**Derivation of CYP3A4 and CYP2B6 Degradation Rate Constants in Primary Human Hepatocytes: A siRNA-Silencing-Based Approach**

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The first-order degradation rate constant (*kdeg*) of cytochrome P450 (CYP) enzymes is a known source of uncertainty in the prediction of time-dependent drug-drug interactions (DDIs) in physiologically-based pharmacokinetic (PBPK) modelling. This study aimed to measure CYP *kdeg* using siRNA to suppress CYP expression in primary human hepatocytes followed by incubation over a time-course and tracking of protein expression and activity to observe degradation. The magnitude of gene knockdown was determined by qPCR and activity was measured by probe substrate metabolite formation and CYP2B6-Glo™ assay. Protein disappearance was determined by Western blotting. During a time-course of 96 and 60 hours of incubation, over 60% and 76% mRNA knockdown was observed for CYP3A4 and CYP2B6, respectively. The *kdeg* of CYP3A4 and CYP2B6 protein was 0.0138 h-1 (± 0.0023) and 0.0375 h-1 (± 0.025), respectively. The *kdeg*derived from probe substrate metabolism activity was 0.0171 h-1 (±0.0025) for CYP3A4 and 0.0258 h-1 (± 0.0093) for CYP2B6. The CYP3A4 *kdeg* values derived from protein disappearance and metabolic activity were in relatively good agreement with each other and similar to published values. This novel approach can now be used for other less well-characterised CYPs.

**Keywords:** Cytochrome P450; drug-drug interactions; degradation rate constant; human hepatocytes; mechanism-based inhibition; physiologically-based pharmacokinetic modelling; small-interfering RNA; half-life; time-dependent inhibition

**Introduction**

Cytochrome P450 (CYP) enzymes are drug-metabolising enzymes (DME) indicated in many clinically relevant drug-drug interactions (DDIs). Any alterations to their activity through induction, inactivation or inhibition can manifest to changes in pharmacokinetic (PK) profiles of victim substrate drug(s) [1,2]. DDIs can bring about an increase or decrease in victim drug exposure, and thus a change in potential for therapeutic and adverse effects. Approaches to assessing DDIs have traditionally been carried out *in vitro* and *in vivo* using preclinical species, and require extrapolation to the human *in vivo* situation [3]. Where preclinical studies indicate DDI liability, studies have also been carried out *in vivo* in humans by measuring PK profiles in clinical trials, however these invoke huge costs and face complications in ethical approval [4]. Increasingly, regulators have advocated the use of *in silico* approaches to predict DDIs to be used either as an alternative to, or alongside ongoing clinical studies [5–7]. Such methods include physiologically-based pharmacokinetic (PBPK) modelling, which integrates physiological system-specific parameters with drug-specific parameters derived from *in vitro* and *in vivo* experiments. Once a model has been adequately verified and deemed ‘fit-for-purpose’, the impact of intrinsic and extrinsic sources of variability in PK can be predicted, including DDIs [8,9].

At the kinetic level, CYP enzyme abundance at steady-state comprises a continuous turnover of proteins that balance zero-order *de novo* synthesis and first-order degradation [10]. However many factors such as drugs, hormones and foods can induce or inhibit CYP expression [11], thus disrupting protein abundance. Accordingly, the time required to return to steady-state will be dependent on its rate of degradation [12]. The CYP enzyme degradation rate constant, *kdeg*, is one important system parameter that gives rise to the timescale of interactions, required for time-dependent DDIs [12]. Owing to the challenges with methodology and sample size, there is a lack of consensus on turnover half-life (*t1/2*) and a paucity of reported *kdeg* values for many of the CYPs involved in clinically relevant DDIs. This places a significant limitation on the accurate prediction of changes in drug concentration-time profiles associated with interactions involving enzyme mechanism-based inhibition (MBI) and/or induction [13]. Inaccurate *kdeg* continues to be a source of error andseveral sources have documented the importance of accurate values in facilitating predictions of DDI magnitudes [13–15].

The measurement of *kdeg* values should ideally be achieved by specific labelling of enzyme in humans *in vivo*, however, this direct method is difficult [16,17] and so surrogate *kdeg* values derived indirectly from *in vitro* approaches are needed. For hepatic CYP3A4 the reported *t1/2* ranges from 10 to 140 hours [18–21]. These *t1/2* values have been measured by three main *in vitro* approaches: (i) measuring CYP apoprotein expression loss in liver models over time [22,23], (ii) induction of CYP enzymes followed by tracking of de-induction recovery profiles [22,24,25] and (iii) Pulse-chase analysis after de-induction [26–28].

Many *in vitro* studies have shown that CYP apoprotein and enzyme levels decline differentially over time in culture [29–31]; enzyme *kdeg* can be derived from tracking the loss of expression assuming that the changes are caused solely by endogenous enzyme degradation mechanisms. In the second aforementioned approach, enzyme turnover is estimated by incubation of the liver with a range of doses of a known inducer compound to reach a maximally-induced new steady-state, then removing the inducer and measuring the time taken to return to the basal expression level using probe substrates. Finally, the pulse-chase method has been frequently used to predict *t1/2* of proteins [10,32,33]. In this approach, the protein of interest is labelled with a radioactive amino acid precursor during pre-incubation, followed by a chase period where an excess of non-radioactive amino acid is added to prevent further incorporation of radiolabelled precursor amino acid. Cells are then harvested and the radioactivity determined over several time points to measure rate of protein disappearance [34,35]. Ramsden *et al.* measured CYP3A4 *kdeg* using siRNA and interleukin-6 (IL-6) to specifically inhibit CYP3A4 protein synthesis prior to tracking loss of enzyme activity [25]. Impeding protein synthesis by inhibiting mRNA production using CYP-specific siRNA during protein or activity measurement may reduce the impact of *de novo* enzyme synthesis, which may distort the true *kdeg* value.

The current study presents a flexible and cost-effective *in vitro* approach to investigate human hepatic CYP *kdeg*. The present method utilises human primary hepatocytes, which allows inter-individual variability in CYP *kdeg* to be assessed, thus differing from other recent human hepatocyte siRNA-based approaches [25]. The *kdeg* value derived for CYP3A4 was within the range of published values that were previously derived using alternative methods. This novel method was successfully applied to derive a *kdeg* value for CYP2B6 and will be useful to inform PBPK models to gain a better prediction of clinically-significant DDIs.

**Materials and Methods**

***Materials***

Cryopreserved primary human hepatocytes (Cat. HMCPIS), cryopreserved hepatocyte recovery medium (CHRM® media), William’s E medium (WEM), plating and supplement medium, cryopreserved human hepatocytes, plating cocktail, maintenance cocktail, 24-well collagen coated plates, Hank’s balanced salt solution (HBSS), TRIzol® reagent and opti-MEM® I media were purchased from Thermo Fisher Scientific (Paisley, UK). Taqman® reagents and assays (CYP3A4: Hs00604506\_m1, CYP2B6: Hs04183483\_g1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Hs02758991\_g1 and hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1): Hs02800695\_m1), reverse transcription products and real-time PCR master mix were purchased from Applied Biosystems (Warrington, UK). ON-TARGETplus siRNA were purchased from Dharmacon, UK. Western blot primary and secondary antibodies, anti-cytochrome P450 3A4 (Cat. ab3572), anti-GAPDH (Cat. ab181602) and goat anti-rabbit secondary antibody (Cat. ab97080) were purchased from Abcam (Cambridge, UK) and anti-cytochrome P450 2B6 (Cat. VMA00171) and goat anti-mouse IgG secondary antibody (Cat. STAR207P) were purchased from Bio Rad (Oxford, UK). Luminata™ Forte Western HRP substrate was bought from Millipore (Watford, UK). TWEEN®20, Bradford Reagent, CelLytic™ M, 1’-hydroxymidazolam, bupropion hydrochloride, (2S,3S)-hydroxybupropion and all other chemicals, unless otherwise noted, were purchased from Sigma-Aldrich (Dorset, UK). Midazolam hydrochloride was purchased from Tocris (Bristol, UK). P450-Glo™ CYP2B6 assay was purchased from Promega (Southampton, UK).

***Cell Culture***

Cryopreserved primary human hepatocytes were thawed and plated as described previously by Roberts *et al.* [36]. The donor demographics are given in Table 1, three hepatocyte donors were used and CYP3A4 and CYP2B6 *kdeg*were derived from 3 replicate experiments from 3 donors. Hepatocytes were seeded at a density of 3 x 105 viable cells per well in 500 μl of WEM plating medium on 24-well collagen-I coated plates for CYP3A4 and CYP2B6 metabolism activity and protein expression analyses. Hepatocytes were seeded at 1 x 105 viable cells per well for mRNA and P450-Glo™ CYP2B6 analyses. Cells were incubated with plating medium for 5 hours at 37°C with 5% (v/v) CO2 in a humidified incubator to allow cell adherence prior to siRNA transfection. After 5 hours of incubation, the plating medium was replaced with maintenance media for untreated control, or dosed with varying siRNA conditions in optiMEM® I medium.

[Insert Table 1 here]

***Small-interfering RNA (siRNA) Treatment***

Using siRNA to suppress CYP proteins was conducted by modification of the method published by Vozza-Brown *et al.* [37]. After 5 hours incubation with hepatocyte plating medium, cells were washed and treated with Dharmacon ON-TARGETplus human CYP3A4 or CYP2B6 SMARTpool siRNA. Positive ON-TARGETplus GAPD human control pool and negative control ON-TARGETplus non-targeting pool were also included (Table 2). A non-siRNA treated control was also included to differentiate between the loss of gene expression due to endogenous degradation and loss due to siRNA treatment. 1.25 μl of transfection reagent Lipofectamine RNAiMAX™ was complexed with 50 or 100 nM ON-TARGETplus CYP3A4 or CYP2B6 siRNA, respectively, in reduced serum opti-MEM® I medium for 30 minutes prior to addition to cell culture. Hepatocytes were exposed to siRNA overnight for 15 hours before the cells were washed and replaced with standard hepatocyte maintenance medium. Cells were subsequently incubated at 37°C with 5% (v/v) CO2 for 48, 72 and 96 hours post CYP3A4 siRNA treatment, and for 12, 24, 36, 48 and 60 hours after CYP2B6 siRNA treatment. CYP3A4 and CYP2B6 metabolic activity, mRNA and protein expression were assessed at these time points. Time 0 hours was taken after 5 hours incubation with plating media and prior to siRNA transfection and maintenance media replacement. Following siRNA transfection, maintenance media was replaced every 24 hours.

[Insert Table 2 here]

***CYP mRNA Knockdown Quantification***

After siRNA treatment, hepatocyte lysates were treated with TRIzol® reagent and total RNA isolation was carried out using the standard extraction protocol by Ambion/Life Technologies. Total RNA concentration and purity were assessed by Nanodrop 2000 (Thermo Scientific, UK) and samples with a 260:280 ratio of over 1.6 were reverse transcribed to cDNA. The relative mRNA expression for the target CYP genes were determined by Taqman real-time reverse transcription polymerase chain reaction (qPCR) method. The first step involved the formation of cDNA with Taqman® reverse transcription kit, as specified in the manufacturer’s instructions (Cat. N8080234). qPCR was performed using Taqman® gene expression assays on an Opticon2 Fluorescence Detector (MJ Research, UK). Quantitation was performed using Bio-Rad Opticon Monitor™ Analysis software (Bio-Rad, UK; version 3.2.32). Following quantitation, relative *CYP3A4* and *GAPDH* mRNA expression against housekeeping gene *HPRT1* and relative *CYP2B6* expression against housekeeping *GAPDH* gene were derived by the Pfaffl method for mRNA quantification [38]. siRNA treatment did not alter the expression of the reference genes.

***CYP Activity Assessment Using Probe Substrates of CYP3A4 and CYP2B6***

Probe substrates for CYP3A4 and CYP2B6 activity were 3 μM midazolam and 500 μM bupropion, respectively, prepared with hepatocyte maintenance medium using <0.5% methanol as a vehicle. CYP3A4 activity was measured as 1′-hydroxymidazolam metabolite formation following incubation with midazolam for 60 min at 37°C with 5% CO2 at 0, 48, 72 and 96 hours after CYP3A4 siRNA transfection. CYP2B6 activity was determined by (2S,3S)-hydroxybupropion formation following incubation with bupropion hydrochloride for 120 min at 0, 12, 24, 36, 48 and 60 hours after CYP2B6 siRNA transfection. For each assay, probe substrates were incubated with 3 x 105 cryopreserved primary human hepatocytes in 500 μl. Reactions were terminated by removal of an equal volume of metabolite-containing media and immediate storage at -80°C. 1′-hydroxymidazolam was extracted from media by the addition of acetone (5:1 v/v) then mixing on a rotator for 30 min. The mixture was then centrifuged for 30 min and the supernatant fraction dried overnight in a Savant SpeedVac rotary evaporator (Thermo Scientific, UK). Hydroxybupropion was extracted from media using a protocol modified from Loboz *et al.* [39]. This involved using 0.5 M carbonate buffer pH 10.8 (100 μL) and 1.5% isoamyl alcohol in heptane (1 ml). The samples were vortexed for 20 s, mixed on a rotator for 20 min and then centrifuged for 15 min. The organic layer was transferred to a tube containing 0.1 M HCl (100 μL) and then vortexed and centrifuged as before. The organic layer was then discarded and the drug-containing aqueous layer was dried in a rotary evaporator overnight. The dried compounds were reconstituted in 150 μL mobile phase A (with 20% ACN v/v) and 100 μL was injected into a Dionex HPLC system (Thermo, UK). Two mobile phases were used for the chromatographic run for each drug compound, mobile phase A for hydroxymidazolam and hydroxybupropion consisted of 25 and 10 mM potassium phosphate buffer at pH 3.14 and 5.5 respectively. Mobile phase B consisted of 100% ACN, in each case. The chromatographic separation was performed using a reverse-phase Fortis C18 column at a flow rate of 1 ml/min in a step-gradient elution. The gradient for 1′-hydroxymidazolam elution were as follows: 80% mobile phase A and 20% mobile phase B from 0.0 to 1.1 min, 60% mobile phase A and 40% mobile phase B at 1.1 to 6.0 min, 55% mobile phase A and 45% mobile phase B at 6.0 to 6.1 min, 20% mobile phase A and 80% mobile phase B at 6.1 to 8.1 min and finally 80% mobile phase A and 20% mobile phase B at 8.1 to 9.0 min. The gradient for (2,3S)-hydroxybuproprion elution were as follows: 80% mobile phase A and 20% mobile phase B from 0.0 to 0.5 min, 40% mobile phase A and 60% mobile phase B at 0.5 to 5.0 min, 20% mobile phase A and 80% mobile phase B at 5.0 to 7.0 min, 80% mobile phase A and 20% mobile phase B at 7.0 to 7.1 min and finally 80% mobile phase A and 20% mobile phase B at 7.1 to 8.1 min. Hydroxymidazolam and hydroxybupropion were detected at 235 and 214 nm, having a retention time of 4.88 and 4.22 min, respectively, using a Dionex UV detector (Thermo, UK). The amount of CYP metabolite formation was derived from a linear standard curve over the concentration range of 0.04-20 μM and 0.02-20 μM for hydroxymidazolam and hydroxybupropion, respectively.

***CYP2B6 Activity Assessment by P450™ Glo Assay***

CYP2B6 is reported to be lowly expressed at around 2-10% of total hepatic CYP abundance [40], thus in anticipation of insufficient probe metabolite formation detectable by UV-HPLC, CYP2B6-Glo™ assay was employed to act as comparison to probe substrate activity assays. Primary human hepatocytes were seeded at 1 x 105 cells/well in 24-well collagen-coated plates in WEM medium and treated with siRNA compounds. CYP2B6 activity was detected at 0, 12, 24, 36, 48 and 60 hours post siRNA transfection with the P450™ Glo assay performed according to the manufacturer’s protocol (Promega, UK). CYP2B6 activity was normalised to activity detected for the untreated control at each incubation time point.

***CYP Protein Quantification***

Protein was extracted from whole cell lysates using CelLytic™ M and total protein was quantified by Bradford protein assay according to manufacturer’s recommendations. 20 μg of total protein was suspended in a reduced sample buffer and separated on a NuPage 4-12% Bis-Tris Gel (Thermo Scientific, UK) by electrophoresis. The proteins were subsequently blotted onto Amersham Protran 0.45 nitrocellulose membrane using a Criterion™ blotter (Bio Rad, UK). The membranes were then blocked in 5% (w/v) BSA in 0.01% (v/v) Tween-tris buffered saline (T-TBS) for 2 hours at room temperature prior to probing for CYP proteins. Membranes were probed with 1:2000 dilution of CYP3A4 (ab3572, Abcam) and 1:1000 dilution of CYP2B6 (VMA00171, Bio Rad) primary antibodies overnight at 4oC in 2% (w/v) BSA in 0.01% (v/v) T-TBS. Secondary antibodies, 1:2000 dilution of goat anti-rabbit HRP preadsorbed (ab97080, Abcam) and 1:10,000 dilution of goat anti-mouse HRP (STAR207P, Bio Rad) were incubated for 2 hours at 4oC in 2% (w/v) BSA in 0.01% (v/v) T-TBS. Membranes were blocked with 10% (w/v) BSA in 0.01% (v/v) T-TBS overnight before GAPDH, which was used as loading control, was probed using primary antibody at 1:10,000 dilution (ab181602, Abcam) for 2 hours at 4oC and secondary antibody at 1:10,000 dilution (ab97080, Abcam), incubated for 1 hour at room temperature in 2% BSA. Visualisation was performed using chemiluminescent detection with Luminata™ Forte Western HRP substrate (Millipore, UK) and quantification was achieved using a Bio Rad GS800 scanner (BioRad, CA) and ImageJ software (NIH).

***Data Analysis***

*Kdeg* and *t1/2* were calculated using GraphPad Prism 6 software (GraphPad Software, CA). Linear regression was used and the percentage of metabolite, fluorescence unit or normalised protein expression, normalised to untreated control at each corresponding time point post siRNA treatment to account for endogenous degradation, was transformed by taking the natural logarithm (*ln*). *T1/2* values for CYP3A4 and CYP2B6, representative of *kdeg*, were derived from the slope (*k*) of the linear regression using the following equation:

**(1)**

Data was plotted with *ln* percentage on the *y*-axis and incubation time (h) on the *x*-axis and *kdeg* taken from the slope (*k*).

**Results**

***Magnitude of CYP mRNA Knockdown***

Following incubation of primary human hepatocytes with CYP3A4 SMARTpool siRNA (50 nM) for 0-96 h, 60 (± 9.4), 70 (± 6.7) and 66 (± 6.7) % CYP3A4 mRNA knockdown was observed after 48, 72 and 96 hours of incubation (Figure 1A). Incubation with CYP2B6 SMARTpool siRNA (100 nM) 0-60 h, 19 (± 20.5), 74 (± 13) and 97 (± 4.7) % mRNA knockdown was achieved at 12, 24 and 60 hours respectively. After 36 and 48 hours incubation, CYP2B6 was undetectable (Figure 1B.). siRNA specific for CYP enzymes show prolonged mRNA suppression following a single dose over the course of 96 hours for CYP3A4 and 60 hours for CYP2B6.

[Insert Figure 1 here]

***Enzyme Activity of CYP3A4 During siRNA Treatment***

After exposure to CYP3A4 targeting siRNA for 48, 72 or 96 hours, 1′-hydroxymidazolam metabolite formation was measured to determine CYP3A4 activity. The percentage of activity at each time point compared to control was fit to linear regression models (Figure 2A.) based on assumed first-order enzyme degradation kinetics. The *kdeg* derived from the linear regression of CYP3A4 activity was 0.0171 h-1 (± 0.0025 SD) and a *t1/2* of 41.1 h (± 6.5).

[Insert Figure 2 here]

***Comparison of Enzyme Activity Analyses of CYP2B6***

CYP2B6 activity was measured by bupropion probe substrate turnover and CYP2B6-Glo™ assay. The amount of (2,3S)-hydroxybupropion metabolite formation measurement and CYP2B6-Glo™ was conducted at 12, 24, 36, 48 and 60 hours after incubation with CYP2B6 targeting siRNA. The percentage of activity at each time point compared to control was fitted to linear regression models (Figure. 3A). The *kdeg*derived from CYP2B6 enzyme activity analyses were 0.0258 h-1 (± 0.0093 SD) and 0.0271 h-1 (± 0.0064 SD), with a *t1/2* of 26.5 h (± 5.7) and 30.0 h (± 13.1), for the probe substrate metabolite method and CYP2B6-Glo™ assay, respectively. Both methods of measuring CYP2B6 enzyme activity were in good agreement.

[Insert Figure 3 here]

***Enzyme Protein Expression During siRNA Treatment***

The protein degradation rate constant was derived from tracking CYP protein decline following siRNA treatment. The relative expression normalised to GAPDH was calculated and the protein density at each time point compared to control was fitted to a linear regression model (Figures 2 and 3). The protein *kdeg* values derived in this way were calculated to be 0.0138 h-1 (± 0.0023 SD) and a *t1/2*of 51.4 h (± 9.6) and 0.0375 h-1 (± 0.025 SD) and a *t1/2*of 33.0 h (± 33.4), for CYP3A4 and CYP2B6, respectively.

[Insert Table 3 here]

**Discussion**

Determining physiologically-relevant *kdeg* values for DMEs are important for the accurate prediction of time-dependent DDIs in PBPK modelling. CYP3A4 is involved in the metabolism of around 50% of all marketed drugs and, due to its broad specificity, the enzyme has been most widely implicated in DDIs [11,41,42]. There are also a growing incidence of CYP2B6-mediated DDIs; the isoform is inducible and highly variable between individuals accounting for 2-10% of overall CYP abundance and an estimated 8% contribution to metabolism of all clinically-used drugs [40,43]. To date, CYP3A4 is the most extensively studied amongst DMEs for *kdeg* [12];values for other CYP and non-CYP enzymes remain scarce and there are currently large disparities or missing values published for the enzymes most involved in complex DDIs and no consensus on the best method of assessment [12].

There is a general consensus that steady-state protein abundance is a balance between protein synthesis and degradation [44,45], *de novo* protein synthesis will distort the degradation rate calculated and should be impeded during the time of measurement. Other common methods for measuring protein degradation rates utilise protein synthesis inhibitor drugs such as cycloheximide and actinomycin D which inhibit cellular protein synthesis mechanisms to stop universal protein production, followed by tracking the disappearance of specific target protein with pulse-chase analysis [34,46,47]. The concern with such an approach is that the chemical inhibitor drugs are cytotoxic and will disrupt normal cellular function including protein degradation pathways that will in turn distort degradation rates [48]. Ramsden *et al.* reported the use of siRNA and interleukin-6 (IL-6) to specifically inhibit CYP3A4 protein synthesis without affecting regular cellular mechanisms [25] and we have used a similar siRNA silencing approach to specifically target CYP proteins. siRNA rapidly silences specific gene expression by cleaving mRNA and therefore inhibiting downstream translation of mRNA into protein products. A pool of siRNA sequences was used in this study because pooling of target gene siRNA sequences was shown to have higher selectivity and reduced off-target effects [49,50]. Work herein has demonstrated that siRNA was able to knockdown CYP enzymes at the protein level as the metabolite forming activity of siRNA-treated hepatocytes was lower than that of non-targeting and untreated controls (supplementary figure 2).

For CYP3A4, a consensus value of hepatic *kdeg* for use in PBPK models has been investigated and subsequently evolved based upon new techniques and on prediction accuracy. For example, 0.0077 h-1 (corresponding to *t1/2* of 90 hours) was derived from meta-analysis of all the published CYP3A values until 2008 [12]. However, Wang found better prediction accuracy for 54 DDIs involving CYP3A MBI interactions using 0.03 h-1 as the *kdeg* value compared to 0.0077 h-1 [51]. Other subsequent studies also found superiority with 0.03 h-1 compared with 0.0077 h-1 [52,53]. Rowland-Yeo *et al.* reported that 0.0193 h-1 produced decreased bias and increased precision in 29 time-dependent metabolic inhibition DDIs compared with 0.0077 h-1 [54] and Mao *et al.* further validated this value and reported that a CYP3A4 *t1/2* of between 25-35 hours (0.0198 – 0.0277 h-1 *kdeg*) yielded the most accurate crizotinib drug interaction predictions [55]. Subsequently, this value has been used in numerous published examples of DDI prediction [56–58]. The most recent estimates for CYP3A4 kdeg were reported by Ramsden *et al.* at 0.024 h-1 (t1/2 of 28.9 h) derived from tracking CYP activity loss in hepatocytes after treatment with IL-6 [25], Dixit *et al.* at 0.014 h-1 (t1/2 of 49 h) using an induction and recovery approach [24] and Takahashi *et al.* at 0.026 h-1 (t1/2 of 26.7 h) using a stable isotope labeled amino acids (SILAC) method [28]. Moreover, Ramsden *et al.* reported comparable estimates of kdeg at 0.032 h-1 for siRNA and 0.038 h-1 for IL-6. Interestingly, the *kdeg* value of 0.018 h-1 (*t1/2* of 38.5 hours) for CYP3A4 derived in this study using the loss of activity method and 0.014 h-1 (*t1/2* of 50.2 hours) derived by tracking protein disappearance was in good agreement with recently reported values. However, there was a larger variation in CYP2B6 *kdeg* calculated from protein compared to activity loss (average SD for protein was 0.70 compared to 0.26 and 0.36 for probe substrate and CYP2B6-Glo™ activity analysis, respectively) and this may be attributed to the semi-quantitative limitations of using Western blot to assess absolute protein abundance. [59]. Furthermore, there was remarkably good agreement of 0.0258 and 0.0271 h-1 for CYP2B6 *kdeg* derived from two different activity measurement approaches, suggesting that tracking the loss in activity rather than in protein may be the better approach for measuring *kdeg*.

To date there are only two studies which have presented a *t1/2* value for CYP2B6. Renwick *et al.* reported a *t1/2* of 32 h based on using immunoblot detection of decreasing protein levels in human liver slices from a single donor [23]. Dixit *et al.* reported a protein activity *t1/2* of 70 h and this was determined by induction of CYP2B6 in primary human hepatocytes to maximal levels then tracking the time taken to recover to basal activity levels [24]. The hepatic CYP2B6 *kdeg* value of 0.0258 h-1 and *t1/2* of 26.9 hours derived in this study is in good agreement with values obtained by Renwick *et al.* [23].This value can now be incorporated into PBPK simulations involving time-dependent CYP2B6 induction interactions and assessed compared to other available values in terms of prediction accuracy, further validating the physiological relevance and methodology for this approach.

It should be noted that CYP2B6 is highly polymorphic and there can be large inter-individual variability in the expression and activity between donors and this presents a limitation to the current study. Another potential limitation to our *kdeg* approach is that the quantification of a given protein with a long *t1/2* in monocultured primary human hepatocytes is likely to be problematic. Although primary human hepatocytes are regarded as the gold standard *in vitro* model for assessing drug metabolism [60], our preliminary studies showed that after 120 hours of incubation CYP2B6 mRNA expression reduced to 7.6% (± 1.3) of the expression level at time 0. This differential decline in CYP2B6 expression is consistent with the well-documented parallel CYP decline over time in cultured human hepatocytes [23,61–63]. Several studies have commented on the longevity and stabilisation of CYP expression in HepatoPac® model and indeed this model was used by Ramsden *et al.* [25], Dixit *et al.* [24] and Takahashi *et al.* [28] to directly measure CYP *kdeg*. Thus, for proteins with *t1/2* over 120 hours an alternative liver model with prolonged CYP expression, such as the HepatoPac® model may be preferred. There are several conflicting reports regarding the longevity and expression of DMEs in monocultured primary hepatocytes. For example, Rodriguez-Antona *et al.* reported significant mRNA decrease (to below 20%) after 24 hours of culture whereas protein levels were detectable at 72 hours [61]. Heslop *et al.* were able to detect multiple CYP mRNA expression up to 168 hours in culture [62]. Runge *et al.* showed detectable CYP protein expression at 30 days of culture. Hutzler *et al.* reported comparable aldehyde oxidase activity in cryopreserved hepatocytes to freshly isolated hepatocytes after 24-48 h of culture, which suggests reasonable hepatic activity up to 48 h of culture [64]. It is therefore clearly difficult to define a cut-off; the current studies indicate that primary human hepatocytes can be used successfully up to 96 hours but caution should be adopted for investigating proteins beyond this time point. Where prior knowledge indicates that *t1/2* is likely to be less than 96 hours, the use of monocultured primary hepatocytes offers a cost-effective and robust approach but variability across systems and experiments warrant validation for an appropriate cut-off for each experimental set-up.

In summary, a novel and cost-effective method for measuring CYP protein degradation rates using primary human hepatocytes was validated by comparison to published values of CYP3A4 *kdeg* and was employed to determine a *kdeg* for CYP2B6. These data provided will further insight into metabolic enzyme degradation rates and will be useful to inform PBPK models aimed at investigating DDIs.

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Authorship contributions are as such, C. Chan wrote the manuscript. C.Chan, A. Owen, and L. Almond participated in experimental design. C. Chan conducted the experiments with recommendations from O. Roberts, R. K. Rajoli, N. J. Liptrott and M. Siccardi. Data was analysed by C. Chan and A. Owen.

**Conflict of Interest**

The authors report no declaration of interest.

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**Indication of Figures and Tables**

***Table Titles***

Table 1. Donor information for cryopreserved primary human hepatocytes used.

Table 2. Details of the ON-TARGETplus SMARTpool siRNA

Table 3. *Kdeg* and *t1/2* (h) values calculated from the degradation slopes and the combined average and standard deviations using the exponential decay equation.

***Figure Captions***

Figure 1. Impact of siRNA on *CYP3A4* and *CYP2B6* mRNA expression.(A) Changes in CYP3A4 mRNA expression over time. PHH were dosed and incubated with CYP SMARTpool siRNA for 15 h in reduced serum optiMEM I media. After siRNA incubation, media was replaced and cells were incubated for 48, 72 and 96 hours in 37oC 5% CO2. (B) Changes in CYP2B6 mRNA expression. Cells were incubated for 12, 24, 36, 48 and 60 hours after initial siRNA dosing. CYP mRNA expression was determined at each time point by RT-PCR. The level of mRNA expression is given as a percentage of untreated control at each time point. Time 0 was taken after initial cell seeding, where hepatocytes were rested for 5 h prior to siRNA transfection. After 5 h resting, RNA was immediately extracted and mRNA transcripts were quantified by RT-PCR. Data represents mean ± SD from n=3 independent experiments from three donors.

Figure 2. Determination of the CYP3A4 degradation rate constant in primary human hepatocytes. (A) CYP3A4 activity measured by 1′-hydroxymidazolam formation. (B) CYP3A4 protein degradation quantified by Western blot.(C) Representative Western blot showing honor Hu1591, with CYP3A4 expression normalised to that of GAPDH. (D) The CYP3A4 protein density ratio normalised to GAPDH shown for siRNA-treated hepatocytes and corresponding untreated control. Time 0 was taken at 5 h after initial seeding of primary human hepatocytes in 24-well collagen-coated plates prior to siRNA transfection, where activity and protein expression was determined. 1′-hydroxymidazolam metabolite was quantified in the culture supernatant following incubation with midazolam to determine CYP3A4 activity and protein lysates were extracted and quantified by Western blotting. Time 0 is taken as 100% protein expression of activity. CYP3A4 activity and protein is normalised to the untreated control at each time point. The natural logarithm (*ln*) of the percentage activity or protein expression of the untransfected control is given and the slope of the loss of enzyme activity and protein expression was used to calculate the protein *t1/2*, which is equal to the rate of degradation. Data represents mean ± SD from n=3 independent experiments from three donors.

Figure 3. Determination of the CYP2B6 degradation rate constant in primary human hepatocytes. (A) CYP2B6 activity measured by (2,3S)-hydroxybupropion formation. (B) CYP2B6 activity as determined by Promega CYP2B6-Glo™ assay. (C) CYP2B6 protein degradation quantified by Western blot. (D) Representative Western blot showing donor Hu8241, density was normalised to GAPDH. (E) The CYP2B6 protein density ratio normalised to GAPDH shown for siRNA-treated hepatocytes and corresponding untreated control. Time 0 was taken at 5 h after initial seeding of primary human hepatocytes in 24-well collagen-coated plates, prior to siRNA transfection, where activity and protein expression was determined. CYP2B6 activity were either determined by quantifying (2,3S)-hydroxybupropion metabolite formation in the culture supernatant following incubation with bupropion or by Promega CYP2B6-Glo™ assay. Proteins were extracted and quantified by Western blotting. Time 0 was taken as 100% protein expression or activity. CYP2B6 activity and protein is normalized to the untransfected control at each time point. The natural logarithm (*ln*) of the percentage protein expression or activity of the untransfected control is given and the slope of the loss of enzyme activity and protein expression was used to calculate the protein *t1/2*, which is equivalent to the rate of degradation. Data represents mean ± SD from n=3 independent experiments from three donors.

**Table 1. Donor information for cryopreserved primary human hepatocytes used.**

|  |  |  |  |
| --- | --- | --- | --- |
| Demographics | Donor 1 | Donor 2 | Donor 3 |
| Donor ID | Hu1591 | Hu1824 | Hu8241 |
| Age | 29 | 66 | 60 |
| Gender | Male | Female | Male |
| Race/Ethnicity | Caucasian | Caucasian | Caucasian |
| Characteristics | Rare alcohol user. No tobacco or drug use | Tobacco use. No alcohol or drug use | Tobacco and alcohol user. No drug use |
| Cause of death | Not reported | Not reported | Cardiac related |

**Table 2. Details of the ON-TARGETplus SMARTpool siRNA**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Catalog No. | Amount (nmol) | Sequence |
| Non-targeting | D-001810-10-05 | 50 | 5′-UGGUUUACAUGUCGACUAA-3′  5′-UGGUUUACAUGUUGUGUGA-3′  5′-UGGUUUACAUGUUUUCUGA-3′  5′-UGGUUUACAUGUUUUCCUA-3′ |
| GAPD | D-001810-10-05 | 50 | 5′-GUCAACGGAUUUGGUCGUA-3′  5′-CAACGGAUUUGGUCGUAUU-3′  5′-GACCUCAACUACAUGGUUU-3′  5′-UGGUUUACAUGUUCCAAUA-3′ |
| CYP3A4 | L-008169-01-0005 | 50 | 5′-CAUCCCAAUUCUUGAAGUA-3′  5′-GUGGAAAACUCAAGGAGAU-3′  5′-GAACUGAAGCUCUUAUUAU-3′  5′-CCAACUGUCUCGAUGCAAU-3′ |
| CYP2B6 | L-011168-01-0005 | 100 | 5′-GGGGAUAUGGUGUGAUCUU -3′  5′-GGGAGAUUGAACAGGUGAU -3′  5′-UGCAGGAAAUCAAUGCUUA -3′  5′-AAACAUCUCUAAAGCCUGA -3′ |

**Table 3. *Kdeg* and *t1/2* (h) values calculated from the degradation slopes and the combined average and standard deviations using the exponential decay equation.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Parameter** | **CYP3A4** | | | **CYP2B6** | | |
| Donor 1 | Donor 2 | Donor 3 | Donor 1 | Donor 2 | Donor 3 |
| *t1/2*(h) derived from activity determined by probe substrate metabolite formation | 36.2 | 38.7 | 48.5 | 20.8 | 24.2 | 45.0 |
| *kdeg*(h-1) derived from activity determined by probe substrate metabolite formation | 0.0191 | 0.0179 | 0.0143 | 0.0333 | 0.0287 | 0.0154 |
| Average *t1/2*(h) | 41.1 (± 6.50) | | | 30.0 (± 13.1) | | |
| Average *kdeg*(h-1) | 0.0171 (± 0.0025) | | | 0.0258 (± 0.0093) | | |
| *t1/2*(h) derived from activity determined by CYP-Glo™ Assay | - | - | - | 27.8 | 20.1 | 31.4 |
| *kdeg*(h-1) derived from activity determined by CYP-Glo™ Assay | - | - | - | 0.0249 | 0.0344 | 0.0221 |
| Average *t1/2*(h) | - | | | 26.5 (± 5.73) | | |
| Average *kdeg*(h-1) | - | | | 0.0271 (± 0.0064) | | |
| *t1/2*(h) derived from protein determined by Western blot | 62.5 | 45.6 | 46.2 | 11.9 | 15.5 | 71.5 |
| *kdeg*(h-1) derived from protein determined by Western blot | 0.0111 | 0.0152 | 0.0150 | 0.0582 | 0.0447 | 0.0097 |
| Average *t1/2*(h) | 51.4 (± 9.56) | | | 33.0 (± 33.4) | | |
| Average *kdeg*(h-1) | 0.014 (± 0.0023) | | | 0.038 (± 0.025) | | |

**Figure 1.**

**Figure 2.**



0

48

72

96

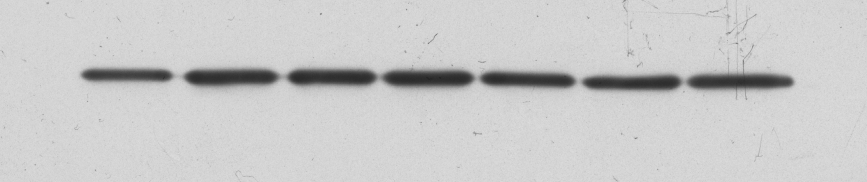
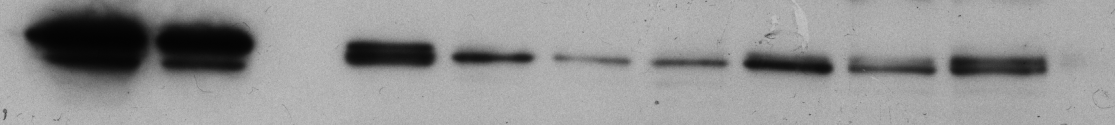
48

CYP3A4 (57 kDa)

GAPDH (37 kDa)

siRNA treated (h)

Untreated (h)



72

96

**C**

****

**D**

**Figure 3.**





**E**

**D**

0

12

24

36

48

12

24

36

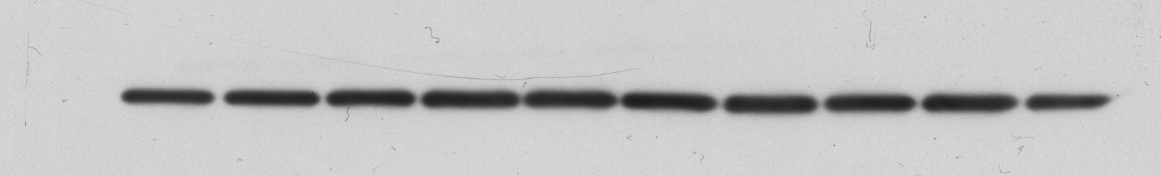
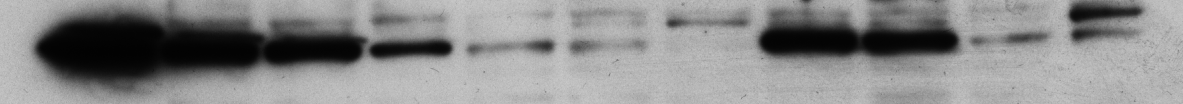
48

CYP2B6 (56 kDa)

GAPDH (37 kDa)

siRNA treated (h)

Untreated (h)



60

**Supplementary Figures**



**Supplementary Figure 1.** The analysis of cell viability of cryopreserved primary hepatocytes by PrestoBlue® assay. The vehicle control, NTC, refers to non-targeting siRNA pool. The relative fluorescence intensity (RFU) reported was normalised to background fluorescence. Time 0 represents hepatocyte culture at 5 h resting after initial seeding in 24-well collagen-coated plates with no CYP or NTC siRNA transfection. Data shown from n=1 experiment.



**A**

**B**

**Supplementary Figure 2.** CYP450 activity as determined by probe substrate metabolite formation. (A) CYP3A4 activity determined by 1′-hydroxymidazolam formation. (B) CYP2B6 activity determined by (2,3S)-hydroxybupropion formation. Time 0 represented hepatocyte culture at 5 h resting after initial seeding in 24-well collagen-cated places with no CYP or NTC siRNA transfection. Data shown as mean ±SD from n=3 independent experiments in three hepatocyte donors.