Metabolite formation kinetics and intrinsic clearance of phenacetin, tolbutilamide, alprazolam and midazolam in adenoviral P450 transfected HepG2 cells, and comparison with hepatocytes and in vivo

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Running title: Intrinsic clearance studies in adenoviral P450 systems

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Abbreviations: Ad-CYP, adenovirus expressing CYP; CLint, intrinsic clearance; CLmax, maximum activated clearance; GFP, green fluorescence protein; HLM, human liver microsomes; MOI, multiplicity of infection; P450, cytochrome P450; pfu, plaque forming units; RAF, Relative Activity Factor
Abstract

Cryopreserved human hepatocytes and other in vitro systems often underpredict in vivo intrinsic clearance (CLint). The aim of this study is to explore the potential utility of HepG2 cells transduced with adenovirus vectors expressing a single P450 enzyme (Ad-CYP1A2, Ad-CYP2C9 or Ad-CYP3A4) for metabolic clearance predictions. The kinetics of metabolite formation from phenacetin, tolbutamide, and alprazolam and midazolam, selected as substrates probes for CYP1A2, CYP2C9 and CYP3A4, respectively, were characterised in this in vitro system. The magnitude of the $K_M$ or $S_{50}$ values observed in Ad-CYP cells was similar to those found in the literature for other human liver-derived systems. For each substrate, CLint (or CLmax), values from Ad-CYP systems were scaled to human hepatocytes in primary culture using the relative activity factor (RAF) approach. Scaled Ad-CYP CLint values were about 3-6-fold higher (for phenacetin O-deethylation, tolbutamide 4-hydroxylation and alprazolam 4-hydroxylation) or lower (midazolam 1'-hydroxylation) than those reported for human cryopreserved hepatocytes in suspension. Comparison with the in vivo data reveals that Ad-CYP cells provide a favourable prediction of CLint for the substrates studied (in a range of 20-200% in vivo observed CLint). This is an improvement as regards to the consistent underpredictions (<10% to 50% in vivo observed CLint) found in cryopreserved hepatocyte studies with the same substrates. These results suggest that the Ad-CYP cell is a promising in vitro system for clearance predictions of P450-metabolised drugs.
Introduction
The introduction of a new drug into the market is not only a complex, time-consuming and costly process, but also a risky business. Thus, there is an urgent need for strategies that identify and discard compounds with inappropriate properties at earlier stages of drug discovery. As unsatisfactory pharmacokinetic properties are a major reason for the attrition of candidate drugs in early clinical trials, the importance of pharmacokinetics predictions in the optimisation and selection of drug candidates is increasingly recognized (Ansede and Thakker, 2004). In particular, great efforts have been made to develop fast, reliable experimental strategies to predict human hepatic metabolic clearance (Ito and Houston, 2005).

Human liver microsomes (HLM) have been mainly used for this purpose (Iwatsubo et al., 1997; Obach, 1999; Riley et al., 2005). However, underestimation of human clearance using this in vitro system is common for many drugs (Ito and Houston, 2005; Hallifax et al., 2005). More recently, human hepatocytes have also been explored as an alternative in vitro system for clearance predictions. Unlike HLM, intact hepatocytes contain a full complement of metabolic enzymes (Phase I and Phase II) and physiological cofactors, as well as intact cell membranes and transporter proteins. Thus, hepatocytes provide an in vitro system for integrated metabolism and transport studies, and might reflect the in vivo situation more closely than subcellular models. One major limitation for the widespread use of human hepatocytes for clearance studies is the scarcity of high-quality fresh human liver required for cell harvesting. Technical advances in freezing protocols have made cryopreserved human hepatocytes available from commercial suppliers, which has notably enabled their application for hepatic clearance studies (Bachmann et al., 2003; Naritomi et al., 2003; McGinnity et al., 2004).

Compared to microsomes, the use of hepatocytes appears to be a significant improvement in the accuracy of clearance predictions because of the reduced bias (Brown et al., 2007). However, cryopreserved hepatocytes are not fully quantitative for predictions as a systematic underprediction of the in vivo value is obtained (Hallifax et al., 2005; Brown et al., 2007).

Recombinant cytochrome P450 (P450) enzymes have also been proved as in vitro systems for human metabolic clearance predictions (Proctor et al., 2004; Stringer et al., 2009). A previous study showed improved reliability of cDNA-expressed P450 enzymes in relation to HLM or hepatocytes (Stringer et al., 2009). A potential advantage of the high P450 levels of recombinant systems is enhanced assay sensitivity,
which could be particular helpful for those drugs primarily eliminated by minor P450 forms or for lower clearance drugs. Compared with hepatocytes, however, one major drawback is the lack of intact membranes and transport mechanisms.

The adenovirus vector strategy has been successfully used for introducing target genes into hepatocytes and other liver-derived cells (Castell et al., 1997; Bai and Cederbaum, 2004; Naiki et al., 2004; Vignati et al., 2005; Aoyama et al., 2009). A major characteristic of this approach is that protein expression can be modulated as a function of the level of adenovirus infection. In the present study, we investigated the suitability of a cell system based on an adenoviral-mediated expression of individual P450 enzymes (Ad-CYP) in HepG2 cells as an alternative in vitro system for predicting the metabolic clearance of drugs. The HepG2 cell model was selected for the study because of its human liver origin, their content in NAPH-cytochrome P450 reductase and cytochrome b5 (Rodriguez-Antona et al., 2002; Gonzalez and Korzekwa, 1995) and because it has been reported as an adequate host system for the transient expression of P450 enzymes (Vignati et al., 2005; Aoyama et al., 2009). The kinetic characteristics of model substrates for CYP1A2 (phenacetin), CYP2C9 (diclofenac) and CYP3A4 (alprazolam and midazolam) have been analysed in intact Ad-CYP cells and compared with the corresponding data in human hepatocytes.
Materials and Methods

Chemicals. Diclofenac, testosterone, and resorufin were purchased from Sigma (Madrid, Spain). 7-Ethoxyresorufin was obtained from Molecular Probes Europe BV (Leiden, The Netherlands). 4'-Hydroxydiclofenac, 6β-hydroxytestosterone, midazolam, 1’-hydroxymidazolam, phenacetin and acetaminophen were supplied by Ultrafine (Manchester, UK). Tolbutamide and 4-hydroxytolbutamide were provided by Toronto Chemical Research (Canada). Alprazolam was supplied by Sigma-Adrich (Poole, UK) and 4-hydroxyalprazolam was purchased from Enzo Life Sciences (Exeter, UK). β-glucuronidase/arylsulfatase and the High Pure PCR Product Purification Kit was obtained from Roche (Barcelona, Spain). All other chemicals were of an analytical grade.

Construction of recombinant adenoviruses. CYP3A4 cDNA (2.1 kb) was released from the mammalian expression vectors pCI-CYP3A4 (Jover et al., 1996). The coding cDNA sequence of human CYP1A2 was obtained from human liver mRNA through high-fidelity RT-PCR, and CYP2C9 cDNA from cells previously generated in our laboratory (Bort et al., 1999). Primers were designed using a specific primer analysis software (Oligo 4.0), and the "wild-type" allele CYP1A2 or CYP2C9 sequences were selected from the GeneBank/EMBL database. Both the forward and reverse primers sequences are listed in Table 1. The amplified PCR products were purified by column chromatography (the High Pure PCR Product Purification Kit, Roche) and the presence of a unique DNA band of the expected size was confirmed by agarose gel electrophoresis. Purified CYP cDNAs were double digested with the appropriate restriction enzymes and ligated into the adenoviral pAC/CMVpLpA plasmid (Gomez-Foix et al., 1992) which was previously digested with the same restriction enzymes to provide a directional cohesive cloning. Constructs were confirmed by restriction enzyme analysis and were sequenced to ensure that the cloned CYP cDNA was 100% homologous with the described wild-type sequence.

Each recombinant plasmid pAC/CMVpLpA containing CYP cDNA (transfer vector) was cotransfected with pJM17 (McCroy et al., 1988) into 293 cells (AdE1A-transformed human embryonic kidney cells) by calcium phosphate/DNA co-precipitation to obtain a recombinated adenovirus expressing each P450 isoform (named Ad-CYP1A2, Ad-CYP2C9 and Ad-CYP3A4) (Becker et al., 1994). The 293 cells should be co-transfected when they are subconfluent, typically at approximately 80%
After isolating viral DNA from the infected 293 cells, the presence of the cDNA insert can be confirmed by preparing viral DNA and quantitative PCR. The resulting viruses were plaque-purified, expanded into a high-concentration stock and titrated by plaque assay. The titer obtained was in the range of $10^8$-$10^{10}$ plaque-forming units (pfu). A positive control of infection was performed by using an increasing MOI (Multiplicity Of Infection, defined as pfu/cell) of a human adenovirus expressing green fluorescence protein (GFP), a protein originally isolated from the jellyfish that fluoresces green when exposed to blue light. This adenoviral vector (Ad-CMV-GFP) was kindly provided by Dr. AM Gómez-Foix and was constructed as described elsewhere (Ferrer-Martínez et al., 2004). Since GFP is easily visualized under fluorescence microscopy, this adenovirus was used to determine the transduction efficiency and to optimise the viral infection condition in HepG2 cells.

**Culture of HepG2 cells and adenovirus infection.** HepG2 cells were cultured in Ham’s F-12/Leibovitz L-15 (1:1 v/v) (Gibco BRL, Paisley, UK) supplemented with 7% newborn calf serum (Gibco BRL, Paisley, UK), 50 U penicillin/ml and 50 μg streptomycin/ml. For subculturing purposes, cells were detached by treatment with 0.25% trypsin/0.02% EDTA (Gibco BRL, Paisley, UK) at 37°C. For adenovirus infection studies, cells were seeded in 24-well plates (0.3-1.8 x 10^5 cells/well) and 48 h later adenovirus-containing medium was added. After 24h, cells were shifted to adenovirus-free medium and cultured for additional 24 h prior to incubations with P450 substrates.

**Incubation conditions.** Assays were performed by incubation of Ad-CYP HepG2 cells cultured in 24-well plates with selected substrates. For each assay, optimal conditions were selected in terms of incubation time, cell density and adenovirus dose to ensure that they were within the linear range (see Table 2 for detailed conditions). Substrates were added in dimethyl sulfoxide or methanol, at such concentrations that the final solvent concentration did not exceed a 0.5% (v/v) when added to the incubation medium. Metabolite formation kinetics studies were performed in 3-4 independent Ad-CYP-transduced cell cultures using at least seven concentrations of the substrates. All incubations were performed in duplicate.

In brief, a volume of 250 μl of warmed (37°C) incubation medium (Na_2HPO_4, 1 mM, 137 mM NaCl, 5 mM KCl, 0.5 mM MgCl_2, 1 mM CaCl_2, 10 mM glucose, and 10 mM...
Hepes pH 7.4 buffered solution) containing the appropriate substrate concentration was added to each well. Immediately, an aliquot of 100 μl was taken (time 0) from each well, centrifuged at 1000 x g, and the supernatant (80 μl) was stored at -80°C. After incubating cells with the substrate at 37°C for the appropriate time (Table 2), aliquots of the incubation medium were taken and centrifuged as described above. Prior to metabolite analysis, samples were incubated with β-glucuronidase and arylsulfatase for 2 h at 37°C to hydrolyse any glucuronide and/or sulfate conjugates formed (Donato et al., 1993). Hydrolysis reactions were stopped by diluting (1:2) in the quenching solution (cold acetonitrile). Samples were collected and stored at –80°C until analysis.

To quantitatively compare the data obtained from Ad-CYP HepG2 cells with those from human hepatocytes, we used a Relative Activity Factor (RAF), calculated as the ratio of a specific marker activity in primary cultured human hepatocytes to the same activity in Ad-CYP cells:

\[
\text{RAF} = \frac{\text{Activity in human hepatocytes (pmol/10^6 cells/min)}}{\text{Activity in Ad-CYP cells (pmol/10^6 cells/min)}}
\]

RAF was defined by Crespi (1995) to obtain quantitative comparisons between cDNA-expressed P450s and HLM by assuming that the correlations obtained from the marker substrate can be extrapolated to other substrates for the same P450 enzyme. 7-Ethoxyresorufin O-deethylation, diclofenac 4'-hydroxylation and testosterone 6β-hydroxylation were used as marker reactions for CYP1A2, CYP2C9 and CYP3A4, respectively. Marker activities were measured in each Ad-CYP HepG2 preparation. Data from hepatocytes are an average of at least twelve independent cultures (Donato et al., 1999, 2006). For both Ad-CYP HepG2 and primary human hepatocytes, activities were assayed by direct incubation of cell monolayers with 10 μM 7-ethoxyresorufin, 10 μM diclofenac or 200 μM testosterone for 60, 120 or 60 min, respectively (Donato et al., 2006).

**Metabolite determinations.** Acetaminophen (desethylphenacetin) (CYP1A2), 4-hydroxytolbutamide (CYP2C9), 4-hydroxydiclofenac (CYP2C9), 4-hydroxyalprazolam (CYP3A4) and 1'-hydroxymidazolam (CYP3A4) formed and released into the culture medium were quantified by high performance liquid chromatography tandem mass spectrometry (LC/MS/MS). This system comprised a Micromass Quattro Micro (Waters, Milford MA, USA) triple quadrupole mass spectrometer in the electrospray
ionisation mode, interfaced with an Alliance 2795 HPLC (Waters, Milford MA, USA). Chromatography was performed at 35ºC, and an aliquot (20 µl) of incubation extract was injected into a Teknokroma C18 column (100 mm × 2.1 mm, 3µm particle size) at a flow rate of 0.4 ml/min. The mobile phase was 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The proportion of acetonitrile was increased linearly from 0 to 90% in 6 min, and then the column was allowed re-equilibration at the initial conditions for 4 min. The column eluent was directed to an electrospray ionisation interface without splitting, operating at 320ºC and using nitrogen as cone gas (50 l/h). The MS/MS analysis was carried out with a triple quadrupole analyzer operating in the multiple reaction monitoring mode using argon as collision gas (Table 3).

Resorufin formed during the CYP1A2 activity assay and 6β-hydroxytestosterone (CYP3A4) were quantified fluorometrically (Donato et al., 1993) and by HPLC analysis (Donato et al., 2006), respectively.

**In vitro kinetics.** The initial rate of metabolite formation with substrate concentration was fitted to either the Michaelis-Menten equation (phenacetin, Equation 1), a substrate inhibition model (tolbutamide and midazolam, Equation 2) or the Hill equation (alprazolam, Equation 3), by non-linear regression analysis using GraFit 5 (Michaelis-Menten/Hill; Erithacus Software, Horley, Surrey, UK) or SigmaPlot (substrate inhibition; Systat Software Inc., London, UK). The appropriateness of the model was determined by visual inspection, distribution of residuals and standard error of parameters.

\[
v = \frac{V_{\text{max}} \cdot S}{K_M + S}
\]  
Equation 1

\[
v = \frac{V_{\text{max}}}{1 + \left(\frac{K_M}{S} + \left(\frac{S}{K_i}\right)\right)}
\]  
Equation 2

\[
v = \frac{V_{\text{max}} \cdot S^n}{S_{50}^n + S^n}
\]  
Equation 3
where $S$ is the substrate concentration, $K_i$ is the substrate inhibition constant, $S_{50}$ is the substrate concentration at half the maximum velocity, $n$ is the Hill coefficient denoting sigmoidicity.

**Intrinsic clearance.** $CL_{int}$ was determined from the ratio of $V_{max}$ and $K_M$ for Michaelis-Menten/substrate inhibition kinetics. For atypical kinetics, the equivalent to $CL_{int}$, $CL_{max}$ (maximum activated clearance) was determined using Equation 4 (Houston and Kenworthy, 1999)

$$CL_{max} = \frac{V_{max}}{S_{50}} \cdot \frac{n-1}{n(n-1)^{1/n}}$$

Equation 4

The $CL_{int}$ and $CL_{max}$ values were scaled to in vivo units using the scaling factors of 120 x $10^6$ hepatocytes/g liver (Hakooz, et al., 2006) and 21.4 g liver/kg body weight (Davis and Morris, 1993).

Although no attempt was made to measure non-specific binding in the present study, previous reports indicate that the concentration of unbound drug in the incubation medium for alprazolam, midazolam (Hallifax et al., 2005) and tolbutamide (Brown et al., 2007) would have been no more than marginally affected by such binding at the concentrations used; although no equivalent data for phenacetin was available, the log P of this compound indicates marginal binding also.

**Comparison with human hepatocyte and in vivo clearances.** The $CL_{int}$ and $CL_{max}$ values were compared with the equivalent values obtained using the commonly used suspended human cryopreserved hepatocyte system for phenacetin (Stringer et al., 2008), tolbutamide (Brown et al., 2007), alprazolam (Brown et al., 2007) and midazolam (Brown et al., 2007) after multiplication by the appropriate RAF (above). The $CL_{int}$ and $CL_{max}$ values scaled to in vivo were compared with the equivalent human in vivo $CL_{int}$ values for phenacetin (Stringer et al., 2008), tolbutamide (Brown et al., 2007), alprazolam (Brown et al., 2007) and midazolam (Brown et al., 2007).
Results

Cells transient transfection efficiency. Adenoviruses can rapidly and efficiently infect human cells, and by using an appropriate MOI, almost 100% of cells can express the transgene after a short exposure to the virus. Infection of cells with Ad-GFP enabled research into the cellular distribution of the transduced gene. The majority of Ad-GFP-transfected HepG2 cells clearly expressed GFP while untransduced cells or cells treated with wild-type adenovirus were uniformly translucent (Fig. 1). The fluorescence intensity of the HepG2 monolayer increased homogenously with the amount of Ad-GFP. Similarly, the expression of CYP1A2, CYP2C9 or CYP3A4 can be modulated in a simple manner by using Ad-CYP vectors. The toxic effects of the virus to HepG2 cells were tested and only the non-toxic concentrations of Ad-CYPs were used in metabolism studies. Specific increases in specific P450 activities were observed in the HepG2 cells treated with increasing concentrations (MOI) of the Ad-CYP constructs (data not shown).

Incubation time and cell density optimisation for metabolic assays. The linearity of each substrate’s metabolic rates was tested in terms of Ad-CYP doses, cell density and incubation time. Phenacetin O-deethylolation was linear with Ad-CYP1A2 up to 11 MOI and with time up to 10 min. The tolbutamide 4-hydroxylation rate was linear up to 24 MOI of Ad-CYP2C9 and for 45 min. The formations of 4-hydroxyalprazolam and 1’- hydroxymidazolam in Ad-CYP3A4 cells were linear up to 16 and 66 MOI and for 120 min and 30 min of incubation, respectively. For the four substrates, metabolite formation was linear up to a cell density of 1.8 x10^5 cells/well.

Metabolite formation kinetics and intrinsic clearance. The kinetic characteristics of phenacetin, tolbutamide, alprazolam and midazolam metabolism were examined in Ad-CYP1A2-, Ad-CYP2C9- or Ad-CYP3A4-transduced HepG2 cells. Acetaminophen formation by Ad-CYP1A2 cells followed simple Michaelis-Menten kinetics (Fig. 2A), whereas tolbutamide hydroxylation formation (Ad-CYP2C9) followed Michaelis-Menten kinetics with a minor substrate inhibition (Fig. 2B). Autoactivation kinetics or substrate inhibition kinetics was observed for 4-hydroxyalprazolam (Fig. 2C) or 1’- hydroxymidazolam (Fig. 2D) formation rates in Ad-CYP3A4 cells, respectively. The kinetic parameter values obtained for these models (V_{max}, K_M, K_{si}, S_{50} and n) are summarised in Table 4. Metabolite formation kinetics was quantitatively similar to
previous reports for microsomes or suspended hepatocytes: $K_M$ for acetaminophen formation was 12.3 µM (compared with 9, 31 µM (Tassaneeyakul et al., 1993; Venkatakrishnan et al., 1998); $K_M$ for tolbutamide hydroxylation was 29.8 µM (compared with 112 µM, Brown et al., 2007)); $K_M$ for 1'-hydroxymidazolam was 4.59 µM (compared with 6, 11 µM, Hallifax et al., 2005, 2008) and $S_{50}$ for 4-hydroxyalprazolam was 659 (compared with 310, 560 µM, Hallifax et al., 2005, 2008).

For acetaminophen formation by Ad-CYP1A2, 4-hydroxytolbutamide by Ad-CYP2C9 and 1'-hydroxymidazolam by Ad-CYP3A4, $CL_{int}$ (ratio of $V_{max}$ and $K_M$) was 39.6 ± 24.5, 2.34 ± 0.317 and 50.4 ± 31.1 µl/min/10^6 cells, respectively (Table 4). The equivalent maximal clearance for 4-hydroxyalprazolam by autoactivation, $CL_{max}$, was 2.17 ± 0.682 µl/min/10^6 cells (Table 4).

Comparison with suspended human hepatocytes and in vivo clearances. The RAF for Ad-CYP and human primary cultured hepatocytes was 2.1, 0.29 and 0.98 or 0.46 for Ad-CYP1A2, -2C9 and -3A4 (16 or 33 MOI), respectively (Table 5). The $CL_{int}$ (or $CL_{max}$) values from Ad-CYP were scaled using the appropriate RAF to give 83.2, 0.679, 2.13 and 23.2 µl/min/10^6 cells (Table 5), the equivalent to the cultured hepatocyte model. For comparisons with the commonly used suspended cryopreserved hepatocyte system, values of 13.5, 0.148 and 0.818 µl/min/10^6 cells for phenacetin O-deethylation, tolbutamide 4-hydroxylation and alprazolam 4-hydroxylation, respectively, were obtained from the literature (Table 5). The RAF-scaled Ad-CYP $CL_{int}$ values were approximately between 3–6-fold higher than the corresponding hepatocyte values (6.2-, 4.6- and 2.5-fold, respectively). In contrast, the RAF-scaled $CL_{int}$ for midazolam 1'-hydroxylation was 3.4-fold lower than that observed in suspended hepatocytes (77.9 µl/min/10^6 cells).

In vivo $CL_{int}$ was 615, 3.6, 3.7 and 314 ml/min/kg for phenacetin, tolbutamide, alprazolam and midazolam, respectively (Table 5). The equivalent scaled $CL_{int}$ (using RAF based on cultured hepatocytes) for the Ad-CYP system was 214, 1.74, 5.47 and 59.6 ml/min/kg (Table 5), respectively, therefore, the in vitro/in vivo prediction of total in vivo $CL_{int}$ (based on the single CYP pathways) by this system was 0.3, 0.5, 1.50 and 0.2. For comparison, $CL_{int}$ previously reported for the commonly used suspended hepatocyte system was 34.7, 0.38, 2.1 and 200 ml/min/kg (Table 5), which gave in vitro/in vivo predictions of 06, 0.1, 0.6 and 0.6, respectively. Thus, the in vitro/in vivo
prediction of total \textit{in vivo} CL\textsubscript{int} from RAF-scaled Ad-CYP was generally greater than for the suspended hepatocyte system.
Discussion

Prediction of human clearance for P450 substrates has been extensively studied using human liver-derived \textit{in vitro} systems. However, underestimation of human clearance using liver microsomes is common for many drugs (Ito and Houston, 2005; Hallifax et al., 2005). Compared to microsomes, the use of hepatocytes might seem to represent a significant improvement in the accuracy of clearance predictions because of utilisation of the intact cell including uptake phenomena and Phase II metabolic pathways. For this system, a reduced bias has been reported (Brown et al., 2007). However, cryopreserved hepatocytes are not fully quantitative for predictions as a systematic underprediction of the \textit{in vivo} value is obtained (Hallifax et al., 2005; Brown et al., 2007; Riley et al., 2005). Furthermore, wide individual variability, unresolved from bias and imprecision which are inherent in the use of human liver \textit{in vitro} systems, combine to sustain considerable uncertainty in predicting clearance from \textit{in vitro} (Hallifax and Houston, 2009).

Alternative strategies have been proposed for metabolism studies of P450 substrates, among them recombinant adenoviruses has proven as a versatile tool to transfer genes to a broad spectrum of cell types, including hepatic cells. By means of this strategy, high levels of functional transgene expression can be achieved without causing major changes in the expression of other constitutive or inducible hepatic genes (Castell et al., 1997). The HepG2 cells transduced with adenoviral constructs have been used to study either the mechanisms involved in the control of the P450 expression or the role of a particular P450 enzyme in the bioactivation of hepatotoxicants (Bai and Cederbaum, 2004; Vignati et al., 2005; Zangar et al., 2008). However, their utility in the prediction of metabolic clearance has not yet been explored.

In the present study, we examine the potential of using Ad-CYP transfected HepG2 cells for human clearance predictions. For this purpose, metabolite formation kinetics and the intrinsic clearance of phenacetin, tolbutamide, alprazolam and midazolam were analysed in Ad-CYP1A2, Ad-CYP2C9 or Ad-CYP3A4 HepG2 cells. The kinetic characteristics of metabolite formation for all four substrates conformed to previous observations in human liver \textit{in vitro} systems. O-deethylation of phenacetin has been seen to follow two-site Michaelis-Menten kinetics in HLM (Tassaneeyakul et al., 1993; Venkatakrishnan et al., 1998) with the high affinity site attributed to CYP1A2; $K_M$ in Ad-CYP1A2 HepG2 cells was within the previously observed range for the high affinity site. 4-Hydroxylation of tolbutamide has been reported to follow both simple
Michaelis-Menten kinetics and Michaelis-Menten kinetics with substrate inhibition – the latter as observed in this study (Brown et al., 2007; Hallifax et al., to be submitted); $K_M$ for 4-hydroxytolbutamide formation in Ad-CYP2C9 HepG2 cells was low compared with human hepatocytes, but within 4-fold. Michaelis-Menten kinetics with substrate inhibition for 1’-hydroxymidazolam formation has also been previously observed in HLM (Kronbach et al., 1989; Perloff et al., 2000) or human hepatocytes (Hallifax et al., 2005; Hallifax et al., unpublished results) with $K_M$ in Ad-CYP3A4 similar to previously reported data, although this kinetic characteristic may have resulted from inactivation reported for midazolam (Khan et al., 2002). Autoactivation kinetics of alprazolam 4-observed in Ad-CYP3A4 HepG2 was in accordance with atypical kinetics previously demonstrated in HLM (Rawden et al., 2005), cryopreserved and fresh human hepatocytes (Hallifax et al., 2005; Hallifax et al., 2008); Ad-CYP3A4 HepG2 $S_{50}$ for this pathway was of a similar magnitude to that observed in these human liver systems. This conformity with human liver P450 characteristics gives a qualitative indication of the potential of the Ad-CYP HepG2 system.

The intrinsic clearances of the Ad-CYP HepG2 cells, which were normalised to cultured hepatocytes using RAFs based on the metabolic rate at a single concentration of the marker probe, were in the range of 3-fold lower to 6-fold higher than reported for human cryopreserved hepatocytes. Accordingly, the prediction of in vivo $CL_{int}$ was greater from the Ad-CYP HepG2 system for 3 of the 4 substrates and $CL_{int}$ for all the substrates was in the range of 20–150% of in vivo. It was assumed that the contribution of other (minor) pathways of clearance for these substrates would not significantly affect this comparison based on approximation of prediction of total clearance. Considering that the prediction of in vivo $CL_{int}$ from cryopreserved hepatocytes has been extensively reported as poor for P450 substrates in general (Hallifax and Houston, 2009), these findings indicate a potentially useful level of performance for $CL_{int}$ of the P450 substrates in the Ad-CYP HepG2 system. Given that a limited set of compounds have been examined, further studies involving other probe substrates would substantiate this finding.

Ad-CYP systems exhibit some of the advantages of hepatocytes (membrane integrity, endogenous cofactors, integrated drug-metabolising pathways), as well as additional ones such as adjustable levels of expression target enzymes. One major drawback of hepatocyte suspensions is the loss of viability which limits their use to short incubation times (2-4 h). Ad-CYP cells have a longer lifespan and show sustained levels of specific
P450 activities at up to 3-4 days post-infection (Aoyama et al., 2009), which contrasts with the early and marked decay in P450 activities observed in human hepatocyte cultures (Rodriguez-Antona et al., 2002). However, a limitation of the Ad-CYP system is that the expression of the enzyme of interest is transient and a new cell transfection is required for each experiment. To correct potential batch-to-batch differences in transfection efficiency, marker activities for RAF calculation were assayed in each Ad-CYP HepG2 cell preparation.

The use of a mixture of recombinant adenoviruses allows the expression of more than one P450 enzyme (Aoyama et al., 2009). Using the Ad-CYP constructs herein described, we prepared HepG2 cells simultaneously expressing CYP1A2, CYP2C9 and CYP3A4. The activity level of each P450 in the co-transfected cells was similar to that found in individually transfected cells (unpublished results). Moreover, Ad-CYPs can be conveniently combined to reach the desired level and relative ratio of the expression of each P450 in the cells (Aoyama et al., 2009, European Patent). Thus, this strategy could allow the generation of customised Ad-CYP cell preparations to express functional levels of multiple P450s that are representative of the average activity in humans or that mimic a particular population group (i.e., extensive or poor metabolizers). In this sense, the utility for clearance predictions of the Ad-CYP HepG2 cells reproducing interindividual variability in P450 activities found in human populations should be further explored as it could reduce the prediction bias compared with other in vitro systems (i.e., HLM or cryopreserved hepatocytes). In summary, the current study suggests Ad-CYP HepG2 as a potential in vitro model for clearance predictions of drug metabolised by P450 enzymes.
References


Footnotes.

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Figure legends

**Fig. 1.** HepG2 cells transduced with increasing amounts of Ad-GFP. Increased green fluorescence was achieved as a higher number of MOIs was used. Fluorescence was measured after 48 h of Ad-GFP exposure.

**Fig. 2.** Typical velocity-substrate concentration profiles in Ad-CYP HepG2 cells for A) phenacetin (CYP1A2) B) tolbutamide (CYP2C9) C) alprazolam (CYP3A4) D) midazolam (CYP3A4).
Table 1. The primer oligonucleotides used to clone the genes

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequences 5’ to 3’</th>
<th>Fragment Length (pb)</th>
<th>Target Ref cDNA</th>
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<td>CYP1A2 FP-KpnI</td>
<td>gcaGGTACCgttggtacagATGgcatt</td>
<td>1627</td>
<td>NM_000761</td>
</tr>
<tr>
<td>CYP1A2 RP-KpnI</td>
<td>aaaGGTACCgaacaagggctgagtc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C9 RP-EcoRI</td>
<td>gGAATTCCggcttcaATGgattcttetgtgg</td>
<td>1485</td>
<td>NM_000771</td>
</tr>
<tr>
<td>CYP2C9 FP-XbaI</td>
<td>cgTCTAGAccttetccagacaggaatgaa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The enzyme restriction sites for cloning purposes are indicated in capital letters.

ATG translation start codon coding for the starting amino acid of the protein (Met) is indicated in capital letters.
Table 2. Incubation conditions for model compounds kinetic assays in Ad-CYP HepG2 cells.

<table>
<thead>
<tr>
<th>P450 enzyme</th>
<th>Reaction</th>
<th>Substrate concentration</th>
<th>Cell density</th>
<th>Ad-CYP dose</th>
<th>Incubation time</th>
<th>Marker reaction for RAF calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylation</td>
<td>0.1-100 μM</td>
<td>120,000 cells/well</td>
<td>4 MOI/well</td>
<td>5 min</td>
<td>7-Ethoxyresorufin O-deethylation</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide 4-hydroxylation</td>
<td>1-1000 μM</td>
<td>120,000 cells/well</td>
<td>24 MOI/well</td>
<td>15 min</td>
<td>Diclofenac 4’- hydroxylation</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Alprazolam 4-hydroxylation</td>
<td>1-1000 μM</td>
<td>120,000 cells/well</td>
<td>16 MOI/well</td>
<td>60 min</td>
<td>Testosterone 6β-hydroxylation</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Midazolam 1’-hydroxylation</td>
<td>0.5-1000 μM</td>
<td>120,000 cells/well</td>
<td>33 MOI/well</td>
<td>15 min</td>
<td>Testosterone 6β-hydroxylation</td>
</tr>
</tbody>
</table>
Table 3. Mass spectrometer conditions for MRM metabolite quantification.

<table>
<thead>
<tr>
<th></th>
<th>Acetaminophen (CYP1A2)</th>
<th>4-OH-tolbutamide (CYP2C9)</th>
<th>4-OH-diclofenac (CYP2C9)</th>
<th>4-OH-alprazolam (CYP 3A4)</th>
<th>1’-OH-midazolam (CYP 3A4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Capillary voltage</td>
<td>3</td>
<td>2.8</td>
<td>3</td>
<td>3.05</td>
<td>3.05</td>
</tr>
<tr>
<td>Cone voltage</td>
<td>30</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Desolvation temperature</td>
<td>550</td>
<td>550</td>
<td>550</td>
<td>550</td>
<td>550</td>
</tr>
<tr>
<td>Source temperature</td>
<td>320</td>
<td>320</td>
<td>320</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>Collision energy</td>
<td>32</td>
<td>20</td>
<td>35</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Analyte m/z transition</td>
<td>152→110</td>
<td>287→171</td>
<td>312→230</td>
<td>325→280</td>
<td>342→324</td>
</tr>
</tbody>
</table>
Table 4. Metabolite formation kinetic parameter values in Ad-CYP HepG2 cells.

<table>
<thead>
<tr>
<th>P450</th>
<th>Substrate</th>
<th>$K_M$ (or $S_{50}$)</th>
<th>$K_{si}$</th>
<th>$n$</th>
<th>$V_{max}$</th>
<th>$CL_{int}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μM</td>
<td>μM/min/10⁶ cells</td>
<td>pmol/min/10⁶ cells</td>
<td>μl/min/10⁶ cells</td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>12.3 ± 2.6</td>
<td>448 ± 217</td>
<td>39.6 ± 24.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide</td>
<td>29.8 ± 3.1</td>
<td>7050 ± 4242</td>
<td>69.0 ± 4.7</td>
<td>2.34 ± 0.317</td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Alprazolam</td>
<td>659 ± 560&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6</td>
<td>2160 ± 1330</td>
<td>2.17 ± 0.683&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>4.59 ± 2.47</td>
<td>242 ± 78</td>
<td>185 ± 45</td>
<td>50.4 ± 31.1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Denotes $S_{50}$ value.

<sup>b</sup> Denotes $CL_{max}$ value.
Table 5. Intrinsic clearance in Ad-CYP HepG2 cells and suspended cryopreserved hepatocytes and comparison with *in vivo* intrinsic clearance.

<table>
<thead>
<tr>
<th></th>
<th>Substrate</th>
<th>RAF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Scaled Ad-CYP CL&lt;sub&gt;int&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hepatocyte CL&lt;sub&gt;int&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Ad-CYP predicted CL&lt;sub&gt;int&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Hepatocyte predicted CL&lt;sub&gt;int&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
<th>In vivo observed CL&lt;sub&gt;int&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450</td>
<td>Phenacetin</td>
<td>2.1</td>
<td>83.2</td>
<td>13.5</td>
<td>214</td>
<td>34.7</td>
<td>615</td>
</tr>
<tr>
<td></td>
<td>Tolbutamide</td>
<td>0.29</td>
<td>0.679</td>
<td>0.148</td>
<td>1.74</td>
<td>0.38</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>Alprazolam</td>
<td>0.98</td>
<td>2.13</td>
<td>0.818</td>
<td>5.47</td>
<td>2.1</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>Midazolam</td>
<td>0.46</td>
<td>23.2</td>
<td>77.9</td>
<td>59.6</td>
<td>200</td>
<td>314</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative Activity Factor based on a ratio of marker activities (7-ethoxyresorufin O-deethylation, diclofenac 4′-hydroxylation or testosterone 6β-hydroxylation) in human cultured hepatocytes and Ad-CYP-transduced HepG2 cells.

<sup>b</sup> Scaled using RAF for the primary cultured human hepatocytes.

<sup>c</sup> Data from Brown et al., 2007 (tolbutamide, alprazolam, midazolam) and Stringer et al., 2008 (phenacetin).

<sup>d</sup> Scaled using a hepatocellularity of 120 x 10<sup>6</sup> hepatocytes/g and liver weight of 21.4 g/kg and based on human cryopreserved hepatocytes.
Fig. 2.