Received: 18 May 2022 | Revised: 20 January 2023 | Accepted: 1 February 2023

DOI: 10.1111/cts.13504

ARTICLE

Preclinical assessment of drug–drug interaction of fb-PMT, a novel anti-cancer thyrointegrin αvβ3 antagonist

Kazutoshi Fujioka[1,†] | Mostafa I. Fekry[2,3] | Jeanne M. Rumsey[2] | Toni Steiner[2] | David Thompson[2] | Shaker A. Mousa[1]

[1]NanoPharmaceuticals LLC, Rensselaer, New York, USA

[2]Inotiv, Maryland Heights, Missouri, USA

[3]Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt

Correspondence
Shaker A. Mousa, NanoPharmaceuticals LLC, 1 Discovery Drive, Rensselaer, NY 12144, USA.
Email: shaker.mousa@gmail.com

Abstract

The objective of the current study was to identify potential drug–drug interactions (DDIs) with the drug candidate fb-PMT, a novel anticancer thyrointegrin αvß3 antagonist. This was accomplished by using several in vitro assays to study interactions of fb-PMT with both cytochrome P450 (CYP) enzymes and drug transporters, two common mechanisms leading to adverse drug effects. In vitro experiments showed that fb-PMT exhibited weak reversible inhibition of CYP2C19 and CYP3A4. In addition, fb-PMT did not show time-dependent inhibition with any of the seven CYP isoforms tested, including 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4. Human liver microsomal incubations demonstrated that fb-PMT is stable. Potential transporter-mediated DDIs with fb-PMT were assessed with two ATP binding cassette (ABC) family transporters (P-glycoprotein and breast cancer resistance protein) using Caco2 cells and seven solute carrier family (SLC) transporters (organic cation transporter OCT2, organic anion transporters OAT1 and OAT3, organic anion transporter peptides OATP1B1 and OATP1B3, and the multidrug and toxic extrusion proteins MATE1 and MATE2-K using transfected HEK293 cells). Fb-PMT was not a substrate for any of the nine transporters tested in this study, nor did it inhibit the activity of seven of the transporters tested. However, fb-PMT inhibited the uptake of rosuvastatin by both OATP1B1 and OATP1B3 with half-maximal inhibitory concentrations greater than 3 and less than 10 μM. In summary, data suggest that the systemic administration of fb-PMT is unlikely to lead to DDIs through CYP enzymes or ABC and SLC transporters in humans.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

We did not have any information of the effects of our lead clinical candidate fb-PMT (NP751) on CYP enzymes or transporters that are involved in the different

[†]Deceased.

absorption, distribution, metabolism, and excretion aspects of other concomitants drugs.

WHAT QUESTION DID THIS STUDY ADDRESS?

Fb-PMT was not a substrate for any of the nine transporters tested in this study, nor did it inhibit the activity of seven of the transporters tested. However, fb-PMT inhibited the uptake of rosuvastatin by both OATP1B1 and OATP1B3 with half-maximal inhibitory concentrations (IC_{50s}) less than 10 μM. In summary, data suggest that the systemic administration of fb-PMT is unlikely to lead to drug–drug interactions (DDIs) through CYP enzymes or ABC and SLC transporter in humans.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

Fb-PMT was not a substrate for any of the nine transporters tested in this study, nor did it inhibit the activity of seven of the transporters tested along with little effects on CYP enzymes. However, fb-PMT inhibited the uptake of rosuvastatin by both OATP1B1 and OATP1B3 with IC_{50s} greater than 3–10 μM.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Data suggest that the systemic administration of fb-PMT is unlikely to lead to DDIs through CYP enzymes or ABC and SLC transporters in humans.

INTRODUCTION

Drug-polymer conjugates have been developed for many applications in pharmaceuticals, including anticancer drugs.[1,2] Drug-polymer conjugates are one category of polymer-based therapeutics, such as antibody-polymer conjugates and antibody-drug conjugates.[3] Polymer conjugates of small-molecule drugs have several advantages compared to small-molecule drugs.[4,5] The improved pharmacokinetic parameters include prolonged elimination half-life and enhanced permeation and retention effects that enable us to optimize anticancer therapeutics by enhancement of pharmaceutical activity and reduction of off-target toxicity. We developed fb-PMT as a targeted anticancer drug-polymer conjugate with a high efficacy toward multiple cancers.[6] The pharmacodynamic and pharmacokinetic properties of PEG-TAT conjugated molecules are attributed to the optimized chemical structure of this drug-polymer conjugate.[7–10]

Drug–drug interactions (DDIs) are problematic when a drug candidate enters into clinical applications because they may affect pharmacokinetic and pharmacodynamic properties of other pharmaceuticals administered at the same time.[11] Preclinical assessment of DDIs is, therefore, important to minimize potential adverse effects of drug candidates when they are used in the clinical setting.[12] For example, cytotoxic anticancer drugs, such as platinum complexes and taxanes, have a narrow therapeutic window in which a toxic concentration (TC_{50}) is close to the effective concentration (half-maximal inhibitory concentration [EC_{50}]). If a drug affects the metabolism and distribution of a co-administered, highly toxic anticancer drug, the concentration of the latter may increase above the TC_{50}, resulting in serious adverse effects in the treated patients.[13]

Preclinical evaluation of such drug interactions is critical to avoid the problematic outcome in the translational development of drugs. Cytochrome p450 (CYP) enzymes are essential and important in the metabolism of xenobiotics in mammals, including humans. Most anticancer drugs are hydrophobic in nature and undergo phase I metabolic transformation by CYPs to yield more hydrophilic, and sometimes reactive metabolites.[14] In vitro assays to analyze the inhibitory effects of drugs on CYPs are useful to predict whether a drug candidate has the potential to have a clinically relevant DDI.[15] There are two different common mechanisms to assess DDIs. The first involves metabolism assays evaluating whether the drug is a substrate or inhibitor of the major CYPs involved in metabolic clearance from the liver and whether the drug is capable of CYP induction. CYP reaction phenotyping is a common approach for identifying the enzymes responsible for the metabolism of a drug. In this study, the experimental approach used for CYP reaction phenotyping involved incubations in human liver microsomes (HLMs) in the presence or absence of CYP-specific chemical inhibitors. Furthermore, the ability of the drug to inhibit the major human CYP isoforms was evaluated by assessing potential for time-dependent inhibition (TDI)

using probe substrates. This approach allows the effects of both the parent and metabolites of a drug to be evaluated for any inhibitory effects. CYP induction potential is typically conducted in human hepatocytes by measuring increases in metabolic activity or mRNA expression of specific CYP isoforms in treated cells relative to vehicle control.

The second common mechanism to assess DDIs involves measuring whether the drug is a substrate or inhibitor of drug transporters that are known to be involved in clinical adverse reactions. Drug transporters are involved in the movement of endogenous and exogenous substrates across cell membranes. The inhibition of transporters results in the disrupted homeostasis of those substrates, including anticancer therapeutics. These assays are typically carried out in transfected cell lines expressing the transporter of interest, or in Caco-2 cell lines. Therefore, the result of in vitro CYP reaction phenotyping, CYP inhibition, CYP induction, and transporter assays are useful and insightful to assess the DDI potential of tested drugs in vivo.[16,17]

The objective of this study was to evaluate the DDI potential of our newly developed anticancer drug-polymer conjugate, fb-PMT, using both in silico and in vitro approaches. We initially used the ADMET Predictor software to predict potential CYP metabolites of fb-PMT and the potential to inhibit various CYP isoforms. We hypothesized that although there may be potential CYP metabolites of fb-PMT, either fb-PMT or CYP metabolites of fb-PMT do not show DDI because fb-PMT contains a large fraction of PEG (partial molecular weight: ~1600), which may prevent the access of a fb-PMT molecule into the CYP active sites. We subsequently carried out in vitro CYP reaction phenotyping, CYP inhibition, TDI, and transporter assays. For the CYP assays we evaluated seven isoforms (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) in HLM for the phenotyping and inhibition studies, whereas the CYP induction studies were carried out in cultured human hepatocytes. These assays evaluated the effects of fb-PMT on the expression of CYPs 1A2, 2B6, and 3A4 using mRNA expression levels as the end point. Finally, we conducted in vitro assays to determine whether fb-PMT is a substrate or inhibitor of the following ABC and SLC transporters: P-gp, BCRP, MATE1, MATE2-K, OCT2, OAT1, OAT3, OATP1B1, and OATP1B3. Two different cell lines were used for these experiments: Caco-2 and transfected HEK293 cells.

MATERIALS AND METHODS

Materials

Fb-PMT (NP751) was synthesized by a contract manufacturing organization (DALTON Pharma Services). Pooled (10 donor) HLMs and individual lots of cryopreserved human hepatocytes were purchased from BioIVT. Probe substrates, metabolites, and inhibitors for the CYP reaction phenotyping, CYP inhibition, and drug transporter studies were obtained from Sigma-Aldrich, except as noted otherwise. Cell culture reagents, including fetal bovine serum (FBS), buffers, salts, and other research chemicals were purchased from Sigma-Aldrich or other local suppliers. Dimethyl sulfoxide was obtained from Alpha Aesar. Solvents and internal standards for liquid chromatography tandem mass spectrometry (LC–MS/MS) analyses were obtained from ThermoFisher Scientific or Sigma Aldrich. Caco-2 cells were ordered from Adari Cell Science and shipped pre-plated in 24-well plates (Caco Ready Kit, KRECE-CCR03). HEK293 cells transfected with individual SLC transporters (TranSelect) were obtained as cryopreserved vials from Solvo Biotechnology.

Methods

Six types of studies were carried out to assess the DDI potential of fb-PMT (Table 1). The methods and materials used for each type of study are described below. All in vitro studies were carried out at the Maryland Heights, MO, laboratory of Inotiv, a CRO headquartered in West Lafayette, IN, USA.

In silico CYP-mediated metabolism/inhibition

Potential CYP-mediated metabolism of fb-PMT and possible inhibition of CYP enzymes were assessed in silico using the ADMET Predictor software program, version 9.4 (Simulations Plus). This software generates a list of potential metabolites of the parent compound as well as estimates the likelihood of the parent or metabolite(s) to inhibit individual CYP enzymes.

CYP reaction phenotyping

The extent of fb-PMT metabolism by seven CYP isoforms (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) was determined by a substrate depletion assay in HLM, measuring the concentration of parent compound remaining at various timepoints over the incubation period in the presence and absence of isoform-specific inhibitors. Fb-PMT was prepared at 10 mM in 80:20 methanol: DMSO. A working solution was prepared from this stock at 50× incubation concentration in 25:75 acetonitrile: water to obtain a final incubation concentration of 1 μM fb-PMT. The reaction

TABLE 1 Summary of DDI studies conducted with fb-PMT.

Study	Platform	CYPs or transporters evaluated
In silico CYP-mediated metabolism/inhibition	ADMET Predictor	In silico prediction of potential CYP-mediated metabolism or inhibition
CYP reaction phenotyping	Human liver microsomes	CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4
CYP inhibition/time-dependent inhibition	Human liver microsomes	CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4
CYP induction	Human hepatocytes	CYP1A2, CYP2B6, and CYP3A4
ABC transporter substrate/inhibition	Caco-2 cells	P-gp and BCRP
SLC transporter substrate/inhibition	Transfected HEK293 cells	MATE1, MATE2-k, OATP1B1, OATP1B3, OAT1, OAT3, and OCT2

Note: Six types of DDI assays were carried out in the current study. These include one in silico and five in vitro assays. The platform (in silico software, liver microsomes, hepatocytes and other cell types) used for each study type are given as are the individual CYPs or transporters evaluated in each type of assay.

Abbreviation: DDI, drug-drug interaction.

phenotyping incubation mixtures contained fb-PMT (1 μM), HLM (0.5 mg/mL), $MgCl_2$ (3 mM), potassium phosphate buffer (100 mM, pH 7.4), ± NADPH (2 mM), and ± an isoform-selective inhibitor in a total volume of 500 μL. The incubations were carried out for 30 min at 37°C with 50 μL aliquots taken at timepoints of 0, 3, 7, 12, 20, and 30 min. The final organic solvent concentration was less than or equal to 0.5% (v/v). Separate incubations with probe substrate/inhibitor pairs for each CYP isoform were carried out in parallel to confirm the activity of each CYP isoform in the HLMs used and the activity of the selective inhibitors used. The probe substrates, metabolites, and inhibitors used to measure CYP activities are listed in Table 2. At each timepoint, the 50 μL aliquot was quenched with 150 μL of cold acetonitrile containing internal standard (tolbutamide), then vortexed and centrifuged at 5000 *g* for 10 min. One hundred μL of the supernatants were transferred to another plate with 100 μL water in preparation for LC–MS/MS analysis.

CYP inhibition

The ability of fb-PMT to inhibit the same seven CYP isoforms tested above was evaluated in two types of assays. The first measured CYP inhibition with fb-PMT and probe substrates added at the same time, whereas the second assay format included a 30-min pre-incubation with fb-PMT prior to addition of the probe substrate. The pre-incubation period allows for metabolism to take place and detects whether any inhibitory metabolites are formed. The second assay is referred to as TDI or alternatively as an IC_{50} shift assay.[18,19] The probe substrates and inhibitors used for both the CYP inhibition and TDI assays are shown in Table 2. Note that two probe substrates were used for the CYP3A4 reactions (midazolam and testosterone). The concentrations of fb-PMT tested in the CYP inhibition assays ranged from 0.1 to 100 μM. The CYP inhibition reaction mixtures contained HLM (0.1 or 0.5 mg/mL, depending on CYP isoform), CYP probe substrate, $MgCl_2$ (3.3 mM), potassium phosphate buffer (100 mM, pH 7.4), and NADPH (2 mM) in a total volume of 100 μL. The probe substrate concentrations were near the Km values for both the CYP inhibition and IC_{50} shift assays.[18,19] The final organic solvent concentration was less than 1.5% (v/v). Incubations for CYPs 1A2, 2B6, 2C8, and 2C19 used 0.5 mg/mL HLMs, whereas the other isoforms used 0.1 mg/mL The incubation time varied depending on the isoform – 90 min (CYP2C19) 30 min (CYP1A2, 2B6, and 2C8), and 15 min (CYP2C9, 2D6, and 3A4). The incubations were started by the addition of NADPH. After incubation, the 100 μL incubation mixture was quenched with 100 μL of cold acetonitrile, then vortexed and centrifuged at 3000 rpm for 10 min. One hundred μL of the

TABLE 2 Probe substrates, metabolites, and inhibitors used for CYP phenotyping and inhibition studies.

CYP isoform	Probe substrate	Probe metabolite	Probe inhibitor
CYP1A2	Phenacetin (50 μM)	Acetaminophen	Furafylline (10 μM)
CYP2B6	Bupropion (80 μM)	Hydroxy-Bupropion	PPP[a] (30 μM)
CYP2C8	Amodiaquine (3 μM)	N-Desethyl Amodiaquine	Montelukast (0.2 μM)
CYP2C9	Diclofenac (4 μM)	4′-Hydroxy-Diclofenac	Sulfaphenazole (10 μM)
CYP2C19	S-Mephenytoin (100 μM)	4′-Hydroxy-Mephenytoin	Omeprazole (100 μM)
CYP2D6	Dextromethorphan (10 μM)	Dextrorphan	Quinidine (1 μM)
CYP3A4	Midazolam (3 μM)	1′-Hydroxy-Midazolam	Ketoconazole (1 μM)
CYP3A4	Testosterone (50 μM)	6β-Hydroxy-Testosterone	Ketoconazole (1 μM)

Note: The probe substrates (with final assay concentration), metabolites analyzed by LC–MS/MS, and probe inhibitors (with final assay concentration) used for each of the CYP phenotyping and CYP inhibition assays are listed above.

Abbreviation: LC-MS/MS, liquid chromatography-tandem accurate mass spectrometry.

[a]PPP refers to 2-phenyl-2-(1-piperidinyl)propane.

supernatants were transferred to another plate with 100 μL water in preparation for LC–MS/MS analysis.

Time-dependent inhibition

The TDI assays were performed in an identical manner to the above CYP inhibition assays except for a pre-incubation period in the presence of the substrate (fb-PMT). Furafylline, fluoxetine, and verapamil were used as positive controls to illustrate IC_{50} shift values for CYP1A2, 2C19, and 3A4. The pre-incubations were carried out for 30 min at 37°C in the absence and presence of NADPH (2 mM). Following the pre-incubation period, the appropriate probe CYP substrate was added, and the reaction was initiated by the addition of NADPH. At the end of the incubation period, the samples were processed as described above for LC–MS/MS analysis. A fold shift of greater than 1.5 is considered a significant shift and the compound is classified as a TDI.

CYP induction

The ability of fb-PMT to induce the expression of CYP enzymes 1A2, 2B6, and 3A4 was assessed in cryopreserved primary human hepatocytes. Three separate lots of hepatocytes were exposed to a six-point concentration curve of fb-PMT, ranging from 0.412 to 100 μM using half-log serial dilutions. Following 48-h exposure to fb-PMT, the hepatocytes in each well were lysed with 400 μL of MR1 lysis buffer containing TCEP (tris(2-carboxyethyl)phosphine) as a reducing agent (Macherey-Nagel). The lysates were transferred to Eppendorf tubes and spun at 11,000 *g* for 1 min. The cleared supernatants (350 μL) were subsequently transferred to deep well plates. NucleoMag beads and MR2 Buffer were added to each of the samples which were then loaded onto a Kingfisher Flex instrument (Thermo Fisher Scientific) for isolation of total RNA. Finally, isolated RNA samples were quantified using a Qubit Flex Fluorometer (Thermo Fisher Scientific) and diluted to a final concentration of 20 ng/μL. The reverse transcription polymerase chain reaction (RT-PCR) reactions were run on an ABI QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific). RT-PCR primer pairs and Taqman probe sets for each of the target genes were obtained from Integrated DNA Technologies. The data were normalized to the expression level of the reference gene cyclophilin A and then compared to vehicle (DMSO) control. Positive control inducers for the three CYP isoforms were omeprazole (1A2, 50 μM), phenobarbital (2B6, 1000 μM), and rifampin (3A4, 50 μM). Criteria for a positive response include greater than or equal to two-fold increase in mRNA expression relative to vehicle control and a concentration-dependent response.

ABC transporter substrate/inhibition

The ABC transporters include P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). The ability of fb-PMT to act as a substrate or inhibitor of P-gp

and BCRP was measured in Caco-2 cells. Following receipt of the Caco-2 plates (24-well Transwell), the cell medium used for shipping was changed to standard Caco-2 cell culture medium (DMEM, 10% FBS, 1% glutamine, and 1% penicillin–streptomycin). The cells were put into a humidified incubator at 37°C in 5% CO_2 until they reached the equivalent of day 21 of cell culture (full differentiation). The integrity of the cell monolayers was checked by measuring transepithelial electrical resistance (TEER) prior to their use in the assays. The TEER values for cells used in this study were greater than 1000 Ω cm^2.

ABC transporter substrate assay

On the day of the study, Caco-2 cell monolayers were washed and then preincubated for 10–30 min with transport buffer (HBSS with 25 mM glucose and 10 mM HEPES, pH 7.4). Test compounds were diluted from DMSO or aqueous stocks and added to either the apical (A) or basolateral (B) chambers in duplicate, whereas fresh transport buffer alone was placed in the receiver chambers. Fb-PMT was tested at three concentrations (1, 3, and 10 μM). For one concentration (3 μM), an inhibitor of P-gp (valspodar) or BCRP (Ko143) was also added. Digoxin and teriflunomide were used as positive control substrates for the assay. Plates were incubated for 2 h, at which time aliquots for analyte quantitation were removed from each chamber and processed for LC–MS/MS. The LC–MS/MS data were used to calculate apparent permeability (Papp) values. The formula used to calculate Papp was $Papp = (dQ/dt)/(A*C_0)$, where dQ/dt is the change in moles per incubation period (nmol/sec), A is the active membrane area (cm^2), and C_0 is the initial donor side concentration. An efflux ratio (ER) was also calculated from the Papp values in both directions according to the formula: ER = Papp (B to A)/Papp (A to B). An ER greater than or equal to two indicates that the compound is a substrate.

ABC transporter inhibition assay

The protocol for the inhibition assays was similar to the uptake assays. On the day of the study, cell monolayers were washed and then pre-incubated for 10–30 min with transport buffer (HBSS with 25 mM glucose and 10 mM HEPES, pH 7.4) in both chambers. Reference inhibitors (valspodar or Ko143) or fb-PMT were diluted from DMSO or aqueous stocks and added to the appropriate wells (both the apical [A] and basolateral [B] chambers) in triplicate and incubated for an additional 30 min. The final DMSO concentration was 0.6%. A six-point concentration range (0.412–100 μM) of fb-PMT was tested in the assay. The assay was initiated by the addition of probe substrate (digoxin or teriflunomide) to the B chamber only and plates were incubated for 2 h, at which time aliquots for analyte quantitation were removed from each chamber and processed for LC–MS/MS.

SLC transporter substrate/inhibition

The SLC transporters implicated in potential DDIs include OAT1, OAT3, OATP1B1, OATP1B3, OCT2, MATE1, and MATE2-K. These transporters function as uptake proteins and are primarily located on the basolateral or canalicular membranes of the liver and kidney. The ability of fb-PMT to act as a substrate or inhibitor of each of these seven SLC transporters was evaluated in transfected HEK293 cells. One day prior to the assay, cryopreserved cells were thawed and seeded on 0.1 mg/mL poly-D-lysine coated 24-well plates at greater than or equal to 300,000 cells/well. Cells were cultured for 24 h in complete medium containing 10% FBS + 0.5% penicillin–streptomycin. A media supplement was added to transporter-expressing cells (0.3 μL supplement per mL of medium) as suggested by the cell line manufacturer. After 24 h, the media was removed and either HBSS or Krebs–Henseleit Buffer (for MATE1 or MATE2-K-expressing cells) was added and incubated at 37°C with gentle shaking for 10 min. For each transporter tested, an identical plate was run using mock transfected cells to determine nonspecific uptake. The probe substrates and concentrations used for the SLC transporter assays were 10 μM rosuvastatin (OATP1B1 and OATP1B3), 10 μM *p*-amino hippuric acid (OAT1), 5 μM estrone 3-sulfate (OAT3), 10 μM MPP^+ (OCT2), and 5 μM metformin (MATE1 and MATE2-K). The reference inhibitors and concentrations used were 1 μM cyclosporin A (OATP1B1 and OATP1B3), 10 μM probenecid (OAT1 and OAT3), and 10 μM cimetidine (MATE1, MATE2-K, and OCT2).

SLC transporter substrate assays

Triplicate reactions were run for each of three concentrations of fb-PMT (1, 3 and 10 μM). In addition, the 3 μM concentration was run with a known inhibitor for each transporter. When present, the inhibitor was incubated for 30 min prior to the start of the assay. For each transporter cell line, fb-PMT or the appropriate substrate was added to the media to start the assay and incubated for 10 min. Ice cold buffer was added at the end of the

incubation and the cells washed three times. Methanol (250 μL) containing internal standard was added to each well to process the samples for evaluation of compound concentrations by LC–MS/MS. RIPA buffer was added to separate (duplicate) wells for protein quantitation using a Qubit Fluorometer. A concentration-dependent increase in uptake that is greater than or equal to two-fold relative to the mock cells at each concentration tested plus reduced uptake in the presence of inhibitor is considered evidence that the test article is a substrate.

SLC transporter inhibition assays

The protocol for the inhibition assays was similar to the uptake assays. Cells were incubated with inhibitors for 30 min prior to substrate addition. A six-point concentration range (0.412–100 μM) of fb-PMT was tested in these assays. Once the substrate was added, cells were incubated for 10 min at 37°C and stopped by the addition of ice-cold buffer and washed three times. Sample prep for LC–MS/MS analysis followed the steps outlined above for the uptake assays.

Bioanalysis by LC–MS/MS

The quantitation of fb-PMT was achieved through liquid chromatography-electrospray ionization-tandem mass spectrometry using a triple-quadrupole mass spectrometer (AB Sciex API-5500) coupled to a liquid chromatography system (AB Sciex Exion LC AD). Analytes were analyzed by gradient elution using a reversed-phase stationary phase (Phenomenex Synergi Polar-RP 2.1 × 50 mm, 4 μm; Phenomenex) maintained at 45°C. Mobile phase (MP) A consisted of 10 mM ammonium formate in 90:10:0.1 water: acetonitrile; formic acid (v/v); and mobile phase (MP) B consisted of 10 mM ammonium formate in 10:30:60:0.1 water: acetonitrile: isopropanol: formic acid (v/v). The initial solvent composition of 40% B was maintained for 0.5 min followed by a linear increase in MP B to 100% over 3 min. The gradient was maintained at 100% B for an additional 1.5 min, with the solvent composition being returned to 40% B over 0.01 min. A final re-equilibration period of 1 min resulted in a total run time per sample of 5.5 min. High-performance liquid chromatography flow rate was 0.8 mL/min; source temperature was 600°C; and mass spectral analyses were performed using Turbo-Ion spray source in positive (5500 V spray voltage). Analysis of other positive control substances used in this study was achieved using the same instrumentation with methods developed internally or obtained from the literature.[7,20]

RESULTS

Computational simulation of fb-PMT metabolism with P450 enzymes

Using the ADMET predictor software, the potential metabolism, and inhibitory properties of fb-PMT (Figure 1a) with selected CYP isoforms are shown in Table 3. The software predicted that fb-PMT can inhibit CYP1A2, CYP2C9, and CYP3A4. It predicted that fb-PMT can be a substrate of CYP2C8; and more than 100 metabolites can be produced by CYP2C8. The majority of the predicted metabolites are hydroxylation of PEG36 and the break down compounds after hydrolysis, such as fb-PEG and HO-PMT (Figure 1b). Other than those, tetrac, des-tetrac-fb-PMT, fluorophenyl aldehyde, des-fluorobenzyl-fb-PMT, and some hydroxylated compounds are predicted to be produced by CYP2C8 metabolism (Figure 1b).

Reaction phenotyping

The contribution of seven human CYP enzymes (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) to the in vitro metabolism of fb-PMT was evaluated at 1 μM in HLM with NADPH in the presence and absence of specific CYP enzyme inhibitors. The disappearance of fb-PMT was monitored over six timepoints up to 30 min to assess NADPH-dependent metabolism. The results from these experiments indicate that fb-PMT is stable and does not show measurable clearance in HLM under the incubation conditions used in this study (Table 4). Therefore, the impact of the specific CYP inhibitors tested was not calculable. CYP probe substrates were utilized in separate incubations as positive controls to demonstrate the formation of selective CYP metabolites from each of the seven CYP probe substrates as well as inhibition by an isoform-selective inhibitor. The fraction metabolized ($F_{m,CYP}$) for the probe substrates ranged from 0.701 to 1.0 indicating good activity for each isoform (data not shown).

CYP inhibition assays

Reversible and time-dependent inhibition (or IC_{50} shift) of the same seven P450 enzymes (1A2, 2B6, 2C8, 2C9 2C19, 2D6, and 3A4 isoforms) in HLM by fb-PMT were studied using eight probe substrates. Both midazolam and testosterone were used as CYP3A4 substrates. The results of the CYP inhibition assays are shown in Tables 5 and 6. Fb-PMT showed weak reversible inhibition of CYP2C19-dependent metabolism of S-mephenytoin with an IC_{50} of 78.4 μM. Modest inhibition of CYP3A4-dependent testosterone

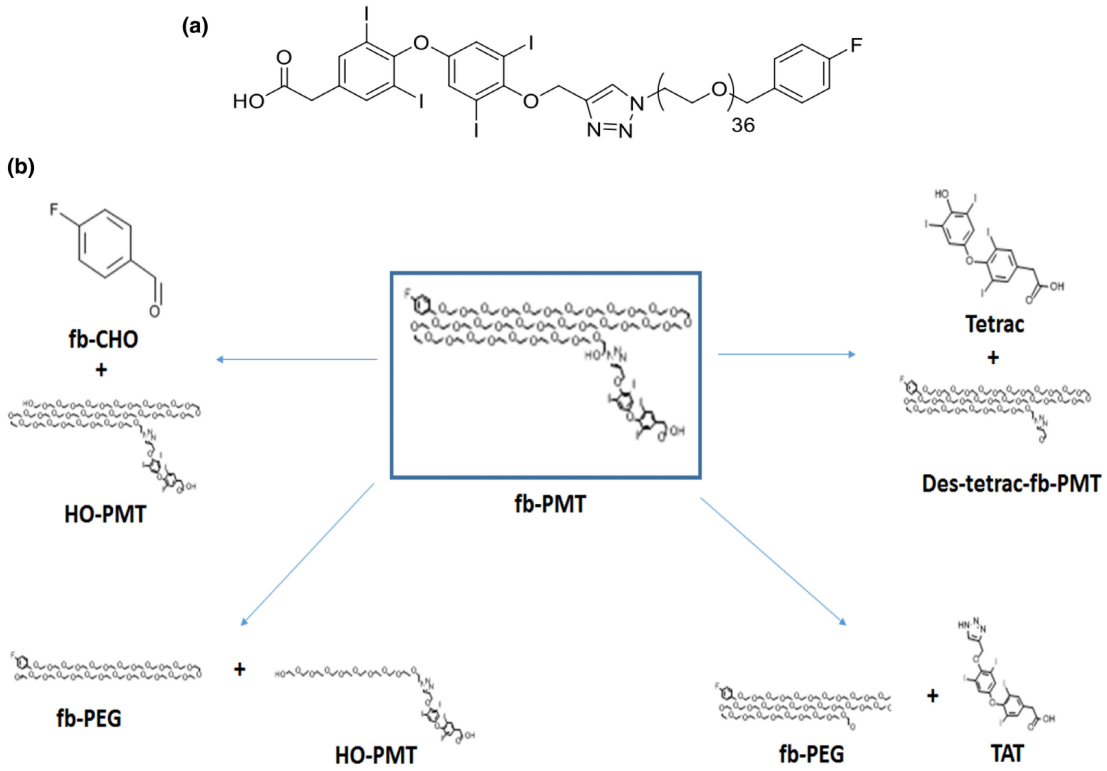

FIGURE 1 (a) Chemical structure of fb-PMT and (b) Selected metabolites of fb-PMT predicted by the computer simulation.

TABLE 3 Computational prediction of fb-PMT metabolism with CYP enzymes.

CYP isoform parameter	Prediction for fb-PMT
CYP1A2_Inhibition	Yes
CYP2C8_Substrate	Yes (77%)
CYP2C9_Inhibition	Yes
CYP2C19_Inhibition	No
CYP2D6_Inhibition	No
CYP3A4_Inhibition	Yes
CYP3A4_Inhibition _ Midazolam	Yes (88%)
CYP3A4_Inhibition _ Testosterone	No (78%)
CYP3A4_Ki_Midazolam	4.223
CYP3A4_Ki_Testosterone	0.001

Note: Fb-PMT was analyzed by the ADMET Predictor software program for its potential to act as a substrate or inhibitor of several CYP isoforms. Fb-PMT was suggested to be a substrate of CYP2C8 and to inhibit several CYPs, including 1A2, 2C9, and 3A4. The CYP3A4 inhibition was evaluated with both midazolam and testosterone as substrates.

metabolism was also observed, but the IC_{50} was greater than 100 μM. No effect of fb-PMT was observed with midazolam, however. Fb-PMT had no observable inhibitory effect on the other CYP isoforms tested (Table 5).

Similarly, in the TDI assays, a weak inhibitory effect was observed only for CYP3A4-dependent testosterone oxidation. The IC_{50} values without and with NADPH were 51.7 and 80.1 μM, respectively; resulting in an IC_{50} shift of less than one (0.6). This demonstrates that there is no NADPH-dependent increase in inhibitory potency for fb-PMT on CYP3A4. Due to a lack of inhibition on the other CYPs. no IC_{50} shifts for fb-PMT could be calculated (Table 6). The data for CYP3A4-dependent metabolism of testosterone are also shown in Figure 2 for both fb-PMT (no effect) and verapamil (positive IC_{50} shift).

CYP induction

The ability of fb-PMT to induce CYP1A2, CYP2B6, and CYP3A4 was assessed in three individual lots of human hepatocytes. Induction was measured using mRNA as the end point following 48 h exposure to the test compounds. Fold-induction values versus vehicle control for all concentrations of fb-PMT tested were less than or equal to 1.25 across all three lots of hepatocytes. Data from a representative lot is shown in Table 7. These data demonstrates that fb-PMT has little potential to induce the major human CYPs.

ABC transporter assays

Fb-PMT was tested as a potential substrate of human P-gp and BCRP-mediated transport in Caco-2 cells. There was

TABLE 4 Fb-PMT (1 μM) mean percent remaining (30 min) and fraction metabolized in human liver microsomes ± CYP inhibition.

CYP isoform	Inhibitor	% Remaining		Estimated $f_{m,CYP}$ in HLM[b]
		Mean	SD	
–	No inhibitor	102	22	–
1A2	Furafylline	96.1	23.2	NC
2B6	PPP[a]	108	19	NC
2C8	Montelukast	110	20	NC
2C9	Sulfaphenazole	110	18	NC
2C19	Omeprazole	97.8	16	NC
2D6	Quinidine	94.6	15.4	NC
3A4	Ketoconazole	101	2	NC

Note: Fb-PMT was incubated with HLM for 30 min and the overall metabolism was determined to be negligible. In the presence of specific inhibitors for each of the seven CYPs tested, no significant metabolism could be detected. The compound was considered stable under the reaction conditions used in this assay.

Abbreviation: HLM, human liver microsome.

[a]PPP refers to 2-phenyl-2-(1-piperidinyl)propane.

[b]fm, CYP refers to the estimated fraction metabolized (calculated by comparison of the clearance of the no inhibitor control to the clearance with CYP inhibitor), whereas NC indicates that the result could not be calculated due to the lack of measurable clearance with or without inhibitors.

TABLE 5 IC_{50s} of fb-PMT and positive controls (specific reversible CYP inhibitors) following incubation in HLM.

CYP isoform	Substrate	Inhibitor	IC_{50} (μM)
CYP1A2	Phenacetin	Furafylline	0.986
		Fb-PMT	–
CYP2B6	Bupropion	PPP[a]	10.2
		Fb-PMT	–
CYP2C8	Amodiaquine	Montelukast	2.40
		Fb-PMT	–
CYP2C9	Diclofenac	Sulfaphenazole	0.196
		Fb-PMT	–
CYP2C19	S-Mephenytoin	Omeprazole	0.662
		Fb-PMT	78.4
CYP2D6	Diclofenac	Quinidine	0.0964
		Fb-PMT	–
CYP3A4	Midazolam	Ketoconazole	0.0554
		Fb-PMT	–
	Testosterone	Ketoconazole	0.0794
		Fb-PMT	>100

Note: CYP inhibition studies were carried out in HLM for each of the seven isoforms shown in the table. Inhibitors (fb-PMT or isoform-specific inhibitors) were pre-incubated with the reaction mixtures for 30 min prior to the start of the reactions (addition of 2 mM NADPH). Formation of metabolites from each probe substrate were measured by LC–MS/MS and IC_{50s} were calculated where indicated. Each known inhibitor blocked the formation of metabolite from the probe substrate as expected. However, fb-PMT had only a modest effect on CYP2C19 and CYP3A4 (testosterone) with IC_{50s} above 75 μM.

Abbreviations: HLM, human liver microsome; IC_{50}, half-maximal inhibitory concentration; LC-MS/MS, liquid chromatography-tandem accurate mass spectrometry.

[a]PPP refers to 2-phenyl-2-(1-piperidinyl)propane.

no evidence for fb-PMT acting as a substrate for either P-gp or BCRP (Table 8). The positive controls (digoxin for P-gp, and teriflunomide for BCRP) both demonstrated net transport in the basolateral to apical (B to A) direction as expected. Digoxin gave an efflux ratio of 11.5, whereas in the presence of the selective P-gp inhibitor, valspodar, the

TABLE 6 IC_{50} shift of fb-PMT and positive controls (irreversible CYP inhibitors) following incubation in HLM.

CYP isoform	Substrate	Inhibitor	IC_{50} (μM) −NADPH	IC_{50} (μM) +NADPH	IC_{50} shift
CYP1A2	Phenacetin	Furafylline	1.26	0.084	15.0
		Fb-PMT	>100	>100	NC
CYP2B6	Bupropion	Fb-PMT	>100	>100	NC
CYP2C8	Amodiaquine	Fb-PMT	>100	>100	NC
CYP2C9	Diclofenac	Fb-PMT	>100	>100	NC
CYP2C19	S-Mephenytoin	Fluoxetine	64	19.8	3.2
		Fb-PMT	>100	>100	NC
CYP2D6	Diclofenac	Fb-PMT	>100	>100	NC
CYP3A4	Midazolam	Verapamil	55.3	1.43	38.7
		Fb-PMT	>100	>100	NC
	Testosterone	Verapamil	128	0.984	130.1
		Fb-PMT	51.7	80.1	0.6

Abbreviations: HLM, human liver microsome; IC_{50}, half-maximal inhibitory concentration; NC, not calculable; TDI, time-dependent inhibition.

Note: IC_{50} shift assays (or TDI) were performed to determine whether inhibitory metabolites of fb-PMT are formed that are capable of inhibiting any of the CYP isoforms under evaluation. The incubations were carried out for 30 min in the presence or absence of 2 mM NADPH. Three known TDIs were included as positive controls to demonstrate an IC50 shift (Furafylline for CYP1A2, Fluoxetine for CYP 2C19, and Verapamil for CYP3A4, midazolam, and testosterone). These three compounds showed IC_{50} shifts of 15, 3.2, 38.7, and 130.1, respectively. In contrast, Fb-PMT only showed inhibition for CYP3A4 and the shift in IC_{50} was <1, indicating no TDI occurred.

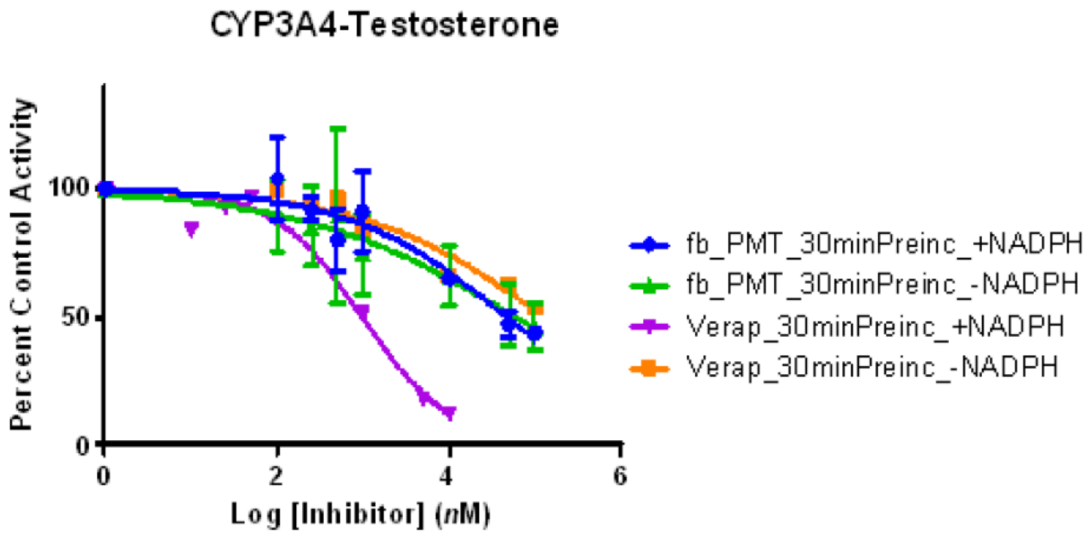

FIGURE 2 Time-dependent inhibition (TDI; IC_{50} Shift) of CYP3A4 in HLM by fb-PMT using testosterone as a probe. TDI of CYP3A4-mediated metabolism of testosterone by fb-PMT and verapamil (positive control). Incubations were carried out in the presence and absence of 2 mM NADPH. There was no apparent shift in IC_{50} for fb-PMT while there is a significant shift for verapamil in the presence of NADPH. HLM, human liver microsome; IC_{50}, half-maximal inhibitory concentration; TDI, time-dependent inhibition.

efflux ratio was reduced to unity (1.08). Similarly, the efflux ratio for teriflunomide was 6.06 in the absence and less than one (0.681) in the presence of Ko143, a selective inhibitor of BCRP. In contrast, fb-PMT showed low permeability in both directions, and at each concentration tested gave an efflux ratio of less than one, demonstrating there was no interaction with either P-gp or BCRP. In addition, neither valspodar nor Ko143 had any effect on the Papp value of 3 μM fb-PMT.

Fb-PMT was also tested as a potential inhibitor of human P-gp and BCRP activity in Caco-2 cell monolayers using digoxin or teriflunomide as a reference substrate. None of the six concentrations of fb-PMT caused concentration-dependent inhibition of digoxin or

TABLE 7 CYP induction data.

Sample ID	Fold induction		
	CYP1A2	CYP2B6	CYP3A4
Vehicle control	1.01	1.01	1.00
0.412 μM fb-PMT	0.932	0.943	0.864
1.23 μM fb-PMT	1.07	0.940	0.957
3.7 μM fb-PMT	1.09	0.831	0.943
11.1 μM fb-PMT	1.03	0.698	0.858
33.3 μM fb-PMT	0.941	0.605	0.842
100 μM fb-PMT	1.14	0.547	0.786
50 μM Omeprazole	60.7	–	–
1000 μM PB	–	12.2	–
50 μM Rifampin	–	–	9.40

Note: Three separate lots of cultured human hepatocytes were exposed to a six-point concentration range of Fb-PMT (0.412–100 μM) for 48 h and mRNA extracted for assessment of possible induction of CYP1A2, CYP2B6, and CYP3A4 genes. Gene expression was quantified by reverse transcription polymerase chain reaction (RT-PCR) and expressed as fold induction relative to vehicle (DMSO) control. Cyclophilin A was used to normalize mRNA levels prior to analysis. Results from one lot of human hepatocytes are shown in the table. No evidence of induction was observed in this lot or any lot of hepatocytes tested with fb-PMT. Omeprazole, phenobarbital (PB), and rifampin were used as positive controls.

teriflunomide uptake (Figure 3a,b). Therefore, fb-PMT is not an inhibitor of either P-gp or BCRP. The probe substrates digoxin and teriflunomide showed efficient transport in the B to A direction, which was reduced by valspodar or Ko143, respectively.

SLC transporter assays

The results of the OATP1B1 and OATP1B3 substrate and inhibitor assays with fb-PMT are shown below. The results from other SLC transporter assays are not shown as fb-PMT was not observed to be a substrate or inhibitor. When tested as a potential substrate for human OATP1B1 and OATP1B3, concentration-dependent intracellular accumulation of fb-PMT was observed in both the mock and transporter-transfected cells (Figure 4a,b). However, at all three test concentrations (1, 3, and 10 μM) there was no significant difference in uptake of fb-PMT observed between the two cell lines. In addition, incubation of fb-PMT at 3 μM in the presence of the reference inhibitor cyclosporin A (CysA) did not alter uptake in either transporter cell line. Therefore, fb-PMT is not a substrate of either OATP1B1 or OATP1B3. The probe substrate rosuvastatin demonstrated both OATP1B1- and OATP1B3-dependent uptake in the respective cell lines. In OATP1B1 cells, CysA reduced rosuvastatin uptake from 20.6 ± 1.23 to 0.02 ± 0 pmol/min/mg, whereas in OATP1B3 cells, CysA reduced uptake from 26.6 ± 3.09 to 3.09 ± 0.721 pmol/min/mg.

TABLE 8 Assessment of fb-PMT as a substrate of P-gp or BCRP transporters.

Treatment	Papp (A–B)	Papp (B–A)	Efflux ratio
10 μM Digoxin	1.35	15.5	11.5
10 μM Digoxin +1 μM Valspodar	4.46	4.80	1.08
1 μM Teriflunomide	5.51	33.4	6.06
1 μM Teriflunomide +2 μM Ko143	18.9	12.8	0.681
1 μM fb-PMT	4.44	0.521	0.117
3 μM fb-PMT	1.51	0.457	0.302
3 μM fb-PMT + 1 μM Valspodar	1.27	0.442	0.348
3 μM fb-PMT + 2 μM Ko143	1.13	0.523	0.462
10 μM fb-PMT	0.937	0.625	0.667

Note: Fb-PMT was tested as a substrate for P-gp and BCRP in Caco-2 cells. Test compounds were added to either the apical or basolateral chamber and incubated for 2 h, after which the concentrations of test compounds were measured in both the donor and receiver chambers. Digoxin and Teriflunomide were used as positive controls for P-gp and BCRP, respectively. Both positive controls gave an efflux ratio of ≥2 indicating, they were substrates, while fb-PMT did not. In addition, transporter-specific inhibitors reduced the efflux ratios to ≤1.0. In contrast, the apparent permeability (Papp) values shown in the table are low for fb-PMT in both directions, and the efflux ratios were less than one at all three test concentrations. Neither inhibitor had a notable effect on the efflux ratio of the 3 μM fb-PMT concentration, confirming that fb-PMT is not a substrate for either transporter. Papp values ($\times 10^{-6}$ cm/s) represent means of duplicate measurements.

Fb-PMT was also tested as a potential inhibitor of human OATP1B1 and OATP1B3 activity in HEK293 cells using rosuvastatin as a reference substrate. Fb-PMT caused a significant concentration-dependent inhibition of rosuvastatin uptake in both the OATP1B1 and OATP1B3 cells (Figure 5a,b), The IC_{50s} were determined to be 8.58 μM for OATP1B1 and 3.45 μM for OATP1B3 using Prism GraphPad software.

A summary of the results from all ABC and SLC transporters tested is shown in Table 9. Fb-PMT was not a substrate for any of the ABC or SLC transporters tested and only showed inhibition against the two OATP transporters as described above. In summary, there is a potential for fb-PMT to inhibit OATP1B1 and OATP1B3 transporter activity depending on plasma concentrations reached in vivo.

DISCUSSION

This study was designed to evaluate the potential DDIs of fb-PMT, a novel drug-polymer conjugate with therapeutic

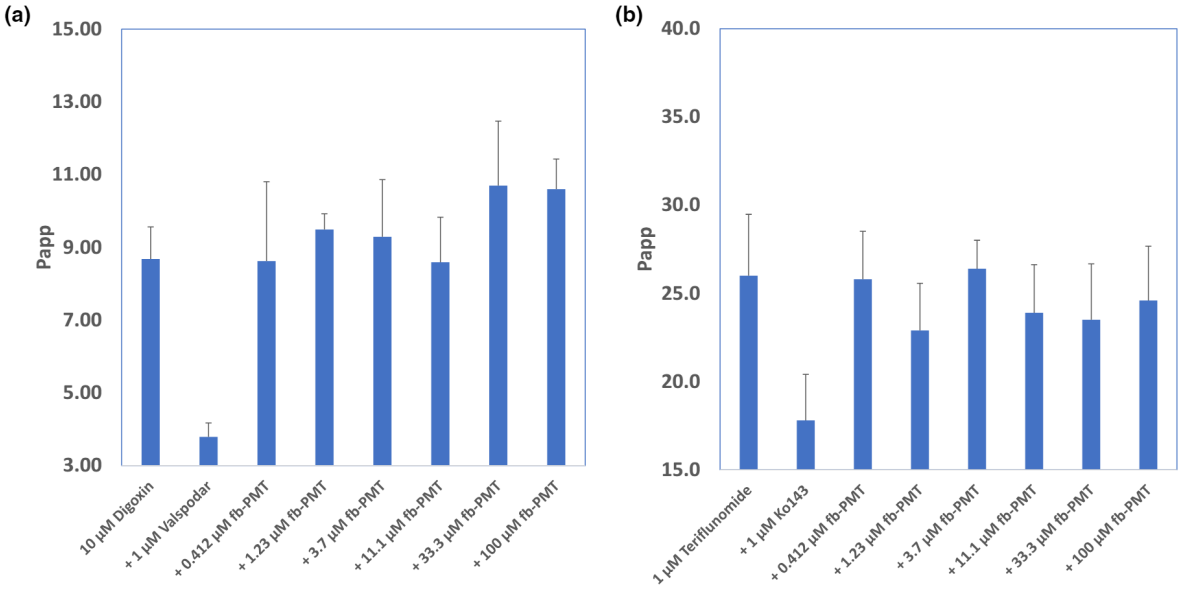

FIGURE 3 Effect of fb-PMT on digoxin and teriflunomide uptake in Caco-2 Cells. Fb-PMT was tested for its ability to inhibit P-gp (a) or BCRP (b) transporter activity in Caco-2 cells. Digoxin and teriflunomide were used as the probe substrates for these assays, whereas valspodar and Ko143 were used as the inhibitors. Inhibitors were added to the incubations for 30 min prior to the start of the assay. Fb-PMT was evaluated over a concentration range of 0.412–100 µM. All compounds were added to the basolateral chamber and incubated for 2 h following the addition of probe substrate. The value obtained in the presence of the known inhibitor was used as the 100% inhibition. Fb-PMT did not inhibit either transporter in a concentration-dependent manner. Papp, apparent permeability.

potential as an anticancer thyrointegrin αvß3 antagonist. The most common mechanisms for DDIs involve metabolic or transporter-mediated effects. The in vitro studies conducted therefore included in silico and microsomal assessment of CYP metabolism, CYP inhibition, CYP induction, and the evaluation of fb-PMT as a substrate or inhibitor of several ABC and SLC transporters. The in silico evaluation of fb-PMT as a potential substrate of inhibitor of CYPs suggested several effects that were not confirmed by the in vitro microsomal studies. The inconsistency of the computational prediction, which predicted inhibitory properties of fb-PMT on CYP1A2, CYP2C9, and CYP3A4, and the results of the in vitro microsomal CYP inhibition assays may be attributed to the limited power of computational prediction and the unique physicochemical property of fb-PMT in water, which is different from small molecules. Presumably, the PEG moiety of fb-PMT is highly hydrated in aqueous media[21]; and the steric effect and the hydrophilic nature of hydrated PEG may prevent the acceptance of the drug-PEG conjugate as a substrate of CYP enzymes. We previously reported that a tetrac moiety in fb-PMT is hindered with PEG moiety in water judged by the change in chemical shift of tetrac in D_2O,[6] and the result of chemical shift change supports the poor substrate property of fb-PMT with CYP enzymes. Despite the disagreement between the computational simulation and the IC_{50} shift, fb-PMT showed weak inhibition of CYP3A4, especially testosterone oxidation activity; suggesting some of the tetrac moiety in fb-PMT is accessible to CYP active sites. Although the tetrac moiety of fb-PMT is shielded by hydrated PEG in aqueous media, the results of IC_{50} shift and anticancer activity suggest that it is capable of binding to active sites of target proteins, such as integrins and CYPs, to antagonize or inhibit their activities.

The results of the CYP reaction phenotyping assay suggested that none of the seven human CYP enzymes (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 isoforms) contribute to the metabolism of fb-PMT. The compound was stable over the time-period measured (30 min). The possibility exists that fb-PMT may be cleared by CYPs at a much slower rate than could be detected in these incubations, but, overall, our results demonstrate that the compound is very stable in the presence of microsomal enzymes and NADPH. In addition, results of CYP inhibition assays demonstrated that fb-PMT showed only weak inhibitory properties against CYP enzymes. Of the seven CYPs tested, fb-PMT only showed effects with CYP2C19 (IC_{50} = 78.4 µM) and CYP3A4 (testosterone only, $IC_{50} > 100$ µM). Results from the TDI assay confirmed the lack of inhibitory effect on most CYPs and detected no potential for TDI. There was also no evidence for induction of CYP1A2, 2B6, or 3A4 in human hepatocytes. Together, these results demonstrate that fb-PMT exhibits a very low potential for DDIs based on CYP enzymes. There was little or no evidence of

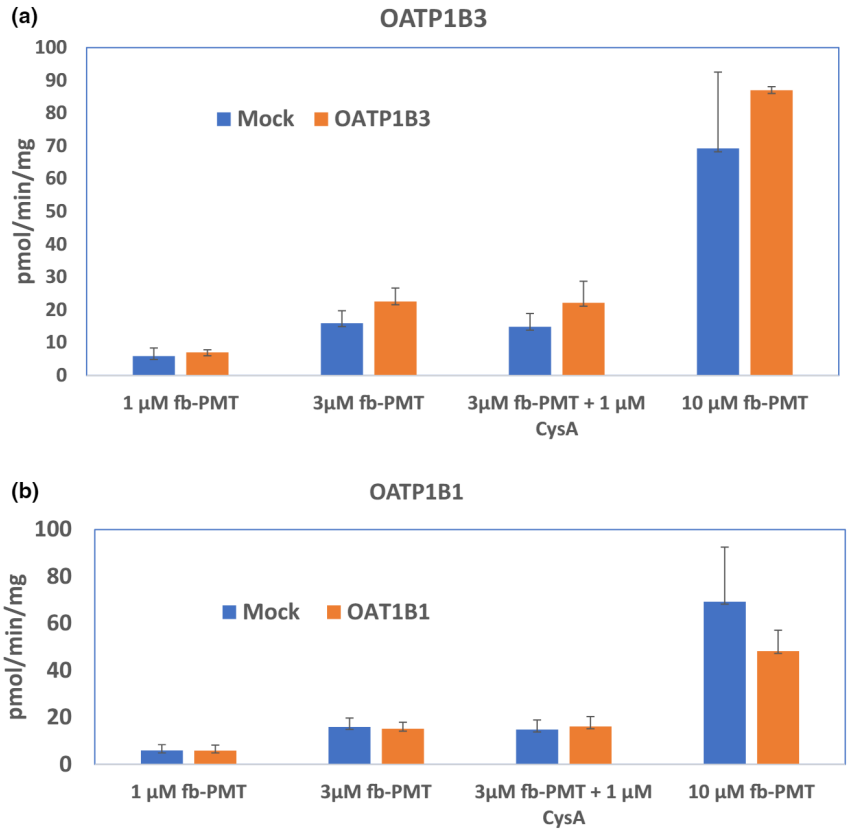

FIGURE 4 Transport of fb-PMT by human OATP1B1 and OATP1B3. Fb-PMT was evaluated as a substrate in both OATP1B1 and OATP1B3 in HEK293 cell lines expressing these transporters. Mock transfected cells were used as a control for non-specific uptake. Test compounds were incubated for 10 min and then extracted for measurement of total uptake. Three concentrations of fb-PMT were tested in these assays (1, 3, and 10 µM). A known inhibitor of OATP uptake (cyclosporin A) was added to the 3 µM incubations. No evidence was observed that fb-PMT was a substrate for either of the OATP transporters.

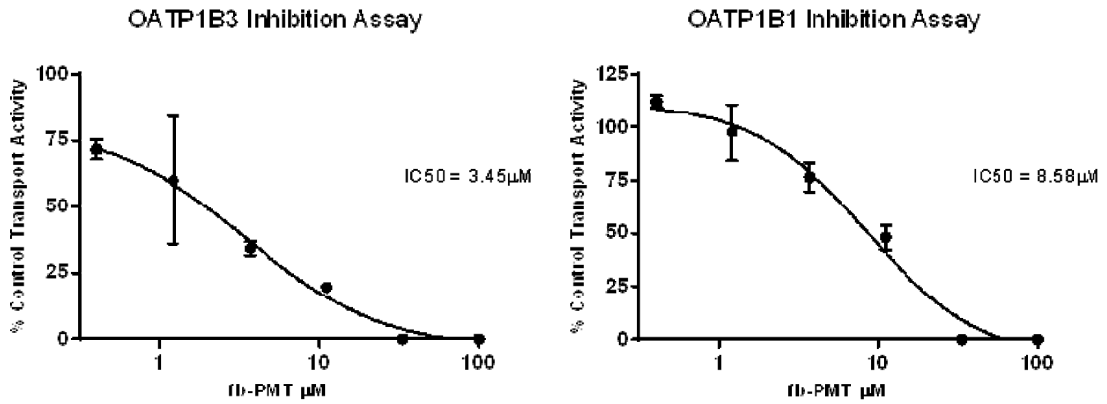

FIGURE 5 Inhibition of OAT1B1 and OATP1B3 by fb-PMT. Fb-PMT was tested as an inhibitor of both OATP1B1 and OATP1B3 in HEK293 cell lines expressing these transporters. Fb-PMT was pre-incubated for 30 min with the cells before the probe substrate was added. Fb-PMT caused a concentration-dependent inhibition of both transporters with $IC_{50s} \leq 10$ µM. IC_{50}, half-maximal inhibitory concentration.

CYP-mediated metabolism, CYP inhibitory activity, or CYP induction potential.

The second major mechanism of DDIs involves drug transporters. Both major drug transporter families (ABC and SLC) were tested with fb-PMT. Fb-PMT was neither a substrate nor inhibitor of the ABC transporters P-gp or BCRP. Similarly, fb-PMT was not a substrate for any of the seven SLC transporters tested. The only notable effects of fb-PMT on SLC transporters were inhibition of OATP1B1 and OATP1B3 function. These two transporters are located on the basolateral surface of hepatocytes and function to uptake drugs from the blood into the liver. They are known to have overlapping substrate specificities; thus, it is not surprising that fb-PMT has a similar effect on both. Known substrates for these transporters include statins, repaglinide, angiotensin II antagonists, ACE inhibitors, and many others, including a variety of endogenous substances. The IC_{50s} for these transporters were both less than 10 µM, which suggests a potential for DDIs depending on the maximum in vivo concentration reached in vivo and the pharmacokinetic (PK) profile of fb-PMT in humans. To date, PK data are

TABLE 9 Summary of fb-PMT interactions with ABC and SLC transporters.

Transporter	Type	Assay platform	Substrate results	Inhibitor results
P-gp	ABC	Caco-2 cells	Non-substrate	Noninhibitor
BCRP	ABC	Caco-2 cells	Non-substrate	Noninhibitor
MATE1	SLC	Transfected HEK293 cells	Non-substrate	Noninhibitor
MATE2-K	SLC	Transfected HEK293 cells	Non-substrate	Noninhibitor
OAT1	SLC	Transfected HEK293 cells	Non-substrate	Noninhibitor
OAT3	SLC	Transfected HEK293 cells	Non-substrate	Noninhibitor
OATP1B1	SLC	Transfected HEK293 cells	Non-substrate	Inhibitor, IC_{50} = 8.58 μM
OATP1B3	SLC	Transfected HEK293 cells	Non-substrate	Inhibitor, IC_{50} = 3.45 μM
OCT2	SLC	Transfected HEK293 cells	Non-substrate	Noninhibitor

Abbreviation: IC_{50}, half-maximal inhibitory concentration.

available only from rodents and suggests that fb-PMT is rapidly cleared and would be unlikely to reach or stay at levels that might affect the OATP transporters. Actual plasma concentrations of fb-PMT were much lower (nM levels) than required for effects on the OATP transporters. However, until human data are obtained, this will remain hypothetical. In conclusion, the current study demonstrates a low potential for adverse DDIs with fb-PMT. Because of this property, fb-PMT is a preferred anticancer drug candidate compared with other drugs acting in the same therapeutic area. Fb-PMT administered alone at different doses demonstrated high efficacy and low toxicity in various cancer models. It can also be used in combination with other anticancer drugs or any other therapeutics with minimal anticipated DDIs. The application of fb-PMT to clinical anticancer therapy is promising from a viewpoint of DDI.

AUTHOR CONTRIBUTIONS

S.A.M. and D.T. wrote the manuscript. S.A.M., J.M.R., and T.S. designed the research. K.A.F., M.I.F., and T.S. performed the research. K.A.F., M.I.F., J.M.R., and D.T. analyzed the data.

ACKNOWLEDGMENTS

The authors wish to thank co-author Kazutoshi Fujioka, who unfortunately passed before the publication of this work. His guidance and mentorship were much appreciated.

FUNDING INFORMATION

No funding was received for this work.

CONFLICT OF INTEREST STATEMENT

S.A.M. holds several patents on anticancer compounds assigned to NanoPharmaceuticals LLC and founder of the company. All other authors declared no competing interests for this work.

ORCID

Shaker A. Mousa https://orcid.org/0000-0002-9294-015X

REFERENCES

1. Pasut G, Veronese FM. PEG conjugates in clinical development or use as anticancer agents: an overview. *Adv Drug Deliv Rev*. 2009;61(13):1177-1188.
2. Li C, Wallace S. Polymer-drug conjugates: recent development in clinical oncology. *Adv Drug Deliv Rev*. 2008;60(8):886-898.
3. Kopeček J, Yang J. Polymer nanomedicines. *Adv Drug Deliv Rev*. 2020;156:40-64.
4. Ekladious I, Colson YL, Grinstaff MW. Polymer–drug conjugate therapeutics: advances, insights, and prospects. *Nat Rev Drug Discov*. 2019;18:273-294.
5. Camacho KM, Menegatti S, Mitragotri S. Low-molecular-weight polymer-drug conjugates for synergistic anticancer activity of camptothecin and doxorubicin combinations. *Nanomedicine (Lond)*. 2016;11(9):1139-1151.
6. Hay BA, Godugu K, Darwish NHE, et al. New Thyrointegrin αvβ3 antagonist with a scalable synthesis, brain penetration, and potent activity against glioblastoma multiforme. *J Med Chem*. 2021;64(9):6300-6309. doi:10.1021/acs.jmedchem.1c00350
7. Fujioka K, Godugu K, Mousa SA. Pharmacokinetics and biodistribution of a novel anticancer thyrointegrin αvβ3 antagonist: triazole modified tetraiodothyroacetic acid conjugated to polyethylene glycol (P-bi-TAT). *AAPS Open*. 2021;7:2. doi:10.1186/s41120-021-00036-z
8. Godugu K, Rajabi M, Mousa SA. Anti-cancer activities of Thyrointegrin $\alpha_v\beta_3$ antagonist mono- and bis-triazole Tetraiodothyroacetic acid conjugated via polyethylene glycols in glioblastoma. *Cancers (Basel)*. 2021;13(11):2780. doi:10.3390/cancers13112780
9. Sudha T, Godugu K, Glinsky GV, Mousa SA. Triazole modified Tetraiodothyroacetic acid conjugated to polyethylene glycol, a Thyrointegrin $\alpha_v\beta_3$ antagonist as a radio- and chemo-sensitizer in pancreatic cancer. *Biomedicine*. 2022;10(4):795. doi:10.3390/biomedicines10040795
10. Darwish NHE, Glinsky GV, Sudha T, Mousa SA. Targeting Thyrointegrin αvβ3 using fluorobenzyl polyethylene glycol conjugated Tetraiodothyroacetic acid (NP751) in acute

myeloid leukemia. *Front Oncol*. 2022;11:793810. doi:10.3389/fonc.2021.793810
11. Fowler S, Morcos PN, Cleary Y, et al. Progress in prediction and interpretation of clinically relevant metabolic drug-drug interactions: a minireview illustrating recent developments and current opportunities. *Curr Pharmacol Rep*. 2017;3:36-49.
12. McLeod HL. Clinically relevant drug-drug interactions in oncology. *Br J Clin Pharmacol*. 1998;45(6):539-544.
13. Sazonova EV, Kopeina GS, Imyanitov EN, Zhivotovsky B. Platinum drugs and taxanes: can we overcome resistance? *Cell Death Dis*. 2021;7:155.
14. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther*. 2013;138(1):103-141.
15. Fowler S, Morcos PN, Cleary Y, et al. Progress in prediction, and interpretation of clinically relevant metabolic drug-drug interactions: minireview illustrating recent developments and current opportunities. *Curr Pharmacol Rep*. 2017;3(1):36-49.
16. Li P, Lu C, Balani SK, Gan LS. A refined cytochrome P540 IC_{50} shift assay for reliably identifying CYP3A time-dependent inhibitors. *Drug Metab Dispos*. 2011;39(6):1054-1057. doi:10.1124/dmd.111.038208 Erratum in: Drug Metab Dispos. 2011 Sep;39(9):1777.
17. de Ron L, Rajaraman G. Assessment of the time-dependent inhibition (TDI) potential of test compounds with human liver microsomes by IC50 shift method using a non-dilution approach. *Curr Protoc Pharmacol*. 2012; Chapter 7:Unit7.14.
18. Uwimana E, Ruiz P, Li X, Lehmler HJ. Human CYP2A6, CYP2B6, AND CYP2E1 Atropselectively metabolize polychlorinated biphenyls to hydroxylated metabolites. *Environ Sci Technol*. 2019;53(4):2114-2123.
19. Parkinson A, Kazmi F, Buckley DB, et al. An evaluation of the dilution method for identifying metabolism-dependent inhibitors of cytochrome P450 enzymes. *Drug Metab Dispos*. 2011;39(8):1370-1387.
20. Fujioka K, Hay BA, Godugu K, Mousa SA. Pharmacokinetics of fluorobenzyl polyethylene glycol conjugated tetraiodothyroacetic acid (NP751), a novel anticancer thyrointegrin $\alpha_v\beta_3$ antagonist. *Front Pharmacol*. 2022;28:902141. doi:10.3389/fphar.2022.902141
21. Obach RS, Walsky RL, Venkatakrishnan K. Mechanism-based inactivation of human cytochrome P450 enzymes and the prediction of drug-drug interactions. *Drug Metab Dispos*. 2007;35:246-255.

How to cite this article: Fujioka K, Fekry MI, Rumsey JM, Steiner T, Thompson D, Mousa SA. Preclinical assessment of drug–drug interaction of fb-PMT, a novel anti-cancer thyrointegrin αvβ3 antagonist. *Clin Transl Sci*. 2023;00:1-15. doi:10.1111/cts.13504