3. (A) A Dixon plot of OATP1B3 transport activity with various 8-FcA concentrations in the presence of different concentrations of nilotinib from two independent experiments. Nilotinib exposure was conducted with a 15-minute preincubation followed by a 10-minute coincubation. Intersecting lines at the x-axis indicate noncompetitive inhibition. (B) Influence of nilotinib (0–10 μM) preincubation only or pre- and coincubation with 25 μM of 8-FcA on OATP1B3-dependent transport activity in overexpressing HEK293 cells. Values represent the mean ± S.D. of three independent experiments. (C) Uptake of 8-FcA (25 μM) in OATP1B3 overexpressing HEK293 cells following various time points of removing nilotinib (10 μM). Values represent mean ± S.D. from three independent experiments, and transport was normalized to cells exposed to vehicle (DMSO) alone. (D) Detection of V5-tagged OATP1B3 at the membrane surface using biotinylation or GAPDH from whole cell lysates, extracted from HEK293 cells or OATP1B3-overexpressing HEK293 cells (HEK293-OATP1B3) that were previously exposed to vehicle (DMSO) or nilotinib (10 μM) for 15 minutes.

OATP1B1 activity (Zimmerman et al., 2013; Hu et al., 2014; Leblanc et al., 2018), including nilotinib being the most potent inhibitor among numerous FDA approved TKIs (Hayden et al., 2021). The former study also reveals a substantial similarity of TKIs capable of reducing OATP1B1 and OATP1B3-mediated transport, including dabrafenib, cabozantinib, ibrutinib, gilteritinib, lapatinib, and nilotinib (Hayden et al., 2021), as well as TKIs that have no impact on OATP1B-dependent transport, such as ruxolitinib and lorlatinib. The ability to simultaneously inhibit OATP1B1 and OATP1B3 may provide insight into cases of hyperbilirubinemia outlined in the prescribing information of these drugs, which is an event common when both transporters are inactive (van de Steeg et al., 2012).

Nilotinib was previously identified as a weak OATP1B3 substrate (Zimmerman et al., 2013), although the 1.5-fold increased uptake is below the two-fold cutoff set by the FDA to classify drugs as substrates (US Department of Health and Human Services, FDA). Further investigation is required to assess the impact of OATP transporters on the pharmacokinetics of nilotinib, however, kinetic analysis included in the current study indicates that the mechanism by which nilotinib influences OATP1B3-dependent transport is predominantly noncompetitive with a low IC_{50} (Figs. 2 and 3). Further work is also needed to clarify the mechanism of inhibition, including whether nilotinib or other TKIs can irreversibly bind to OATP1B3. However, many of the TKIs capable of inhibiting OATP1B1 (Hayden et al., 2021) and OATP1B3 (Fig. 1), namely nilotinib, bosutinib, cabozantinib, dabrafenib, ibrutinib, and ponatinib can reduce activity of LYN (Uitdehaag et al., 2014; https://lincs.hms.harvard.edu/db/datasets/20053/), a kinase that we previously reported to be expressed in the liver (Hayden et al., 2021) and recently confirmed is expressed in hepatocytes (Fig. 4). Consistent with these properties, we found that knockdown of LYN expression significantly reduced OATP1B3 function (Fig. 6). While these observations may not be surprising, considering the ~80% homology between OATP1B1 and OATP1B3, the knowledge that suppression of LYN can influence these transporters simultaneously implies that such inhibitors can potentially increase systemic concentrations of coadministered substrates clinically. Future investigation is warranted to confirm this hypothesis.

Although the inhibitors identified in this study are largely consistent with previous work, some discrepancies are noted. Lapatinib was identified as a potent OATP1B3 inhibitor

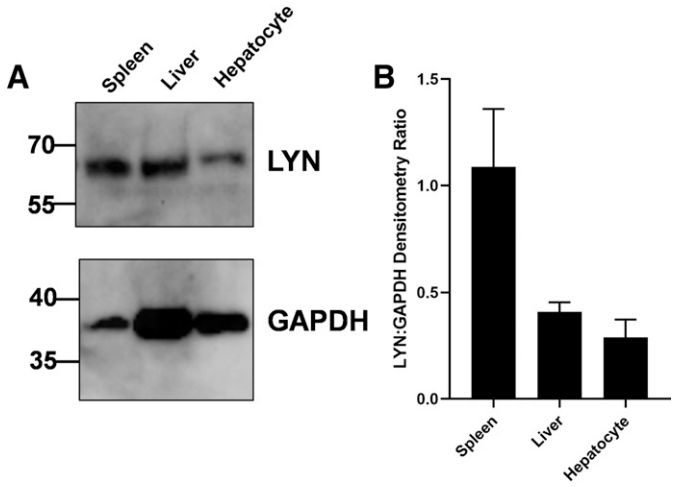

Fig. 4. Expression of LYN kinase or GAPDH in human spleen, liver, and hepatocyte lysates visualized by Western blot analysis (A). Densitometry of Western blot analysis, combined from three independent experiments of LYN kinase normalized to GAPDH expression. (B)

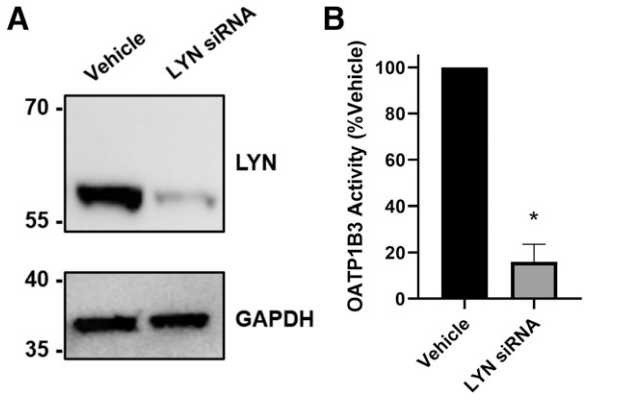

Fig. 6. Regulation of OATP1B3-mediated transport by LYN. (A) Impact of LYN-targeting siRNA (12.5 nM) or vehicle (lipofectamine) on protein expression after 48-hour exposure. (B) Influence of siRNA targeting LYN (48-hour exposure) on OATP1B3-dependent uptake of 8-FcA in OATP1B3-overexpressing HEK293 cells (25 μM 8-FcA for 30 minutes), compared with cells treated with vehicle (lipofectamine) alone. * represents statistical significance as determined by a paired t test ($P < 0.05$). Values represent mean ± S.D. from three independent experiments.

(Fig. 1), while it is unable to influence LYN kinase (Kundu et al., 2013; Uitdehaag et al., 2014). Based on the ability of OATP1B1 to increase intracellular lapatinib concentrations, and its prediction as an OATP1B3 substrate (Kotsampasakou et al., 2015), it is anticipated that lapatinib is acting as a competitive inhibitor at the concentration used in this study. Our findings are also in contrast to Khurana et al., who reported that vandetanib is a more potent OATP1B3 inhibitor than nilotinib (Khurana et al., 2014). This conflict may be attributed to use of different cell models, substrates, or incubation conditions; however, previous work has revealed that inhibition of OATP1B1 by different TKIs is substrate independent, (Leblanc et al., 2018; Hayden et al., 2021) and this may translate to OATP1B3 as well. Nonetheless, another difference between our study and Khurana et al. involves the latter not including preincubation conditions, which we found to be a significant factor in promoting OATP1B inhibition (Hayden et al., 2021). Finally, our findings are also in conflict with vemurafenib being a reported OATP1B3 inhibitor (Kayesh et al., 2021), which may also be due to different substrates and uptake conditions. In fact, Kayesh et al. found that serum was required for vemurafenib to inhibit OATP1B3. Interestingly, we also found that the presence of serum could increase nilotinib-mediated inhibition of OATP1B3 (Fig. 2B). Regardless of the above incongruities, further examination of other OATP1B inhibiting TKIs is required. For example, while we expect that LYN inhibition is the dominant contributor to OATP1B1 and OATP1B3 function, clarification of differences in the ability of TKIs to inhibit these transporters needs to be further investigated. Noticeable differences of TKIs that inhibit OATP1B1 or OATP1B3 activity include imatinib (69.96% versus 25.86%), and ponatinib (64.20% versus 27.88%) (Hayden et al., 2021). These inconsistencies may, however, be the result these TKIs being differentially recognized as substrates between these two transporters (Zimmerman et al., 2013).

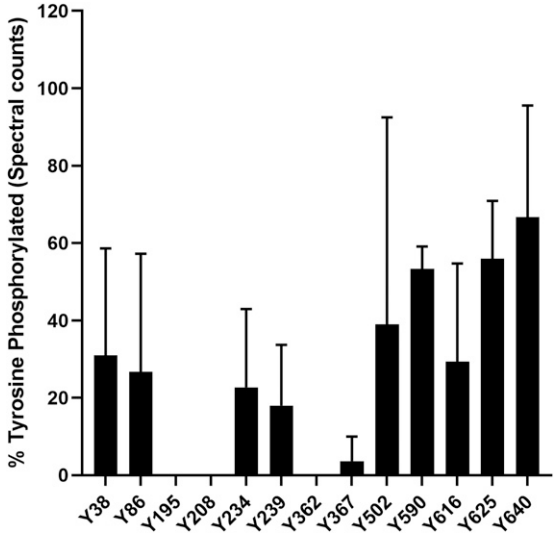

Fig. 5. Phosphorylation of OATP1B3 measured by LC-MS analysis. Data are represented by the percentage of phosphorylated tyrosines (based on spectral counts), located within OATP1B3 peptides that were detected by LC-MS/MS. Values represent mean ± S.D. from three independent experiments.

While the current study provides potential clarity into reported DDIs with TKIs, our findings also provide new questions that should be explored. One in particular involves how the interplay of post-translational modifications collectively mediate OATP1B3 activity. While the exact mechanism is unclear, OATP1B3 function is reportedly reduced by protein kinase C (PKC) activation (Powell et al., 2014). Similar to our observations, manipulation of PKC did not alter OATP1B3 membrane expression or localization (Fig. 3D). However, this mechanism is obviously independent from our findings, considering that nilotinib does not influence PKC at concentrations used in our study (Uitdehaag et al., 2014), and inhibition of tyrosine kinase activity reduces OATP1B3 function, as opposed to transport activation that follows PKC inhibition. Regardless, further insight into how phosphorylation modulates OATP1B3 is required. According to mass spectrometry databases and prediction software, OATP1B3 is phosphorylated at numerous amino acids (Martin Svoboda, 2011; Hornbeck et al., 2015). Previous investigations have confirmed its phosphorylation (Powell et al., 2014), and our study provides evidence that phosphorylation of OATP1B3 is detectable at several tyrosine sites (Fig. 5), including tyrosine 640. We

previously reported that OATP1B1 activity is dependent on tyrosines 640 and 645, which are phosphorylated (Hayden et al., 2021). While tyrosine 645 is not conserved in OATP1B3, tyrosine 640 is located near a predicted LYN recognition sequence and may regulate transport function (Kundu et al., 2013; Kundu et al., 2014). Therefore, further investigation into how nilotinib and LYN regulates OATP1B3 tyrosine phosphorylation is warranted. Future considerations should also include assessment of OATP1B3 phosphorylation in human hepatocytes and manipulation of LYN in humanized mouse models to measure changes in substrate disposition.

In conclusion, the findings outlined in our study identified a novel regulatory mechanism for OATP1B3-dependent transport that involves LYN, a kinase that is sensitive to multiple TKIs. These results indicate that TKIs have the potential to promote adverse events or DDIs through simultaneous inhibition of OATP1B1 and OATP1B3 if sufficient concentrations to inactive LYN are achieved. Moreover, our findings that nilotinib-induced inhibition of OATP1B3 activity is retained in the presence of serum (Fig. 2B) implies that reaching clinically relevant concentrations to inhibit LYN activity is conceivable. While the latter findings are limited to a single hepatocyte donor and will need further verification, the clarity of tyrosine kinase activity regulating solute carrier function provides potential mechanistic insight into various DDIs involving OATP1B substrates and TKIs, along with various cases of hyperbilirubinemia. Furthermore, the knowledge of Src-kinase activity modulating OATP1B3 activity adds to previous reports of Src kinases regulating activity of other solute carriers, including OATP1B1 and organic cation transporter 2 (Sprowl et al., 2016). LYN was also recently shown to regulate the Na+/K+-ATPase pump (Okumu et al., 2020). Therefore, the current study collectively lends support to the role of Src kinases as regulators of endogenous and xenobiotic uptake or disposition which should be further investigated in vivo and in clinical studies.

Authorship Contributions

Participated in research design: Sprowl.

Conducted experiments: Hove, Anderson, Hayden, Pasquariello, Gibson, Shen, Jin.

Performed data analysis: Hove, Anderson, Hayden, Pasquariello, Gibson, Shen, Qu, Jin, Miecznikowski, Hu, Sprowl.

Wrote or contributed to the writing of the manuscript: Hove, Anderson, Sprowl.

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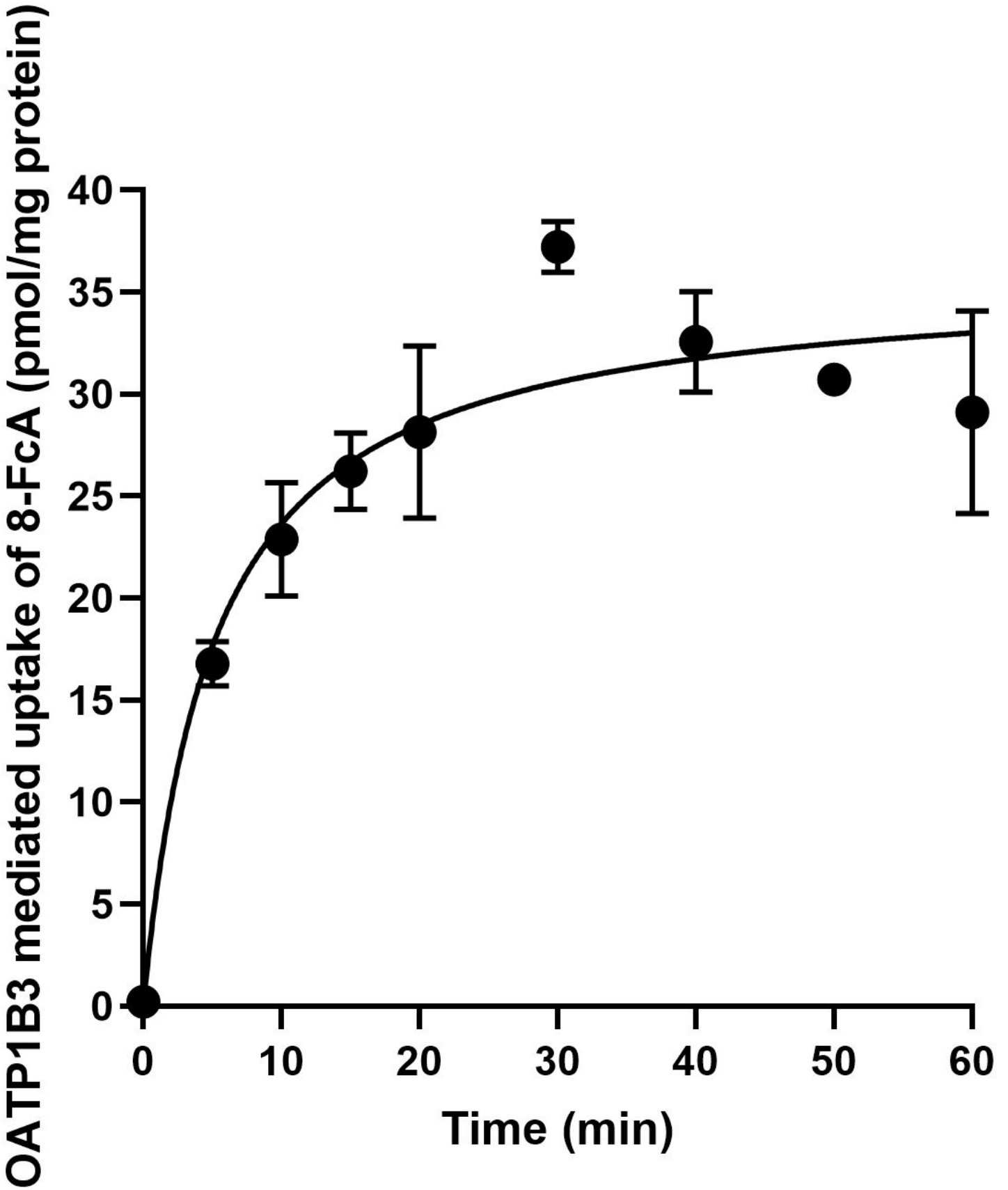
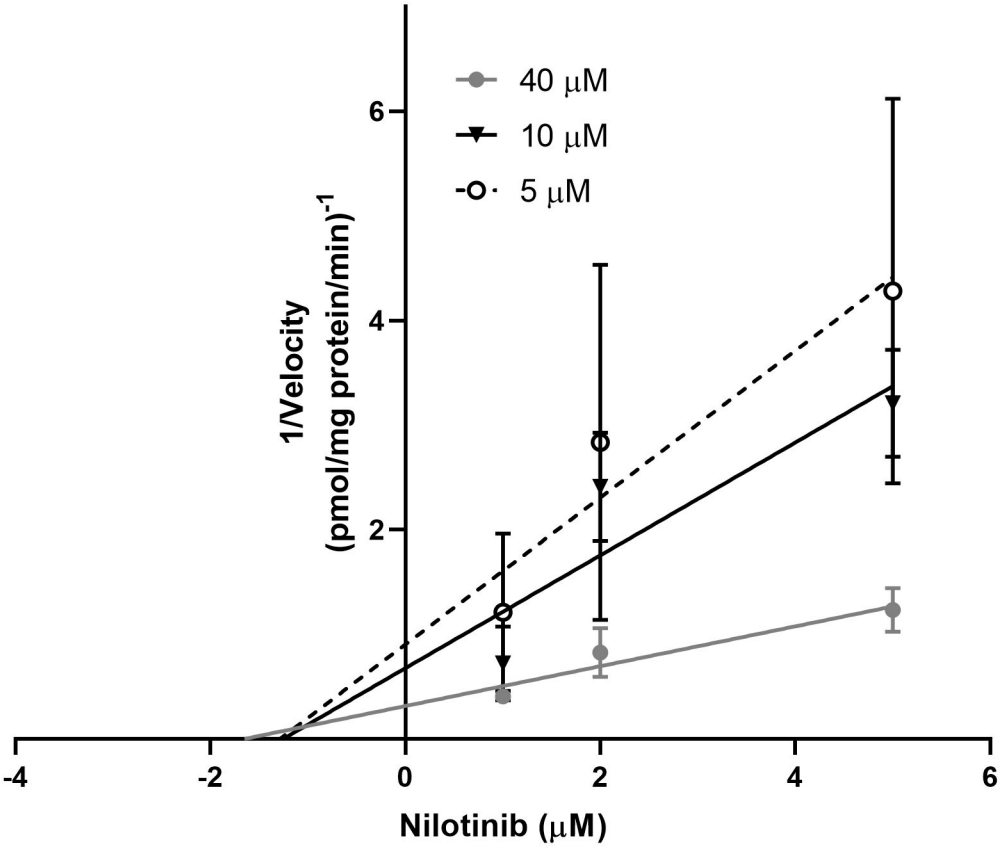
Supplemental Fig. 1



Supplemental Fig. 2



Supplemental Materials

Influence of Tyrosine Kinase Inhibition on OATP1B3-mediated Uptake

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Supplementary Figure Legends

Supplemental Fig. 1. OATP1B3-mediated uptake of 8-FcA (25 μM) in overexpressing HEK293 cells at various time points. Data is represented by intracellular pmol of 8-FcA and normalized to protein (mg). Values represent mean ± SD from three independent experiments.

Supplemental Fig. 2. Kinetic analysis of nilotinib-mediated inhibition of OATP1B3. A Dixon plot of OATP1B3 transport activity with various 8-FcA concentrations in the presence of different concentrations of nilotinib from three independent experiments. Nilotinib exposure was conducted with a 15-minute pre-incubation followed by a 30-minute co-incubation. Intersecting lines at the x-axis indicate non-competitive inhibition.

Supplemental Table 1. Human hepatocyte donor information

Gender	Race	Age (years)	BMI	Tobacco History	Alcohol History	Drug History	Cause of Death	Other
Female	Caucasian	19	20.4	No	No	Cannabis (unknown amount)	Asphyxiation	Hepatitis B (-) Hepatitis C (-) HIV1&2 (-) HTLV 1&2 (-) CMV (-)

Body mass index (BMI); Negative detection by PCR (-); Human immunodeficiency virus (HIV); Human T-lymphotropic virus (HTLV); cytomegalovirus (CMV)

Supplemental Table 2. Supplier, solubility (in DMSO) and stock concentrations of tyrosine kinase inhibitors prepared in DMSO utilized for further dilutions throughout the study.

Tyrosine Kinase Inhibitor	Vendor	DMSO Solubility (mM)	Concentration prepared in DMSO (10 mM)
Afatenib	Sigma-Aldrich	139.44	10
Acalabrutinib	AstaTech Inc	199.78	10
Alectinib	AChemBlock	6.22	1
Avapritinib	Medchemexpress LLC	200.58	10
Axitinib	Tocris Bioscience	67.28	10
Baricitinib	AstaTech Inc	199.24	10
Bosutinib	Selleck Chemical LLC	188.52	10
Binimetinib	Medchemexpress LLC	199.44	10
Brigatinib	Selleck Chemical LLC	5.14	1
Cabozantinib	AChemBlock	199.40	10
Ceritinib	Ark Pharm Inc	7.17	1
Cobimetinib	Ark Pharm Inc	188.21	10
Crizotinib	Selleck Chemical LLC	51.07	10
Dabrafenib	Apexbio Technology LLC	192.47	10
Dacomitinib	Medchemexpress LLC	104.27	10
Dasatinib	Ark Pharm Inc	200.82	10
Encorafinib	Medchemexpress LLC	185.18	10
Erdafitinib	Medchemexpress LLC	199.31	10
Entractinib	Apexbio Technology LLC	178.37	10
Erlotinib	Apexbio Technology LLC	198.25	10
Fostamitinib	Apexbio Technology LLC	199.84	10
Fedratinib	Medchemexpress LLC	190.59	10
Gefitinib	Ark Pharm Inc	199.15	10
Gilteritinib	Medchemexpress LLC	18.09	10
Ibrutinib	Tocris Bioscience	199.77	10
Imatinib	Sigma-Aldrich	200.57	10
Lapatinib	Apexbio Technology LLC	172.10	10
Larotrectinib	Selleck Chemical LLC	200.73	10
Lenvatenib	Ark Pharm Inc	93.71	10
Lorlatinib	Medchemexpress LLC	199.31	10
Neratinib	Ark Pharm Inc	10.77	10
Nilotinib*	Sigma-Aldrich	66.10	10
Nintendanib	Sigma-Aldrich	16.68	10
Osimertinib	Medchemexpress LLC	198.15	10
Pazopanib	Sigma-Aldrich	201.13	10
Pexidartinib	Medchemexpress LLC	198.65	10
Ponatinib	Selleck Chemical LLC	187.77	10
Regorafenib	Apexbio Technology LLC	198.83	10
Ruxolitinib	Ark Pharm Inc	199.11	10
Sorafenib	Tocris Bioscience	200.08	10
Sunitinib	Sigma-Aldrich	62.74	10
Tofacitenib	Sigma-Aldrich	198.48	10

Trametinib	Apexbio Technology LLC	162.50	10
Upadacitinib	Medchemexpress LLC	199.81	10
Vandetanib	Sigma-Aldrich	79.94	10
Vemurafenib	Medchemexpress LLC	197.99	10