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Short Communication

Applying Stable Isotope Labeled Amino Acids in Micropatterned Hepatocyte Co-Culture to Directly Determine the Degradation Rate Constant for CYP3A4

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List of Abbreviations

Cytochrome P450, CYP; degradation rate constant, k_{deg} ; drug-drug interaction, DDI; stable isotope labeled amino acids in culture, SILAC; stable isotope label, SIL; half-life, $t_{1/2}$

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Abstract

The rate of enzyme degradation (k_{deg}) is an important input parameter for the prediction of clinical drug-drug-interactions (DDI) that result from mechanism-based inactivation or induction of cytochrome P450s. Currently, a large range of reported estimates for CYP3A4 enzyme degradation exists, and consequently, large uncertainty exists in steady-state predictions for DDI. In the current investigations, stable isotope labeled amino acids in culture (SILAC) was applied to a long-lived primary human hepatocyte culture, HepatoPac, to directly monitor the degradation of CYP3A4. This approach allowed selective isotope labeling of a population of de novo synthesized CYP3A4, and specific quantification of proteins with mass spectrometry to determine the CYP3A4 degradation within the hepatocytes. The k_{deg} estimate was $0.026 \pm 0.005 \text{ h}^{-1}$. This value was reproduced by cultures derived across four individual donors. For these cultures, data indicated that CYP3A4 mRNA and total protein expression (i.e. labeled and not labeled P450s), and activity were stable over the period where degradation had been determined. This k_{deg} value for CYP3A4 was in good agreement with recently reported values that used alternate analytical approaches, but also employed micropatterned primary human hepatocytes as the in vitro model.

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Introduction

Understanding the mechanism of inhibition is important when assessing the magnitude of CYP-mediated DDI. For example, it has been shown that the extent of DDI is underpredicted when a quasi-irreversible inhibitor such as troleandomycin or an irreversible inhibitor such as mibefradil are assumed to block CYP3A4 via a reversible process (Ito et al., 2004; Fahmi et al., 2008).

Quantitative assessment of irreversible inhibition takes into account the concentration of the perpetrator and kinetic parameters that characterize the *in vitro* potency of the perpetrator on the enzyme (Kitz and Wilson, 1962; Silverman, 1995). In addition, because the perpetrator inactivates the protein to affect its steady-state concentration, CYP degradation rate constant (k_{deg}) is another parameter that requires consideration (Mayhew et al., 2000). It is typically assumed that k_{deg} is a constant value; however, sensitivity analysis have demonstrated that differing values of k_{deg} can have a profound effect on the extent of DDI predictions (Galetin et al., 2006; Wang, 2010). Thus, ambiguity in which k_{deg} value is most appropriate for the quantitative assessment of irreversible inhibition remains a confounding factor for high confidence in the prospective prediction of DDI.

A wide range of k_{deg} values have been reported for CYP3A4 (Yang et al., 2008). Because *in vivo* measurement in human is challenging, several *in vitro* matrices have been used to determine k_{deg} values. Though hepatocytes may be the best characterized model for the function and dynamics of the liver, the observed declines in CYP protein expression and activities in regular short-lived cultures have limited their suitability for determining the true half-life of CYP turnover (Correia, 1991). The development and adoption of long-lived hepatocyte models have been prompted by their superiority in determining metabolic stabilities and generating metabolites for low intrinsic clearance compounds (Chan et al., 2013; Ballard et al., 2016).

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Therefore, a long-lived hepatocyte model such as the HepatoPac, which is a specialized co-culture system that sustains CYP activities for several days (Khetani and Bhatia, 2008), may be a more suitable model for characterizing the dynamics of CYP turnover.

Stable isotope labeling by amino acids in cell culture (SILAC) with HepatoPac was applied to determine k_{deg} . SILAC utilizes the cellular machinery for de novo protein synthesis to take up isotopically-labeled essential amino acids from media and incorporate them into the proteome; thus, creating a population of proteins from determined culture period to be identified. Mass spectrometry (MS) is then used to individually monitor the labeled and unlabeled proteins based on mass shifts, and the dynamics of protein expression can be quantified. SILAC was first demonstrated to describe protein up-regulation during muscle differentiation (Ong et al., 2002) and was subsequently applied to investigate protein turnover in yeast (Pratt et al., 2002) and human adenocarcinoma cells (Doherty et al., 2009). CYP3A4 is responsible for metabolizing the largest proportion of drugs and, therefore, has been the focus for many DDI studies. In the current work, CYP3A4 k_{deg} was determined by measuring protein turnover.

Material and Methods

Materials Used. $^{13}C_6$ arginine and $^{13}C_6$ lysine were purchased from Cambridge Isotope Labs. Testosterone, 6 β -hydroxytestosterone (6HT), and d₇-6 β -hydroxytestosterone were purchased from Sigma, Cerilliant (Round Rock, TX) and Corning/Gentest (Woburn, OH), respectively.

Stable isotope labeled peptides (Arg($U^{13}C_6$, $^{15}N_4$)) used as internal standards were custom synthesized with purities >95% by CPC Scientific Inc (Sunnyvale, CA) or New England Peptide (Gardner, MA). All other chemicals were purchased from vendors at the reagent grade or better.

Cell Culture. Human HepatoPac cultures were prepared and maintained by Hepregen (Medford, MA). Four individual donors of hepatocytes (3121A, 3121B, 4202, and 4297, donor information

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provided as Supplemental Table 1) were tested with 32,000 hepatocytes seeded to each well of a 24-well plate. In preliminary experiments, cell samples at days 3 and 7 following set up of the cultures were collected. Increasing incorporation of heavy amino acid was observed between days 3 and 7 and to a much lesser extent between days 7 and 10, indicating that steady-state had been reached by day 7 (data not shown). However, to ensure steady-state conditions, the co-cultures were maintained with the stable isotope label for 10 days before initiating the experiment by replacing with non-stable isotope label. For seeding and maintenance of the cultures until day 10 of culture, the proprietary seeding and maintenance media were prepared by replacing the lysine and arginine with their stable isotope $^{13}\text{C}_6$ labeled (SIL) equivalents. Media changes were conducted every other day. On day 10 of culture, the SIL-containing media was replaced with the non-SIL-containing regular maintenance media and changed every other day. At the termination of a culture, CYP3A4 activities were determined followed by collection of HepatoPac lysates. In triplicate, four wells were pooled in a single volume for each donor at each time point. The media was removed from the culture and the cells in each well were washed with PBS. Collection buffer (200 μL ; 1 mM EDTA, 0.1 mM dithiothreitol, 2 $\mu\text{g}/\text{mL}$ leupeptin, 250 mM sucrose, and 150 mM KCl, pH 7.4) was added to one cell and the well contents were scraped. The cell suspension was transferred to the next well and collection repeated until four wells were pooled. The wells were washed with an additional 200 μL volume of buffer to collect residual cells, and the cell suspension and wash were pooled and stored at -80C until analysis.

CYP3A4 Activity Measurement. CYP3A4 activity was measured by monitoring the formation of 6HT from each cell culture immediately prior to cell collection and lysis as described previously (Halladay et al., 2012). Testosterone solution was prepared in serum-free media at a final concentration of 200 μM . Media was removed from the wells and 300 μL of testosterone

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solution was added to the hepatocytes. The plates were incubated for one hour at 37°C with 10% CO₂. The supernatants were collected and stored at -80°C until analysis. For analysis, the HepatoPac testosterone supernatants were diluted 50x with water:acetonitrile (ACN) (3:1 by volume) that contained 1 μM d₇-6β-hydroxytestosterone as an internal standard. The formation of 6HT was measured by LC-MS/MS by selected reaction monitoring (SRM) in positive ion electrospray mode (305.5→269.1 and 312.5→276.1 (d₇ internal standard)) using a 5500 QTRAP (AB Sciex, Foster City, CA) attached to a CTC PAL autosampler (LEAP Technologies, Carrboro, NC) with a 1290 pump (Agilent, Santa Clara, CA). Samples were injected to a Kinetex XB-C18 1.7-μm, 50 x 2.1 mm, column (Phenomenex, Torrance, CA) and chromatographed with a gradient elution of water and ACN containing 0.1% formic acid. The flow rate was 0.75 mL/min over the total run-time of 3.5 min. Concentrations were calculated by comparing the samples against a calibration curve of 6HT constructed by weighted (1/x²) linear regression of analyte-to-internal standard peak area ratio versus nominal concentrations (0.1-100 μM). In vitro formation rates were calculated from the concentration of metabolite formed in 60 min by 32,000 hepatocytes and scaled to one million hepatocytes.

LC-MS/MS Measurement of P450 Proteins. Pellets of the HepatoPac lysates (125 μL) were prepared by centrifuging aliquots of the samples at 9000x g for 20 min at 4°C. The supernatants were removed, which in pilot experiments showed no detectable P450 protein. The trypsin digestion procedure employed is similar to that reported by other investigators for the absolute quantification of hepatic P450s (Wang et al., 2008; Michaels and Wang, 2014). The sample pellets were resuspended in ammonium bicarbonate buffer (50 mM) and proteins (target 40 μg) were denatured by adding dithiothreitol (13.3 mM final) and heating to 60°C for 60 min. Samples were alkylated with the addition of iodoacetamide (19.3 mM final) and being left in the

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dark at room temperature for 30 min, then digested with trypsin (400 ng, 1:100 trypsin:protein) overnight at 37°C. An internal standard solution of ACN containing 0.2 fmol of $^{13}\text{C}_6$ ^{15}N -labeled peptide (EVTNFLR) was added to the samples and then the samples were centrifuged and the supernatants were removed for analysis. Samples were analyzed by LC-MS/MS using the setup as described for monitoring 6HT. Samples were injected to a Kinetex XB-C18 2.6- μm , 100 x 2.1 mm, column (Phenomenex). The solvent gradient started at 95% A (water containing 0.1% formic acid)/5% B (ACN containing 0.1% formic acid), which was held for 2 min, then increased to 20% B at 25 min and 50% B at 28 min before the column was flushed with 98% B and re-equilibrated at 5% B. The flow rate was 0.65 mL/min over the total run-time was 35 min. Four unique peptides (amino acids 131-141, 244-250, 380-390, and 407-413) were selected to monitor CYP3A4 based on their selectivity, stability, and reproducible release with trypsin digestion. The identity of the peptides and their SRM parameters used for LC-MS/MS protein measurements are provided in Supplemental Table 2. SRM data were imported into Skyline for processing. The most intense SRM was used for quantitation and two other SRMs were used as qualifier transitions (Supplemental Figure 1). To calculate CYP expression, the ratio of the non-labeled or SIL-containing peptide to the internal standard peptide was multiplied by 0.2 fmol (single point calibration) and scaled to one million hepatocytes.

mRNA Determination. Aliquots of the HepatoPac lysates were used for determination of P450 mRNA levels. The cell lysates were combined with 100 μL of Procarta® cell lysis buffer. A QuantiGene Plex 2.0 Assay Kit based on the bead technology (Panel #11477) from Affymetrix (Santa Clara, CA) was used in a similar manner as has been previously described (Halladay et al., 2012). mRNA measurements for CYP3A4 were normalized to those for GADPH, and the normalized expression reported relative to Day 10 for the same hepatocyte donor.

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Data Analysis and Half-Life Calculations. For each sample at a specific time point, the peak areas for corresponding nonlabeled and SIL-containing peptides were summed. The peak area for the SIL-containing peptide, which corresponded to the pre-existing CYP, was then calculated as a percentage of the total CYP. The resulting percentage of existing peptide/protein remaining was transformed by taking the natural logarithm (\ln) and plotted against time. Half-life ($t_{1/2}$) values were derived ($t_{1/2} = -\ln(2)/k$) from the slope of the line (k) determined by linear regression using Prism 6 (GraphPad Software, San Diego, CA). When calculating the slope, each measurement was normalized by the total protein level and degradation was calculated from the percent depletion over time as required for this first order kinetic process. Therefore, determinations of k_{deg} and $t_{1/2}$ were independent of the absolute expression levels since the percentage of population protein (% heavy peptide) were determined and used for calculating the rates of protein turnover.

Results and Discussions

Stability Assessment of HepatoPac Cultures. There have been multiple publications which have demonstrated that P450 levels in HepatoPac cultures reach steady-state by Day 10. For example, HepatoPac co-cultures have been extensively characterized to show that there is sustained and stable expression and activities of P450s by Day 7 (product information sheet). In addition, in their determination of k_{deg} , Ramsden et al., (2015) and Dixit et al., (2016) maintained the HepatoPac cultures for 9 days before starting their treatment. Ramsden et al., provided more detail to confirm that P450 was at steady-state by showing that there were no significant differences in CYP3A4 activity from Day 7-28 (i.e. <20%), but that the biggest differences (i.e. >40%) were observed only between Day 7-8 and after Day 20. In addition to these reports, preliminary experiments from this laboratory demonstrated stable cultures in SILAC conditions

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with visual inspection of cell morphology and measurement of cell viability by ATP presence (in-house data). In the current experiments, mRNA, protein, and activity of CYP3A4 were monitored to confirm the stability of the hepatocyte culture over the experimental time frame (Figure 1 and Supplemental Table 3). The mean CYP3A4 content in HepatoPac was estimated to be 401-703 pmol per million hepatocytes, assuming 32000 hepatocytes per HepatoPac well. The observed rates for 6HT formation among the four donors were 3.5-4.8 nmol min⁻¹ per million hepatocytes. In general, cultures with higher CYP3A4 content had correspondingly higher rates for 6HT formation. It is worth mentioning that since β -glucuronidase and sulfatase were not added to the incubation, the amount of total 6HT may be underestimated. However, the stability of CYP3A4 mRNA and protein give some confidence that 6HT is likely reflecting CYP3A4 activity. These readouts demonstrated that the cultures had achieved a stable expression level and function of P450s by day 10. mRNA and protein expression were not measured directly on the cultures, but instead, following cell lysate collection, pooling, and processing, so inter-sample variability was expectedly higher. This variability, however, would not bias the determination of degradation rates since the measured stable isotope-containing peptide were normalized to the total measured peptide for each sample.

Incorporation of SIL to CYP3A4. At day 10 of HepatoPac culture (initial day of sample collection and protein measurement), the mean incorporation of ¹³C₆-lysine peptides was 84.8%, and for the ¹³C₆-arginine peptide was 66.2%. At day 13 of culture (final day of sample collection and measurement), the incorporation of ¹³C₆-lysine peptides was 15.2%, and for the ¹³C₆-arginine peptide was 6.0%. There were no trends observed that indicated clear differences among donors. Variability among donors in incorporation of SIL (calculated as CV) were less than 5% on day 10 and less than 10% on day 13.

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Determination of CYP3A4 Degradation Rate. The plots for rates of decline in the SIL-containing peptides for the four HepatoPac donors are presented in Figure 2 and a summary of data is provided in Supplemental Table 4. Between the four hepatocyte donors, the variability was minimal and there was good consistency between the three $^{13}\text{C}_6$ -lysine containing peptides. Given the apparent lower and more variable incorporation of $^{13}\text{C}_6$ -arginine, it was not included for calculations of $t_{1/2}$ and degradation rates. The apparent $t_{1/2}$ of CYP3A4 protein in HepatoPac was 29.7 ± 2.3 , and corresponding estimates for k_{deg} was 0.023 ± 0.002 (Table 1).

Degradation rates for CYP3A4 were recently reported by two other research groups (Ramsden et al., 2015; Dixit et al., 2016). Coincidentally, these investigators also utilized HepatoPac. This reflects the recent development and adoption of long-lived primary human hepatocyte cultures. Whereas the protein dynamics were directly measured in cultures in the current study, the other investigators determined k_{deg} values by mRNA and/or activity changes utilizing HepatoPac during the loss and successive recovery of CYP following treatment with suppressors or inducers. Despite these differences, there is remarkable agreement in determinations with the $t_{1/2}$ of CYP3A4 ranging 22-49 h. The convergence of values for k_{deg} CYP3A4 from various approaches and multiple labs provides added confidence in these determinations, especially in light of the 3-fold range of previously reported estimates for CYP3A4 enzyme degradation (Wong, 2011). The limits for the translatability of this k_{deg} value for steady-state predictions of DDI will need to be determined since it currently reflects a single in vitro model and a limited number of hepatocyte donors. However, the current and apparently consensus k_{deg} values should allow future work to focus on systematically evaluating the sensitivity of the predictive models for DDI to other parameters. Towards advancing the understanding of the relevance of the in vitro k_{deg} values in HepatoPac, our laboratory is currently applying SILAC to investigate if there

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is any potential impact of perpetrators such as irreversible inhibitors and inducers on CYP3A4 k_{deg} in this model. In addition, in vivo SILAC experiments are being conducted in animal models expressing human orthologs of P450 proteins to determine k_{deg} values.

The presented methodology has wide applicability to determine degradation rates for many proteins of interest and is amenable to multiplexing to measure several target proteins in each study. The current results reveal HepatoPac to be a suitable stable cell system that can be sustained over several protein half-lives. Adequate incorporation of the SIL into the proteome depends on the rates of protein synthesis, degradation, and turnover. Within a 10-day period of HepatoPac culture stabilization, high incorporation ($\geq 85\%$) of peptides for lysine incorporation was achieved. This incorporation and the subsequent degradation were largely reproducible between hepatocyte donors, which confirmed that the machinery for protein turnover had been well-maintained throughout hepatocyte isolation, preservation, and culture. This also suggested that HepatoPac may be a suitable model to investigate modulation of de novo protein synthesis and degradation by drugs or other treatment and we are currently undertaking such investigations. An observation that should be considered in subsequent experiments is lower and more variable incorporation of arginine compared to lysine. This likely is due to an alternate source of arginine within the hepatocyte such that cells were not restricted to exclusive use of exogenously supplemented arginine, suggesting the limited utility of the arginine label. In the case of CYP3A4 degradation, comparable estimates for k_{deg} between the current and previous methods indicate that mRNA and enzymatic activity may be appropriate surrogate measurements for protein dynamics. However, there may be limitations on the universal application of the previous methods to study protein kinetics. In particular, because the recovery of CYP3A4 expression was monitored following varying the in vitro conditions via inhibition or

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induction, these approaches do not differentiate between pre-existing and newly formed proteins. Therefore, measurement of k_{deg} can be inaccurate if the perpetuating compound affect the protein synthesis rate. Moreover, the previous methods relied on well-characterized selective substrates, inducers, and/or inhibitors of CYP3A4. For proteins that have been rarely studied, these tools may not yet be identified. In these cases, direct protein measurement, as applied in the current study, may be a preferable strategy. Further, the ability to multiplex the peptide analysis, especially if a non-targeted proteomics approach is taken, presents an opportunity to simultaneously obtain a comprehensive profile of cellular protein dynamics.

In this report, the utility of long-lived hepatocyte co-cultures to directly measure the turnover of P450s by applying SILAC and mass spectrometry was demonstrated. In concept, this approach is immediately applicable to other drug metabolizing enzymes and transporter proteins that maintain expression and functionality in these in vitro models. The estimate for the $t_{1/2}$ of CYP3A4 of 29.7 h agrees remarkably well with recently reported values by other investigators who applied HepatoPac technology to monitor protein dynamics by alternate approaches.

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Authorship Contributions:

Participated in research design: Takahashi, Shahidi-Latham, Wong, Chang

Conducted experiments: Takahashi, Wong

Performed data analysis: Takahashi, Wong

Wrote/contributed to the writing of the manuscript: Takahashi, Shahidi-Latham, Wong, Chang

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Figure 1. Representative CYP3A4 stability data for human HepatoPac cocultures through the time period used for P450 degradation rate determinations. Data are mean from n=3 replicates with error bars indicating standard deviations. mRNA expression are normalized to Day 10 (left axis) and CYP3A4 activity (measured as testosterone-6 β -hydroxylation) and protein expression (right axis) are normalized to one million hepatocytes. Protein expression were determined by MS-determined abundance of peptide EVTNFLR relative to stable isotope ($^{13}\text{C}_6$ ^{15}N) labeled peptide used as an internal standard calibrator. Data shown are for one hepatocyte donor (3121A).

Figure 2. P450 degradation as measured by SIL-containing peptide depletion for four donors with protein specific peptides: CYP3A4, (A) SLLSPTFTSGK, (B) EVTNFLR, (C) VEINGMFIPK, (D) YWTEPEK. Labeled peptide abundances are expressed as percentages of the total measured peptide, n=3 per time point with error bars representing standard deviation. Days are marked from the start of the HepatoPac cultures with SIL amino acids supplemented for Days 0 to 10. Each line represents the linear regression for an individual donor from which the slope was used to calculate the protein t $_{1/2}$.

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Table 1. Degradation rates (k_{deg}) and half-life ($t_{1/2}$) determined using HepatoPac-SILAC for CYP3A4. Data are reported as mean and standard deviation calculated from four donors.

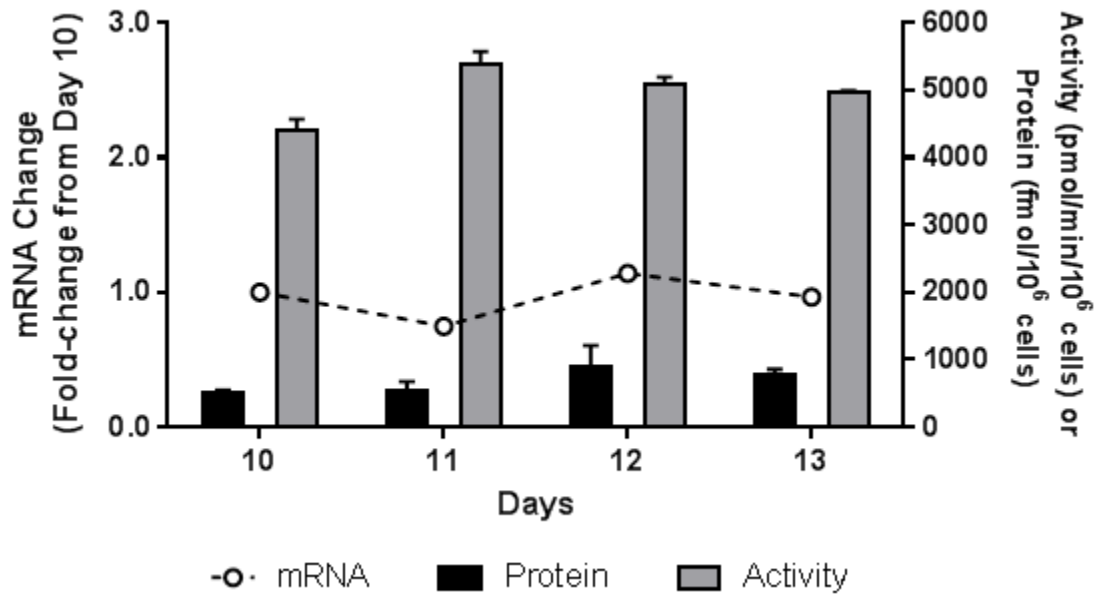
P450	Peptide ^b	k_{deg} (h^{-1})	$t_{1/2}$ (h)
CYP3A4 ^a	SLLSPTFTSGK	0.023 ± 0.002	29.6 ± 1.9
	DVEINGMFIPK	0.023 ± 0.001	29.6 ± 1.0
	YWTEPEK	0.023 ± 0.001	29.8 ± 0.8

^a Average k_{deg} and $t_{1/2}$ determined for the three non-arginine containing CYP3A4 peptides were $0.023 \pm 0.002 \text{ h}^{-1}$ and $29.7 \pm 2.3 \text{ h}$, respectively. The k_{deg} and $t_{1/2}$ for the arginine containing peptide (EVTNFLR) was $0.033 \pm 0.005 \text{ h}^{-1}$ and $21.0 \pm 2.6 \text{ h}$, respectively. Recent k_{deg} and $t_{1/2}$ determinations for CYP3A4 using HepatoPac by mRNA following siRNA or IL-6 inhibition were 0.0240 h^{-1} and 28.9 hr , respectively (Ramsden et al, 2015); and by mRNA following induction were 0.0261 h^{-1} and 26 h , and 49 h by activity following rifampicin induction (Dixit et al, 2016).

^bThe selected reaction-monitoring parameters of the specific peptides used for LC-MS/MS protein measurements are provided in Supplemental Table 2.

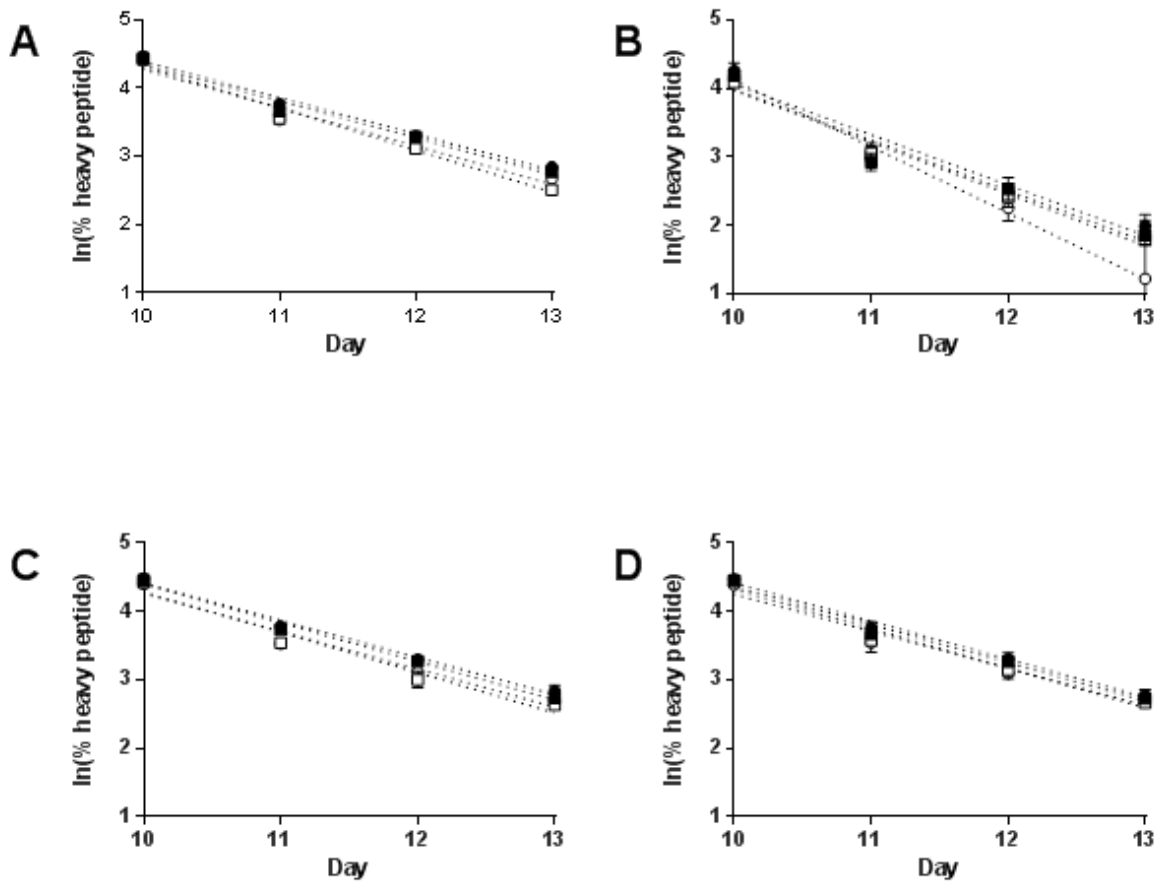
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Figure 1



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Figure 2



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Supplemental Materials

**Applying Stable Isotope Labeled Amino Acids in Micropatterned Hepatocyte Co-Culture
to Determine the Degradation Rate Constant for CYP3A4**

Supplemental Table 1. Donor information for hepatocytes used for HepatoPac cultures.

Donor	3121A	3121B	4202	4297
Sex	Female	Female	Female	Female
Age	52	48	39	54
Height/Weight	65" / 48.2 kg	66" / 59 kg	63" / 90 kg	
Cause of Death	Anoxia, 2nd to Cardiac Arrest	Trauma	Anoxia, 2nd to Cardiac Arrest	Stroke
Ethnicity	Caucasian	Polynesian	Caucasian	Caucasian
Medical History	COPD. No EtOH; Tobacco: 2ppd x 35 years, Drugs: Marijuana Daily	Diverticulitis w/ Surgery. EtOH: Mild	HTN x 3yrs - non-compliant. EtOH: Occasionally; Tobacco: 1/2ppd x 10 years; Drugs: abused Rx narcotics, smoked marijuana.	No drug or tobacco use, 1 glass of wine per day

Supplemental Table 2. Summary of tryptic peptides and their selected reaction-monitoring parameters used for LC-MS/MS protein measurements. Stable isotopes were at the terminal lysine or arginine for stable isotope labeled (SIL) and internal standard (IS) peptides.

P450	Peptide	Q1 (no SIL/SIL)	Q3 (no SIL/SIL)	Observed Retention time (min)	DP	CE
	SLLSPTFTSGK	569/572 ⁺⁺	824/830 ⁺ (y8)	18.4	73	29
	EVTNFLR	439/442 ⁺⁺	650/656 ⁺ (y5)	14.3	63	25
CYP3A4	EVTNFLR (IS)	444 ⁺⁺	660 ⁺ (y5)			
	DVEINGMFIPK	631/634 ⁺⁺	806/812 ⁺ (y7)	25.0	77	32
	YWTEPEK	476/479 ⁺⁺	603/609 ⁺ (y5)	9.2	66	26

+, ++, +++ indicates singly, doubly, and triply-charged ions, respectively.

Supplemental Table 3. CYP3A4 stability data (mRNA, protein expression, and enzymatic activity) for four HepatoPac donors for Days 10-13 of SILAC culture.

CYP3A4 mRNA (GAPDH normalized)				
Day	3121A	3121B	4202	4297
10	0.71±0.03	0.29±0.02		
11	0.53±0.02	0.30±0.03		
12	0.81±0.04	0.52±0.03		
13	0.69±0.05	0.49±0.08		
CYP3A4 protein (fmol per 10 ⁶ cells)				
Day	3121A	3121B	4202	4297
10	510±256	290±51	347±23	183±55
11	607±233	566±102	446±137	362±54
12	909±237	846±197	797±234	594±105
13	787±98	801±183	490±76	465±66
CYP3A4 activity (pmol 6β-hydroxytestosterone mg ⁻¹ min ⁻¹)				
Day	3121A	3121B	4202	4297
10	4406±445	3328±322	3217±293	2380±289
11	5380±721	4479±440	3717±334	2988±217
12	5100±541	4674±604	4340±367	4161±284
13	4961±509	4133±662	5089±273	4368±275

Data are reported as mean±standard deviation. mRNA was determined on n=3 pooled cultures (4 wells per pool) for each day following hepatocyte collection. Protein was determined on n=3 pooled cultures (4 wells per pool) for each day following hepatocyte collection. Activity was determined on n=12 wells for each day prior to hepatocyte collection.

Supplemental Table 4. Calculated $t_{1/2}$ (h) and k_{deg} (h^{-1}) values from individual donors for CYP3A4.

P450	Peptide	Donor	Slope	k_{deg}			$t_{1/2}$		
				(h^{-1})	mean	SD	(h)	mean	SD
CYP3A4	SLLSPTFTSGK	3121A	-0.5410	0.0225	0.023	0.002	30.7	29.6	1.9
		3121B	-0.6174	0.0257			26.9		
		4202	-0.5349	0.0223			31.1		
		4297	-0.5621	0.0234			29.6		
	EVTNFLR	3121A	-0.7411	0.0309	0.033	0.005	22.4	21.0	2.6
		3121B	-0.7566	0.0315			22.0		
		4202	-0.7381	0.0308			22.5		
		4297	-0.9685	0.0404			17.2		
	DVEINGMFIPK	3121A	-0.5653	0.0236	0.023	0.001	29.4	29.6	1.0
		3121B	-0.5892	0.0246			28.2		
		4202	-0.5427	0.0226			30.7		
		4297	-0.5534	0.0231			30.1		
	YWTEPEK	3121A	-0.5588	0.0233	0.023	0.001	29.8	29.8	0.8
		3121B	-0.5778	0.0241			28.8		
		4202	-0.5562	0.0232			29.9		
		4297	-0.5424	0.0226			30.7		
Mean, all CYP3A4 peptides				0.026			27.5		
SD, all CYP3A4 peptides				0.005			4.3		
Mean, three lysine-containing CYP3A4 peptides				0.023			29.7		
SD, three lysine-containing CYP3A4 peptides				0.002			2.3		

Supplemental Figure 1. Representative SRM chromatograms showing CYP3A4 peptide, SLLSPTFTSGK, for one HepatoPac donor over Days 10-13 with SILAC. Top trace shows transitions monitored for non- $^{13}\text{C}_6$ -lysine containing peptide (m/z 569.3 (doubly charged)) and bottom trace shows transitions monitored for “heavy” $^{13}\text{C}_6$ -lysine containing peptide (m/z 572.3 (doubly charged)) for k_{deg} determination.

