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ARTICLE

Formation of CYP3A-specific metabolites of ibrutinib in vitro is correlated with hepatic CYP3A activity and 4β-hydroxycholesterol/cholesterol ratio

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Abstract

Ibrutinib is an orally administered Bruton's tyrosine kinase inhibitor approved for the treatment of B-cell malignancies, including chronic lymphocytic leukemia. Ibrutinib is metabolized primarily via oxidation by cytochrome P450 (CYP) 3A4/5 to M37 (the primary active metabolite), M34, and M25. The objectives of this study were to assess the relationship between formation of the major CYP3A-specific ibrutinib metabolites in vitro and hepatic CYP3A activity and protein abundance, and to evaluate the utility of the endogenous CYP3A biomarker, plasma 4β-hydroxycholesterol (4β-HC) to cholesterol ratio, to predict ibrutinib metabolite formation in individual cadaveric donors with matching hepatocytes. Ibrutinib (5 μM) was incubated with single-donor human liver microsomes ($n = 20$) and primary human hepatocytes ($n = 15$), and metabolites (M37, M34, and M25) were measured by liquid chromatography-tandem mass spectrometry analysis. CYP3A4/5 protein concentrations were measured by quantitative targeted absolute proteomics, and CYP3A activity was measured by midazolam 1′-hydroxylation. Ibrutinib metabolite formation positively correlated with midazolam 1′-hydroxylation in human liver microsomes and hepatocytes. Plasma 4β-HC and cholesterol concentrations were measured in plasma samples obtained at the time of liver harvest from the same 15 donors with matching hepatocytes. Midazolam 1′-hydroxylation in hepatocytes correlated with plasma 4β-HC/cholesterol ratio. When an infant donor (1 year old) was excluded based on previous ontogeny studies, M37 and M25 formation correlated with plasma 4β-HC/cholesterol ratio in the remaining 14 donors (Spearman correlation coefficients [r] 0.62 and 0.67, respectively). Collectively, these data indicate a positive association among formation of CYP3A-specific ibrutinib metabolites in human hepatocytes, hepatic CYP3A activity, and plasma 4β-HC/cholesterol ratio in the same non-infant donors.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Ibrutinib, a first-in-class, oral covalent inhibitor of Bruton's tyrosine kinase, is metabolized extensively by CYP3A4/5. Although variability in ibrutinib exposure

has been attributed largely to CYP3A-mediated metabolism, studies to assess individual CYP3A activity in patients taking ibrutinib are lacking. Plasma 4β-hydroxycholesterol (4β-HC) is an endogenous CYP3A metabolite of cholesterol, and the plasma 4β-HC/cholesterol ratio has been reported as a biomarker of hepatic CYP3A activity. However, whether plasma 4β-HC/cholesterol ratio can be used to predict ibrutinib metabolism is unknown.

WHAT QUESTION DID THIS STUDY ADDRESS?

(1) What is the relationship between individual hepatic CYP3A activity and protein expression and the metabolism of ibrutinib in vitro? (2) Can plasma 4β-HC/cholesterol ratio be used as a biomarker to estimate individual hepatic CYP3A activity and ibrutinib metabolism in vitro in human hepatocytes and plasma from matched donors?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

CYP3A-specific ibrutinib metabolite formation was correlated with individual hepatic CYP3A activity in primary human hepatocytes and 4β-HC/cholesterol ratio in plasma samples from matched non-infant donors.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

These findings provide supportive evidence to explore plasma 4β-HC/cholesterol ratio as a biomarker to estimate CYP3A-mediated hepatic metabolism of ibrutinib in patients. This research may serve as a platform for future clinical studies to inform precision dosing strategies for individualized ibrutinib therapy.

INTRODUCTION

Ibrutinib is an orally administered covalent inhibitor of Bruton's tyrosine kinase (BTK) approved by the US Food and Drug Administration (FDA) for the treatment of various B-cell malignancies, including chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL).[1,2] Ibrutinib is absorbed rapidly after oral administration and reaches its maximum plasma concentration 1–2 h postdose with an elimination half-life of 4–6 h.[3] The oral bioavailability of ibrutinib is low (3%) due to extensive first-pass metabolism.[3,4] Ibrutinib is metabolized primarily by cytochrome P450 (CYP) 3A4/5 and to a lesser extent by CYP2D6.[4] The primary active metabolite, PCI-45227 (M37; Figure 1), which has ~15-fold lower inhibitory activity toward BTK compared to the parent drug, is formed via epoxidation of the ethylene on the acryloyl moiety followed by hydrolysis to the dihydrodiol.[4,5] The other major metabolic clearance pathways of ibrutinib include hydroxylation of the phenyl to monooxygenated metabolite (M35), and opening of the piperidine ring followed by reduction to the alcohol metabolite (M34) and oxidation to the carboxylic acid metabolite (M25; Figure 1).[4]

CYP3A enzymes contribute to the oxidative metabolism of nearly 50% of drugs in clinical use.[6] CYP3A4 is the most abundant P450 enzyme in the adult liver and small intestine whereas CYP3A5 is polymorphically expressed.[7–9] Interindividual variability in CYP3A expression and activity is a major source of variability in pharmacokinetics and therapeutic outcomes.[6,7] Recent studies suggest that variability in CYP3A activity significantly impacts ibrutinib exposure. In healthy volunteers, the intersubject variability (coefficient of variation) in ibrutinib maximum plasma concentration (C_{max}) and area under the curve (AUC) was 100%.[10] Many factors can contribute to interindividual variability in CYP3A-mediated drug metabolism, including genetics, lifestyle, co-administered drugs, and patient characteristics.[6,11,12] Due to non-genetic factors, pharmacogenetic testing may not accurately predict an individual's CYP3A metabolic activity, and practical methods to measure individual CYP3A activity in patients are lacking.[6,13] The current standard for phenotyping CYP3A activity is to determine the clearance of selective exogenous probe substrates, such as midazolam, which requires collection of sequential blood samples and is inconvenient for patients.[14]

There is a growing body of literature exploring the use of endogenous biomarkers to determine CYP3A phenotype.[15] Interestingly, the circulating metabolite 4β-hydroxycholesterol (4β-HC), which is formed by CYP3A-mediated cholesterol metabolism, has been suggested as a promising endogenous biomarker of hepatic CYP3A activity.[15–18] Moreover, plasma 4β-HC can be normalized to cholesterol (plasma 4β-HC/cholesterol ratio) to account for individual variations in cholesterol concentrations.[16] Pharmacokinetic studies in healthy

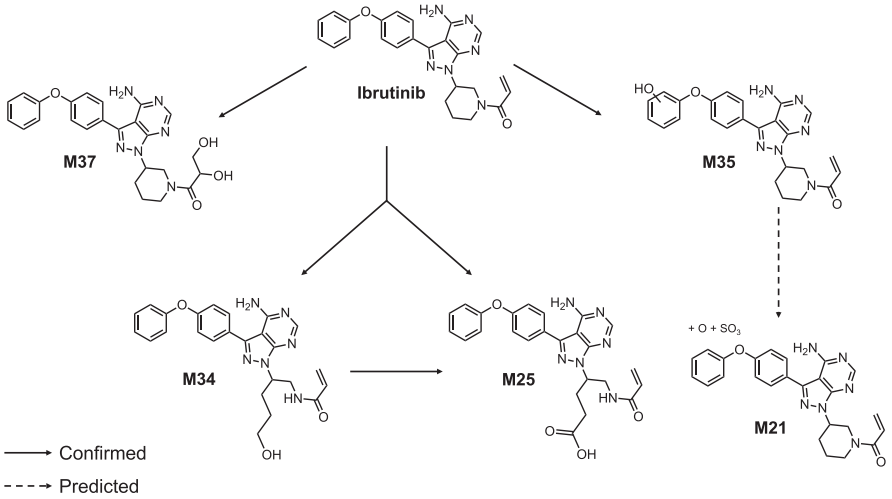

FIGURE 1 Proposed metabolic pathway of ibrutinib to the major metabolites reported in vivo. Simplified metabolic scheme of ibrutinib adapted from Scheers et al.[4]

participants have demonstrated that CYP3A4 inducers and inhibitors increased and decreased plasma concentrations of 4β-HC, respectively.[19–21] In type 2 diabetic and nondiabetic individuals, plasma 4β-HC concentrations and the plasma 4β-HC/cholesterol ratio were significantly correlated with oral midazolam metabolic ratio.[17] A recent study demonstrated that plasma 4β-HC concentrations were associated with the human liver, but not intestinal, microsomal CYP3A activity.[22]

Detailed in vitro investigations to examine the influence of individual hepatic CYP3A activity on ibrutinib metabolism have not been addressed. Moreover, whether plasma 4β-HC/cholesterol can be used as a biomarker to predict individual ibrutinib exposure is unknown. The objectives of this study were to (1) examine interindividual variability in ibrutinib metabolism in vitro using single-donor human liver microsomes and cryopreserved primary human hepatocytes; (2) assess the correlation between ibrutinib metabolite formation and individual hepatic CYP3A activity and protein expression; and (3) evaluate the association of plasma 4β-HC/cholesterol ratio with individual hepatic CYP3A markers (activity and expression) and ibrutinib metabolism in human hepatocytes and plasma from the same matched cadaveric donors. Herein, we demonstrate that CYP3A-specific ibrutinib metabolite formation is correlated with individual hepatic CYP3A activity and CYP3A4 protein concentration in primary human hepatocytes as well as with plasma 4β-HC/cholesterol ratio from matched non-infant donors.

MATERIALS AND METHODS

Chemicals and reagents

Ibrutinib (free base; I-3311) was purchased from LC Laboratories. Deuterium-labeled ibrutinib ([$^2H_5$]ibrutinib, d_5-ibrutinib, 22,561) and PCI 45227 (M37) were purchased from Cayman Chemical. Midazolam (M-908), 1′-hydroxymidazolam (H-902), and d_4-1′-hydroxymidazolam (H-921) were purchased from Cerilliant Corporation. The 4β-HC (700036) and cholesterol (700000P) were purchased from Avanti Polar Lipids. Deuterium-labeled 4β-HC ([$^2H_7$]4β-HC, d_7-4β-HC, H917982) and deuterium-labeled cholesterol ([$^2H_7$]cholesterol, d_7-cholesterol, C432503) were purchased from Toronto Research Chemicals. Dimethyl sulfoxide (DMSO), Optima liquid chromatography mass spectrometry LC–MS grade water, Optima LC–MS grade acetonitrile, Optima LC–MS grade methanol were obtained from Fisher Scientific. All other chemicals and reagents were of analytical grade or higher.

As described previously,[23] an NADPH-regenerating system, consisting of solution A (26 mM $NADP^+$, 66 mM glucose 6-phosphate, 66 mM $MgCl_2$ in water) and solution B (40 U/ml glucose 6-phosphate dehydrogenase in 5 mM sodium citrate), was purchased from Corning Life Sciences. InVitroGRO Krebs–Henseleit buffer (KHB) medium (product no. Z99074) and thawing (HT) medium (product no. 990006) were purchased from BioIVT.

Human liver microsomes

Pooled human liver microsomes from 150 donors, mixed gender (lot number: 38295) were purchased from Corning Life Sciences. Single-donor human liver microsomal samples from 20 donors (15 male and 5 female donors) were purchased from Xenotech. Donor information is provided in Table S1.

Cryopreserved human hepatocytes and matched plasma samples

Pooled cryopreserved human hepatocytes from five donors (LiverPool Cryosuspension, 5-donor, mixed gender,

lot no. YQV) were purchased from BioIVT. Single-donor cryopreserved human hepatocytes and the matched plasma samples (obtained at the time of liver harvest) from 15 donors (7 male and 8 female donors) were also purchased from BioIVT. Demographic information is listed in Table S2.

Measurement of CYP3A activity in single-donor human liver microsomes and hepatocytes

Midazolam 1′-hydroxylation was used as a marker of CYP3A activity, as described previously (see Supporting Information for details).[24]

Ibrutinib metabolism in single-donor human liver microsomes

Preliminary experiments demonstrated that ibrutinib metabolite formation was linear with liver microsomal protein concentration of 0.05 mg protein/ml up to 20 min. Subsequently, ibrutinib (5 μM) was incubated with 20 single-donor human liver microsomal fractions (0.05 mg protein/ml) in 100 mM potassium phosphate buffer (pH 7.4) supplemented with an NADPH-regenerating system for 20 min at 37°C. The reaction volume was 100 μl with a final organic solvent composition of 1% (v/v). Control incubations were performed without NADPH-regenerating system. After the incubation period, reactions were quenched by the addition of two volumes (200 μl) of ice-cold acetonitrile containing d_5-ibrutinib (50 ng/ml, internal standard), mixed with a vortex device, and centrifuged at 3400 ***g*** for 20 min at 4°C. The supernatant was evaporated to dryness using a ThermoSavant SpeedVac. The sample residue was then redissolved with 120 μl of 50:50 mobile phase A/mobile phase B (v/v) (see Supporting Information), mixed with a vortex device, and a 10-μl aliquot was subjected to liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis for measurement of relative levels of metabolites.

Ibrutinib depletion in single-donor human liver microsomes

To evaluate ibrutinib metabolic clearance at clinically relevant concentrations, ibrutinib (1 μM) was incubated with human liver microsomes (0.05 mg protein/ml) from three single donors in 100 mM potassium phosphate buffer (pH 7.4). Donors were White men with similar age (60, 60, and 63 years old) but different CYP3A activity, as measured by midazolam 1′-hydroxylation. Reactions were initiated by addition of the NADPH-regenerating system (final concentration of organic solvent, 0.1% DMSO, 0.9% acetonitrile, v/v) and incubated for 0, 2, 5, 10, 15, 20, 30, and 45 min. At each timepoint, 100-μl aliquots were removed and added to 200 μl of ice-cold acetonitrile containing d_5-ibrutinib (50 ng/ml) to quench the reaction. Samples were processed as described above for ibrutinib metabolism in human liver microsomes.

Ibrutinib metabolism in human hepatocytes

Initial experiments were conducted to examine the linearity of ibrutinib metabolism with respect to time in five-donor pooled cryopreserved human hepatocytes in suspension. Cell viability was determined at the beginning of each experiment using the trypan blue exclusion method. Cells (0.5×10^6 viable cells/ml KHB) were seeded in a 24-well collagen I coated plate at a volume of 0.5 ml and incubated with ibrutinib (5 μM) for up to 30 min at 37°C. M37 formation was linear up to 15 min. M34 and M25 formation was linear throughout the incubation period.

Subsequently, single-donor human hepatocytes (0.5×10^6 viable cells/ml KHB) from 15 donors were incubated in suspension with ibrutinib (5 μM) for 15 min at 37°C. The final incubation volume was 500 μl, and the final organic solvent concentration was 1% (1:9 DMSO/acetonitrile, v/v). After the incubation, an equal volume (500 μl) of ice-cold acetonitrile containing 100 ng/ml d_5-ibrutinib (internal standard) was added to each well. The cell suspension was centrifuged for 20 min at 20,000 ***g*** at 4°C. The clear supernatant was evaporated to dryness under nitrogen gas using a Biotage TurboVap. Samples were reconstituted in 200 μl of 50:50 mobile phase A/mobile phase B (v/v), mixed with a vortex device, and centrifuged for 5 min at 20,000 ***g*** (room temperature). The supernatant was collected, and a 10-μl aliquot was subjected to LC–MS/MS analysis.

LC–MS/MS analysis of ibrutinib and ibrutinib metabolites

Ibrutinib and ibrutinib metabolites were measured by LC–MS/MS analysis using a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer coupled to a Thermo Accela HPLC system (see Supporting Information for details).

Quantitative targeted absolute proteomic analysis of CYP3A proteins

CYP3A protein concentrations in human liver microsomes, and membranes from primary human hepatocytes, from individual donors were quantified by quantitative targeted absolute proteomic (QTAP) analysis using methods described previously (see Supporting Information for details).[25–28]

Quantification of plasma 4β-HC and cholesterol

Plasma concentrations of 4β-HC and cholesterol were determined as described previously with slight modifications (see Supporting Information for details).[29,30]

Data analysis

All statistical analyses were performed using GraphPad Prism 9 software (GraphPad). Results shown in figures represent the mean or mean ± SD of three replicates for each sample unless otherwise indicated.

The depletion rate constants for ibrutinib in single-donor human liver microsomes from three donors were calculated from the slope of the semilogarithmic plot of ibrutinib remaining (measured as peak area ratio to internal standard) versus time (min). Intrinsic clearance (Cl_{int}) values were calculated as follows:

$$Cl_{int}\ (\mu l/min/mg\ protein) = k_{deg} \times (\mu l\ of\ incubation)/(mg\ protein)$$

where k_{deg} is the negative slope of the semilogarithmic plot of the average peak area ratio versus time.[31]

D'Agostino-Pearson and Shapiro–Wilk normality tests revealed that nonparametric evaluation of the data was required. All correlation analyses were performed using Spearman *r* correlation in GraphPad Prism 9 software, and data are graphed on log scale plots. Comparisons of ibrutinib metabolite formation, midazolam 1′-hydroxylation, and plasma 4β-HC/cholesterol ratio by sex (male vs. female) were performed by Mann–Whitney test. Statistical significance was determined at $p < 0.05$.

RESULTS

Ibrutinib metabolism in human liver microsomes from single donors

Individual CYP3A activity was measured by the marker reaction midazolam 1′-hydroxylation in single-donor human liver microsomes from 20 donors (range 25.3–3606.1 pmol/mg protein/min; Figure S1a). The CYP3A4 and CYP3A5 protein abundance data from the same 20 donors are summarized in Table S1.

To investigate the relationship between CYP3A markers and ibrutinib metabolism, formation of ibrutinib metabolites M37 and M34 was measured in human liver microsomes from the same donors (Figure S1b,c). M37 and M34 were formed only in microsomal incubations supplemented with NADPH regenerating system. Representative LC–MS/MS chromatograms of ibrutinib metabolites formed in liver microsomes from donor 710,410 are shown in Figure S2. Chromatographic peaks corresponding to ibrutinib and metabolites M37 and M34 are labeled. Multiple peaks were detected in the M25 channel that were not separated with our LC–MS/MS method; therefore, M25 was excluded from the analysis. M35 was not detected in any of the single-donor human liver microsomal samples examined.

Formation of M37 and M34 positively correlated with midazolam 1′-hydroxylation, microsomal CYP3A4 protein concentration, and, to a lesser extent, CYP3A5 protein concentration (Figure 2).

Depletion of ibrutinib in human liver microsomes from selected single donors

In clinical studies, the mean C_{max} of ibrutinib when given at therapeutic doses was approximately 0.4 μM.[32] The estimated portal vein concentration of ibrutinib was reported to be ~1 μM.[4] Therefore, to evaluate ibrutinib metabolic clearance at a therapeutically relevant concentration, substrate depletion experiments were conducted with ibrutinib (1 μM) using a subset of single-donor human liver microsomes. The human liver microsomal samples selected were from three White male donors (lot numbers 710,410, 710,450, and 810,011) with similar age (ages 60–63 years) but different CYP3A activities, as measured by midazolam 1′-hydroxylation (3606.1, 2613.5, and 268.5 pmol/mg protein/min, respectively). The semilogarithmic plot of percentage (%) ibrutinib remaining was linear over time (0–30 min; Figure 3a). The Cl_{int} values were 322, 248, and 16 μl/mg protein/min, for donors 710,410, 710,450, and 810,011, respectively, representing a 20-fold variation. M37 was formed at similar levels in donors 710,410 and 710,450 but was much lower in donor 810,011 (Figure 3b). M34 formation was highest in donor 710,410 (Figure 3c).

Ibrutinib metabolism in primary human hepatocytes from single donors

To investigate interindividual variation in CYP3A activity and ibrutinib metabolite formation in a more

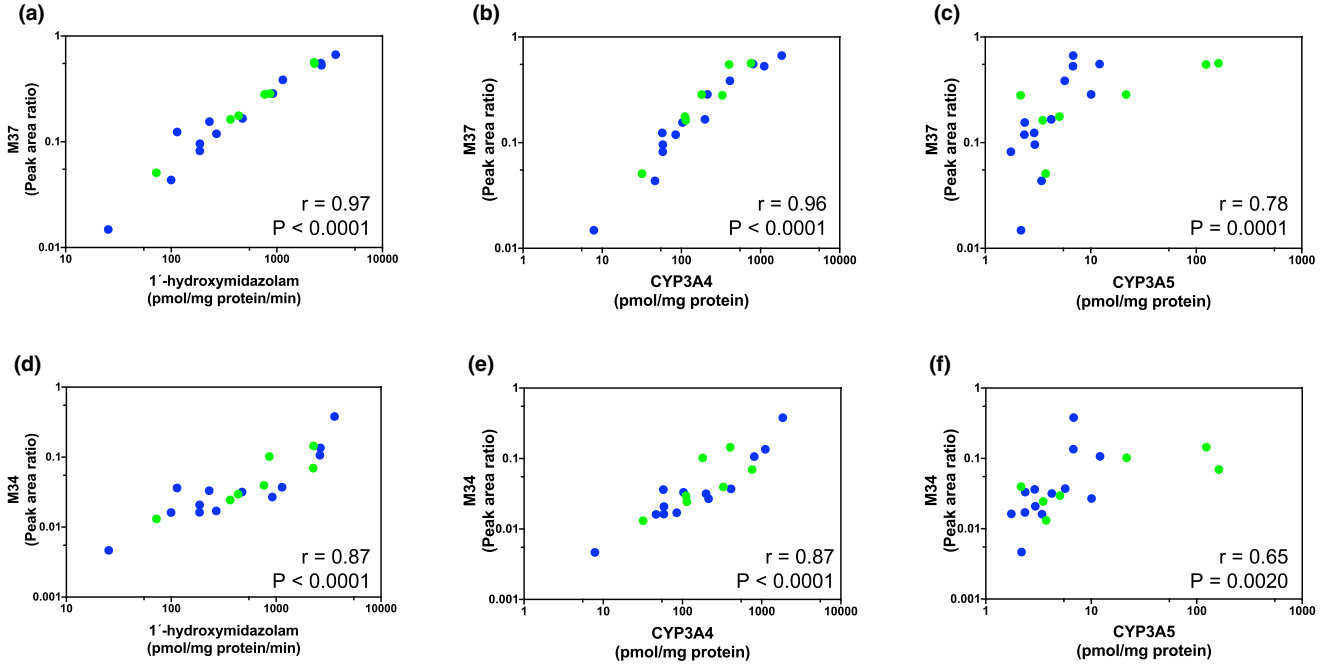

FIGURE 2 Ibrutinib metabolite formation in single-donor human liver microsomes and comparison with CYP3A activity and protein expression. Ibrutinib (5 μM) was incubated with human liver microsomes (0.05 mg protein/ml) from 20 individual donors ($n = 20$) in the presence of NADPH-generating system for 20 min. Formation of M37 and M34 was measured by liquid chromatography-tandem mass spectrometry analysis. Relative levels of metabolite were determined by the ratio of analyte peak area divided by internal standard (d_5-ibrutinib) peak area. Correlation of M37 formation with individual CYP3A activity, as measured by midazolam 1′-hydroxylation (a), CYP3A4 protein content (b), and CYP3A5 protein content (c). Correlation of M34 formation with individual CYP3A activity (d), CYP3A4 protein content (e), and CYP3A5 protein content (f). Spearman correlation analysis was performed using GraphPad Prism 9 Software, and data are graphed on log scale plots. Results are the mean values for each donor from three independent experiments conducted in triplicate. Self-reported race of donors: Blue dots represent Caucasian/White donors. Green dots represent African American/Black donors

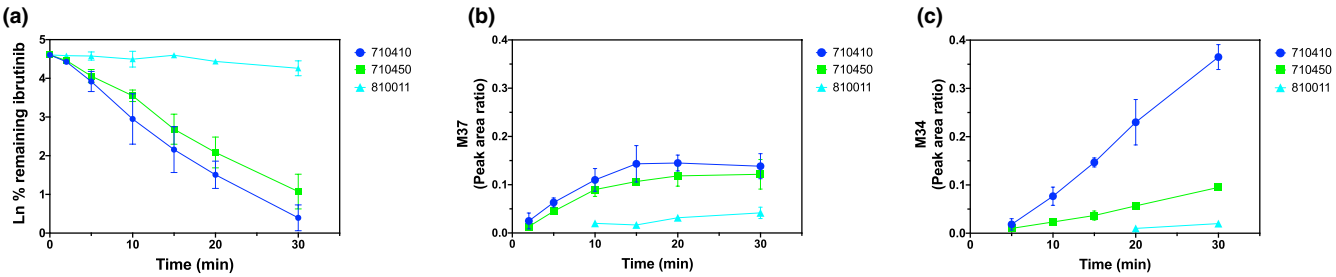

FIGURE 3 Ibrutinib depletion and metabolite formation in single-donor human liver microsomes. Ibrutinib (1 μM) was incubated with human liver microsomes (0.05 mg protein/ml) from three individual donors (3 White male donors: lots 710,410, 710,450, and 810,011) in the presence of NADPH-generating system over time. Samples were analyzed by liquid chromatography-tandem mass spectrometry to determine ibrutinib depletion (a) and formation of M37 (b) and M34 (c). The results shown are from incubations 0, 2, 5, 10, 15, 20, and 30 min. Rates of ibrutinib depletion were calculated using natural log-transformed depletion data (a). Formation of M37 (b) and M34 (c) is presented as the peak area ratio of metabolite to internal standard, d_5-ibrutinib. Results are the mean ± SD for each donor from two experiments conducted in triplicate

physiologically relevant system, we performed a series of experiments using cryopreserved primary human hepatocytes in suspension from 15 individual donors. Donor demographic information (provided by BioIVT) and a summary of CYP3A4, CYP3A5, and CYP3A7 protein concentrations quantified in each donor are presented in Table S2. CYP3A4 protein concentrations varied 135-fold between hepatocyte donors and total CYP3A4 + CYP3A5 protein concentration varied 92.4-fold. CYP3A7 protein (41.7 ± 8.01 pmol/mg protein) was only detectable in donor VYU (1 year old). Midazolam 1′-hydroxylation ranged from 1.32–138.2 pmol/million cells/min between donors

and correlated with CYP3A4 and total CYP3A4 + CYP3A5 protein, but not with CYP3A5 protein content (Figure S3, Table S3). The rates of midazolam 1′-hydroxylation that we measured highly correlated with the lot characterization values for midazolam 1′-hydroxylation reported by the vendor BioIVT (Spearman correlation coefficient $r = 0.95$, $p < 0.0001$; data not shown).

Ibrutinib metabolite formation was examined in the same set of primary human hepatocytes (Figure S4, Table S3). Representative LC–MS/MS chromatograms of ibrutinib metabolites formed in hepatocytes from donor KRX (donor with the highest CYP3A activity) are shown in Figure S5. Formation of M37, M34, and M25 was positively correlated with CYP3A activity (Figure 4a–c). Furthermore, M37 and M25 were strongly correlated with CYP3A4 protein concentration; however, M34 formation was not (Figure 4d–f). Metabolite formation was not associated with CYP3A5 protein concentration (Figure 4g–i).

CYP3A activity (1′-hydroxymidazolam formation) was 3.1-fold higher in hepatocytes from female donors

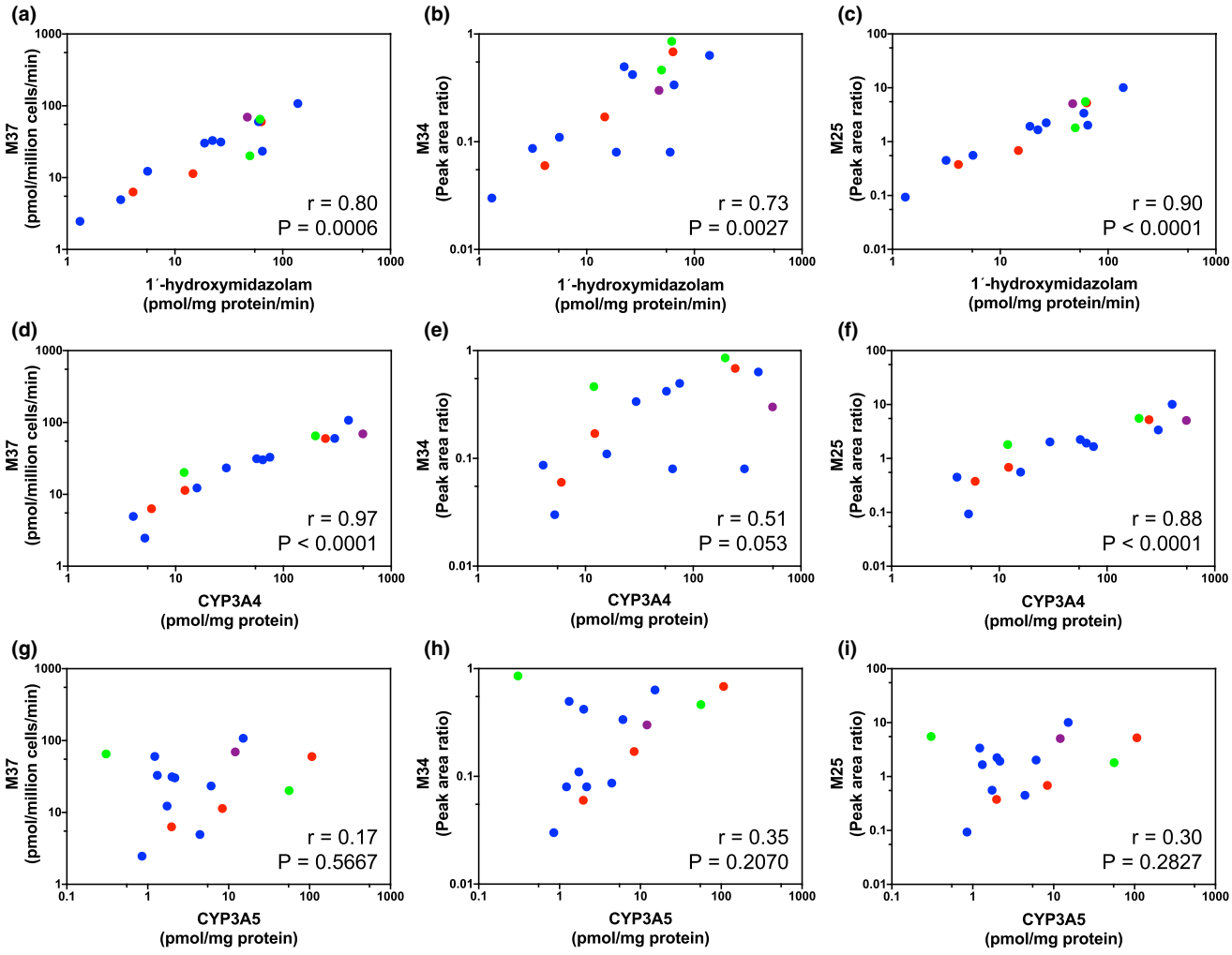

FIGURE 4 Ibrutinib metabolite formation in single-donor human hepatocytes and comparison with CYP3A activity and protein expression. Ibrutinib (5 μM) was incubated with primary human hepatocytes (0.5×10^6 viable cells/ml) from 15 individual donors ($n = 15$) in suspension for 15 min. Formation of M37, M34, and M25 was measured by liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis. Relative levels of metabolite were determined by the peak area ratio of analyte to internal standard, d_5-ibrutinib. Formation of M37 was quantified by LC–MS/MS analysis using a standard curve. Midazolam (2.5 μM) was used as a CYP3A probe substrate, and formation of 1′-hydroxymidazolam was measured by LC–MS/MS analysis. CYP3A4 and CYP3A5 protein concentrations were measured by quantitative targeted absolute proteomic (QTAP) analysis. Correlation of ibrutinib metabolite formation with individual CYP3A activity, as measured by midazolam 1′-hydroxylation (a–c), CYP3A4 protein content (d–f), and CYP3A5 protein content (g–i). Spearman correlation analysis was performed using GraphPad Prism 9 Software, data are graphed on log scale plots. Results are the mean for each donor from one experiment conducted in triplicate. Results for donors VYU and ZWD are the mean from one experiment conducted in duplicate. Self-reported race of donors: blue dots represent Caucasian/White donors; green dots represent African American/Black donors; red dots represent Hispanic donors; and purple dot represents Asian donor

compared with male donors (Figure S6a). Ibrutinib metabolite formation was also higher in hepatocytes from female donors compared with male donors (1.6-fold for M37, 1.8-fold for M34, and 2.1-fold for M25; Figure S6b–d). However, these differences were not statistically significant.

Relationship between plasma 4β-HC/cholesterol ratio and ibrutinib metabolism in hepatocytes from matched donors

Previous clinical studies have demonstrated that plasma 4β-HC concentrations or the plasma 4β-HC/cholesterol ratio is a possible endogenous biomarker of CYP3A activity.[15–18] To evaluate the relationship between plasma 4β-HC/cholesterol ratio and hepatic CYP3A markers, we compared the plasma 4β-HC/cholesterol ratio from individual donors with CYP3A activity and CYP3A4/5 protein concentrations in human hepatocytes from the same matched donors. A representative LC–MS/MS chromatogram of 4β-HC is shown in Figure S7. In addition to 4β-HC, other major hydroxycholesterols in human plasma, such as 4α-HC and 27-HC, were detected. Method validation metrics are shown in Tables S4 and S5. The levels of plasma 4β-HC, cholesterol, and the 4β-HC/cholesterol ratio in individual donors are shown in Table 1. The plasma 4β-HC and cholesterol concentrations and the 4β-HC/cholesterol ratio varied 21.5-fold, 7.1-fold, and 14.6-fold, respectively, between donors.

TABLE 1 Plasma 4β-HC and cholesterol concentration and the plasma 4β-HC/cholesterol ratio in individual donors

Donor	4β-HC (ng/ml)	Cholesterol (mg/ml)	Ratio ($\times 10^4$)
BVT	25.56 ± 1.14	0.94 ± 0.02	0.27
EYN	15.08 ± 0.90	1.10 ± 0.50	0.14
FXF	7.82 ± 0.52	0.73 ± 0.02	0.11
IXN	5.28 ± 0.11	0.78 ± 0.06	0.07
BJY	29.84 ± 2.20	1.65 ± 0.20	0.18
BGY	34.98 ± 0.61	0.96 ± 0.06	0.36
GKP	15.34 ± 0.12	1.32 ± 0.09	0.12
IHB	27.00 ± 3.18	0.67 ± 0.17	0.41
TLK	31.80 ± 0.89	1.70 ± 0.18	0.19
DZS	24.73 ± 0.40	1.25 ± 0.04	0.20
ZWD	29.51 ± 1.34	0.62 ± 0.05	0.48
VYU	14.15 ± 0.50	2.71 ± 0.17	0.05
XKN	21.34 ± 0.80	0.38 ± 0.06	0.56
KRX	113.51 ± 4.00	1.55 ± 0.20	0.73
UJX	47.86 ± 0.81	2.06 ± 0.07	0.23

Abbreviation: 4β-HC, 4β-hydroxycholesterol.

Note: Donors included seven male donors and eight female donors ($n = 15$ total); the median age was 48 years (range 1–63 years). Results represent the mean ± SD of triplicate samples (3 plasma aliquots were processed per donor; see Table S2 for donor demographics).

Correlation analysis revealed a moderate association between plasma 4β-HC/cholesterol ratio and hepatocyte midazolam 1′-hydroxylation among the 15 donors tested (ages 1–63 years; Figure 5a). Plasma 4β-HC/cholesterol ratio did not correlate with CYP3A4, CYP3A5, or total CYP3A4 + CYP3A5 protein concentration (Figure 5b–d) when all 15 donors were evaluated. Next, we performed correlation analysis to determine whether plasma 4β-HC/cholesterol ratio can predict ibrutinib metabolite formation in vitro. We found a moderate correlation between plasma 4β-HC/cholesterol ratio and M25 formation (Figure 6c). M37 and M34 formation was not significantly correlated with plasma 4β-HC/cholesterol ratio (Figure 6a,b).

Age-associated changes in the expression and activity of CYP3A enzymes have been reported to occur during development in neonates and developing infants.[33–37] CYP3A4 activity is reported to reach adult levels at ~1.3 years[36]; therefore, we conducted a secondary analysis excluding the infant donor VYU (1 year old). Exclusion of the infant donor resulted in a significant relationship between plasma 4β-HC/cholesterol ratio, midazolam 1′-hydroxylation, CYP3A4 protein, and total CYP3A4 + CYP3A5 protein concentrations (Figure S8). Furthermore, M37 and M25 formation significantly correlated with plasma 4β-HC/cholesterol ratio (Figure S9a,c), but M34 formation did not correlate with plasma 4β-HC/cholesterol ratio in the remaining 14 donors (Figure S9b).

When comparing donor sex, average plasma 4β-HC/cholesterol ratio was 1.9-fold higher in female donors ($n = 8$) compared with male donors ($n = 7$), but the difference was not statically significant (Figure S10a). However, when donor VYU (1 year old) was excluded from the analysis, female donors ($n = 7$) had significantly higher plasma 4β-HC/cholesterol ratio (2.2-fold) compared with male donors ($n = 7$; Figure S10b).

DISCUSSION

Ibrutinib, an oral covalent inhibitor of BTK used for the treatment of CLL and MCL,[1,2] has low oral bioavailability due to extensive first-pass metabolism by CYP3A, resulting in potential variability in drug response and adverse reactions.[3,4] In the present study, we demonstrated that individual CYP3A activity and expression influence hepatic ibrutinib metabolism. We also evaluated the use of the endogenous CYP3A biomarker plasma 4β-HC/cholesterol ratio as a predictor of ibrutinib metabolism in vitro.

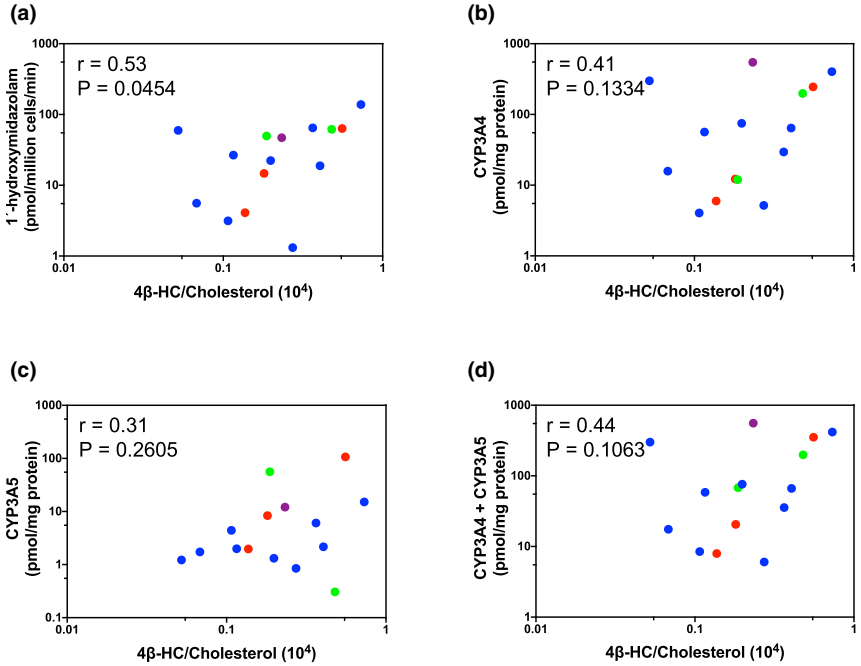

FIGURE 5 Correlation of plasma 4β-HC/cholesterol ratio with CYP3A activity and protein expression in human hepatocytes (ages 1–63 years) from matched donors. Plasma concentrations of 4β-HC and cholesterol from 15 individual donors (ages 1–63 years, $n = 15$) were measured by liquid chromatography-tandem mass spectrometry analysis after derivatization. CYP3A activity was measured by midazolam 1′-hydroxylation, and CYP3A4 and CYP3A5 protein concentrations were measured by quantitative targeted absolute proteomic (QTAP) analysis in primary human hepatocytes from the same matched donors. Correlation of plasma 4β-HC/cholesterol ratio with individual CYP3A activity (a), CYP3A4 protein content (b), CYP3A5 protein content (c), and CYP3A4 + CYP3A5 protein content (d). Results are the mean values for each donor from one experiment conducted in triplicate. Result for donor ZWD is the mean from one experiment conducted in duplicate. Spearman correlation analysis was performed using GraphPad Prism 9 Software, and data are graphed on log scale plots. Self-reported race of donors: blue dots represent Caucasian/White donors; green dots represent African American/Black donors; red dots represent Hispanic donors; and purple dot represents Asian donor

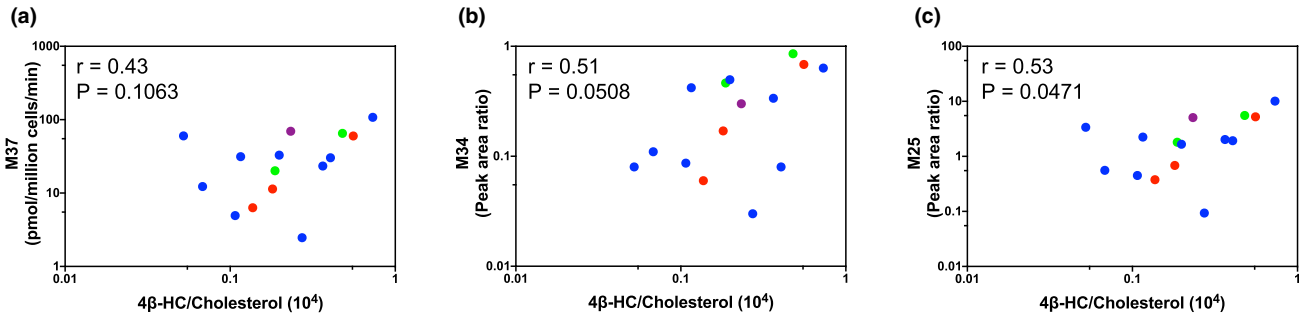

FIGURE 6 Correlation of ibrutinib metabolite formation in single-donor human hepatocytes with plasma 4β-HC/cholesterol ratio. Formation of M37 (a), M34 (b), and M25 (c) in primary human hepatocytes was analyzed for correlation with plasma 4β-HC/cholesterol ratio from the same matched donors. The total number of human hepatocyte donors was 15 ($n = 15$). Plasma concentrations of 4β-HC and cholesterol were measured by liquid chromatography-tandem mass spectrometry analysis after derivatization. Results are the mean values for each donor from one experiment conducted in triplicate. Results for donors VYU and ZWD are the mean from one experiment conducted in duplicate. Spearman correlation analysis was performed using GraphPad Prism 9 Software, and data are graphed on log scale plots. Blue dots represent Caucasian/White donors; green dots represent African American donors; red dots represent Hispanic donors; and the purple dot represents Asian donor

In single-donor human liver microsomes and primary hepatocytes, ibrutinib metabolite formation varied widely between individuals and was highly correlated with hepatic CYP3A activity and CYP3A4 protein concentration. Metabolite formation was moderately correlated with CYP3A5 protein content in human liver microsomes but

not in primary human hepatocytes. These data agree with previous findings indicating that ibrutinib is metabolized primarily by CYP3A4[4] and that CYP3A4 expression varies widely (>100-fold) between individuals.[6] The reported relative contribution of CYP3A5 to the overall ibrutinib metabolism was less than 20% in human liver microsomes.[4] It is possible that secondary (i.e., conjugative) metabolic pathways could be involved in metabolizing ibrutinib in hepatocytes.[4] Collectively, these findings indicate a major role of CYP3A4 and a minor role of CYP3A5 in ibrutinib metabolism.

Plasma 4β-HC is an endogenous CYP3A metabolite of cholesterol, and there is increasing recognition of the potential for the plasma 4β-HC concentrations or normalized 4β-HC/cholesterol ratio to be used as an assessment of hepatic CYP3A activity in clinical settings.[15,17,19,21] Moreover, 4β-HC/cholesterol ratio could be a more accurate marker for hepatic CYP3A activity, especially when plasma cholesterol levels are highly variable between individuals or in patients taking drugs that alter cholesterol concentrations.[15] This led to the hypothesis that plasma 4β-HC/cholesterol ratio could be used to predict individual CYP3A activity and ibrutinib metabolism in vitro. In the present study, plasma 4β-HC/cholesterol ratio moderately correlated with hepatocyte midazolam 1′-hydroxylation. The results from our initial correlation analysis in 15 donors (ages 1–63) revealed a weak to moderate relationship between plasma 4β-HC/cholesterol ratio and ibrutinib metabolite formation in hepatocytes. In a previous clinical study in adult diabetic and non-diabetic subjects, the plasma 4β-HC/cholesterol ratio and the midazolam metabolic ratio varied 6.4-fold and 5.2-fold between subjects, respectively, and the plasma 4β-HC/cholesterol ratio significantly correlated with the midazolam metabolic ratio.[17]

Previous ontogeny studies have demonstrated that age-associated changes in the expression and activity of CYP3A enzymes occur during development.[33–38] CYP3A7 expression and activity are high during early life and rapidly decrease during the first year of life.[33,37,38] Conversely, expression of CYP3A4 is low in the fetus and substantially increases within the first 1–2 years after birth.[33,35] Furthermore, Salem et al. suggested that CYP3A4 metabolic capacity continuously rises from birth to about 1.3 years of age, when it reaches adult levels.[36] Based on these reports, we excluded donor VYU (1 year old) from the analysis and found a significant correlation between ibrutinib metabolite formation (except M34 formation) and plasma 4β-HC/cholesterol ratio. Donor VYU had 5.6-fold lower plasma 4β-HC/cholesterol ratio compared to the average in adult donors; the plasma cholesterol concentration was 2.3-fold higher and the plasma 4β-HC concentration was 2.2-fold lower for donor VYU compared to the average in adult donors. However, the CYP3A4 expression level in donor VYU was relatively high, and midazolam 1′-hydroxylation was 1.5-fold higher in this donor (59.6 pmol/million cells/min) compared with the average in adult donors (38.6 pmol/million cells/min). Furthermore, our data indicate that ibrutinib metabolite formation correlated with CYP3A activity and CYP3A4 protein expression when donor VYU was included in the analysis. The reason for these discrepancies is unclear. It is possible that factors other than CYP3A4 expression contribute to interindividual differences in plasma 4β-HC/cholesterol ratio. CYP3A7 protein (41.7 pmol/mg protein) was detected in donor VYU whereas CYP3A7 was undetectable in the other donors. CYP3A4 and CYP3A7 share 87% amino acid sequence identity and overlapping substrates,[38] and CYP3A7 may contribute to midazolam metabolism in fetal livers.[34] However, CYP3A7 shows a much lower rate of cholesterol metabolism to 4β-HC compared to CYP3A4.[39] Whether CYP3A7 can metabolize ibrutinib will need to be confirmed with future studies.

Previous in vitro studies showed that female subjects have higher CYP3A activity compared with male subjects.[23,40] In the present study, differences in CYP3A activity, as measured by midazolam 1′-hydroxylation, and ibrutinib metabolite formation in human hepatocytes comparing male and female subjects were not statistically significant. Plasma 4β-HC/cholesterol ratio was significantly higher in female subjects compared with male subjects when pediatric donor VYU was excluded from the analysis. The lack of significant differences between male subjects and female subjects with respect to ibrutinib metabolism is consistent with a previous pharmacokinetic study demonstrating similar ibrutinib exposure in male and female patients with CLL.[41] As mentioned earlier, in addition to CYP3A-mediated metabolism, secondary metabolic pathways could be involved in metabolizing ibrutinib in hepatocytes.[4]

A limitation of this study is the small sample size. With the limited donor information provided, we cannot rule out the possibility of altered CYP3A activity or plasma 4β-HC and/or cholesterol concentrations due to medications, dietary supplements, or other factors. In addition, the hepatocyte samples in this study were from cadaveric donors, and the quality of cryopreserved human hepatocytes may vary. It is possible that our findings with hepatocytes in vitro may not reflect in vivo CYP3A expression and activity; however, the variation in CYP3A4 expression observed in the present study is consistent with previous literature.[6] Another limitation is that the ibrutinib concentration (5 μM) tested in the present in vitro study was higher than the expected maximum in vivo concentrations (0.4–1 μM) of ibrutinib when taken at therapeutic doses.[4,32] The Michaelis constant (K_m)

for CYP3A4-mediated metabolism of ibrutinib to M37 was reported to be 11.95 μM for recombinant wild-type CYP3A4[42]; however, the kinetics of ibrutinib metabolism likely vary between individuals. In the subset of human liver microsomal samples ($n = 3$) tested at 1 μM ibrutinib, higher rates of ibrutinib metabolic clearance were associated with high CYP3A activity. Additional studies are needed to determine if individual plasma 4β-HC/cholesterol ratio can predict ibrutinib clearance at therapeutically relevant concentrations.

In addition to the liver, intestinal CYP3A4 is involved in first-pass metabolism of many drugs.[43] This study did not investigate intestinal metabolism of ibrutinib; however, CYP3A-mediated hepatic metabolism has been reported to play a larger role in first-pass metabolism of ibrutinib compared to intestinal metabolism.[22,44] Moreover, plasma 4β-HC is thought to primarily reflect hepatic CYP3A activity.[45]

In summary, our findings indicate that CYP3A-specific ibrutinib metabolite formation is highly correlated with CYP3A activity and CYP3A4 protein concentrations in both human liver microsomes and primary human hepatocytes from individual donors. The plasma 4β-HC/cholesterol ratio was also correlated with the formation of specific ibrutinib metabolites and CYP3A activity in primary human hepatocytes from matched non-infant donors. Future clinical studies are needed to evaluate the utility of plasma 4β-HC/cholesterol ratio to predict individual ibrutinib metabolism and exposure in vivo.

AUTHOR CONTRIBUTIONS

J.L., J.K.F., and K.D.J. wrote the manuscript. J.L. and K.D.J. designed the research. J.L. and J.K.F. performed the research. J.L. and J.K.F. analyzed the data. J.K.F. and P.C.S. contributed new reagents/analytical tools.

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CONFLICT OF INTEREST

The authors declared no competing interests for this work.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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