DMD Fast Forward. Published on November 8, 2006 as doi:10.1124/dmd.106.009423
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DMD #9423

UTILITY OF LONG-TERM CULTURED HUMAN HEPATOCYTES AS AN *IN VITRO* MODEL FOR CYTOCHROME P450 INDUCTION

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Number of text pages	15
Tables	2
Figures	4
References	34
Number of words in the Abstract	240
Number of words in the Introduction	721
Number of words in the Results and Discussion	1339

Abstract

Cytochrome P450 induction may have considerable implications for drug therapy. Therefore, understanding the induction potential of a new chemical entity at an early stage in discovery is crucial to reduce the risk of failure in the clinic and help the identification of non-inducing chemical structures. Availability of human viable tissue often limits evaluation of induction potential in human hepatocytes. A solution is to increase the time period during which the hepatocytes remain viable. In this study we have investigated the induction of several CYP isozymes in long term cultured hepatocytes compared with short term cultured hepatocytes from the same individuals. Short and long-term cultured primary hepatocytes isolated from each individual were cultured on a 96-well format and treated for 24 hours with a range of prototypical CYP inducers and Merck Research Laboratories (MRL) compounds. CYP3A4, 1A1, 1A2, 2B6 and 2C9 mRNA levels were measured using quantitative real-time reverse transcriptase-polymerase chain reaction (TaqMan) from the same cultured hepatocyte wells. CYP3A4, 1A1, 1A2, 2B6 and 2C9 were shown to be inducible in long term cultured hepatocytes. The fold induction varied between donors, and between short and long term cultured hepatocytes from the same donor. However, this variability can be controlled by normalising data from each hepatocyte preparation to a positive control. The use of long term cultured hepatocytes on 96-well plates has proven to be sensitive, robust and convenient for assessing CYP induction potential of new compound entities during drug discovery process.

Introduction

Metabolism by Cytochrome P450 (CYP) is a major route of detoxification for a large number of xenobiotics including pharmaceuticals (Guengerich, 1990). It is now well established that expression of CYP genes can be regulated by a range of chemicals (LeCluyse, 2001). Consequently any changes in the expression of these enzymes are of importance to scientists in the field of drug development, especially if the changes are brought about by treatment with a drug candidate.

An inducing drug may lead to a drug-drug interaction through induction of an alternate enzyme responsible for the clearance of a co-administered therapy thereby resulting a decrease in exposure of this drug (Worboys and Carlile, 2001). Another possible consequence of CYP induction is that blood concentrations of drugs that induce their own metabolism may fall to sub-therapeutic levels (a so-called autoinduction) negating any beneficial effect (Simonsson et al., 2003). Consequently an understanding of the potential for a new chemical entity to elicit an induction response is critical to avoid costly errors in the clinic.

Traditionally the approach taken was to repeatedly dose animals with the test compound followed by analysis of liver CYP enzymes ex vivo. This approach in drug discovery is cumbersome because it requires large numbers of animals, a large amount of test substance, and is very labour intensive, with the additional caveat of lack of relevance to the human situation. Therefore a number of *in vitro* human models have been developed such as they use of *in vitro* assays based on expression

of specific reporter gene constructs for orphan nuclear receptors (NR) such as the Pregnane-X-receptor (PXR) (Ogg et al., 1997). Although these assays have the potential of screening large numbers of compounds, this approach runs the risk of false negatives due to the multiple mechanisms that may contribute to an induction phenomenon and serves to highlight the importance of hepatocytes as a tool.

Primary human hepatocytes are considered the 'gold standard' for *in vitro* testing of the induction potential of drug candidates (Kostrubsky et al., 1999;Li et al., 1997;Silva et al., 1998). However, several disadvantages are associated with the use of this model system. Hepatocytes for primary cultures have to be isolated from the liver every time they are required. As human liver availability is limited and unpredictable, planning of experiments is rendered difficult. Additionally, it is difficult to assess the quality of hepatocyte preparations that are to be used as primary cultures. One possibility to overcome these difficulties is to develop long-term culture systems which additionally would provide flexibility on the timing of experiments.

Several systems for long-term culture have been described in the last 20 years. These include co-culture of hepatocytes with a rat liver epithelial cell line (Guillouzo et al., 1985), the use of extracellular matrix components (Dunn et al., 1991), and the use of a specific additive such as dimethyl sulfoxide (Isom et al., 1985). The long term culture system used in this study has the advantage of not requiring the use of additive cells or a matrix component preparation and is therefore, as easy to use as the conventional culture systems. This system has been shown to maintain liver phenotypic characteristics for at least 35 days (Ferrini et al., 1997; Lanford et al., 1989). Previous work using long term cultured human hepatocytes suggested that human hepatocytes

maintained in the modified long term Lanford's medium for several weeks were suitable for investigating xenobiotic metabolism (Guyomard et al, 1990). The use of long term cultured hepatocytes to investigate CYP gene expression was previously reported (Pichard-Garcia et al., 2002). In this study we have extended this approach by investigating the usefulness of long-term cultured human hepatocytes as a high throughput *in vitro* model to study the induction of CYP in drug discovery. To account for any idiosyncratic differences, hepatocytes obtained from each donor were split into two batches for short and long term culture on 96-well collagen-coated plates. CYP1A1, 1A2, 2B6, 2C9 and 3A4 mRNA levels were measured using quantitative PCR after treatment of the hepatocytes for 24 hours with a range of prototypical CYP inducers and MRL compounds. The study showed that induction of these CYP isoforms can be measured from as few as 30,000 cells per well. It also demonstrated that long term culture is a useful tool for investigating CYP induction of NCE in the drug discovery process.

Materials and Methods

Chemicals

Collagen I coated 96 well supports were obtained from AES (Combourg, France). Williams E culture medium, glutamine, penicillin and streptomycin for the isolation and culture of the hepatocytes were from Invitrogen (Cergy, France). Bovine insulin and hydrocortisone succinate were from Sigma (St Quentin Fallavier, France), and fetal calf serum was obtained from Dutscher (Brumath, France). Williams E culture medium, L-glutamine and penicillin/streptomycin for hepatocyte treatment were obtained from Invitrogen (Paisley, UK). Gentamycin, dexamethasone (DEX), rifampicin (RIF), phenobarbital (PB), pregnenalone 16α-carbonitrile (PCN), clotrimazole (CLOT), omeprazole (OME), and sterile dimethyl sulfoxide (DMSO) were from Sigma (Poole, UK). Nucleic acid purification lysis solution was obtained from Applied Biosystems (Warrington, UK).

Isolation of Hepatocytes and Culture Conditions

Human hepatocytes were isolated from surgical wastes by Biopredic (Rennes, France) using the two-step dissociation method as described previously (Guguen-Guillouzo et al., 1986). The cells were seeded on collagen I 96 well coated supports at a density of 0.17 million cells per cm^2 (Figure 1). Hepatocytes were plated in Williams medium E supplemented with glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 µM bovine insulin, and 10% (v/v) fetal calf serum (Short term cultured medium). Cells

were allowed to attach for 24 h, at which time the media was replaced with either a long term (LT) culture medium (Ferrini et al., 1997;Lanford et al., 1989) or a short term (ST) culture medium composed of Williams E medium supplemented as described above but the fetal calf serum was replaced with 5 x 10^{-5} M hydrocortisone hemisuccinate. Short term culture hepatocytes were then shipped at room temperature to the Neuroscience Research Centre (Harlow, UK) for treatment. Long term culture hepatocytes were maintained in the long term culture medium for two weeks before shipping. Prior to shipping of the hepatocytes, phenacetin deethylase and nifedipine oxidase activities were measured according to published methods (Wortelboer et al., 1990). Long and short term hepatocytes were cultured from 3 different individuals as shown in figure 1.

Hepatocyte Treatment Protocol

Upon arrival of the cells at the Neuroscience Research Centre, the media was replaced with Williams medium E containing 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 µg/ml gentamycin, 100 nM dexamethasone, and 1 µM insulin. The cells were maintained in an atmosphere of 95% air and 5% CO_2 at 37°C for an additional 24 h. Hepatocytes were then treated (n = 3 wells/treatment) with prototypical inducers including 10 to 50 µM RIF, 100 to 1000 µM PB, 5 to 10 µM PCN, 25 to 100 µM DEX, 2.5 to 10 µM CLOT, and 25 to 100 µM OME for 24 h. The hepatocytes were also treated with novel Merck compounds (A to E) at concentrations ranging from 0.1 to 10 µM (Figure 1). All compounds were dissolved in sterile DMSO which was added to the culture medium at a final concentration of 0.1%. DMSO was added as one treatment to act as a dose vehicle control. At the end of the

induction period, the medium was aspirated and replaced with 125 µl/well lysis solution for storage at -80° before analysis for CYP mRNA levels.

Extraction of Total RNA.

Total RNA was extracted from hepatocytes was performed in a 6700 Nucleic acid Workstation using manufacturer's solutions and protocols (PE Applied Biosystems, UK). On extraction, treatment with an Absolute RNA wash was included to eliminate DNA and inhibitory proteins.

TaqMan QRT-PCR

All primers and probes were submitted to the National Center for Biotechnological Information (NCBI) for nucleotide comparison using the basic logarithmic alignment search tool (BLASTn) search for short, nearly exact sequence to ensure specificity. Primers and probes were synthesized by Applied Biosystems (UK), where probes were 5' – and 3' – labelled with the FAM and TAMRA reporter dyes, respectively. Additional primers and probes were generated as shown in Table 1. the human 18s primer/probe set was purchased from Applied Biosystems (UK) and used per manufacturer's instructions. A two-step RT-PCR reaction was conducted by reverse-transcribing an aliquot of total RNA (~ 50 ng) to cDNA on the Workstation using the high capacity cDNA archive kit as per manufacturer's instructions (PE Applied Biosystems UK). Using the Workstation samples were diluted 1:20 and final plates prepared for analysis on 9700 real time QPCR, instrument (PE Applied Biosystems UK).

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Primer and probe concentrations were 300 and 200 nM respectively for all isoforms. PCR was performed using TaqMan Universal PCR Master Mix and TaqMan ribosomal RNA control kits according to manufacturer's recommended protocols (PE Applied Biosystems, UK). RT-qPCR was performed using the ABI PRISM 9700 Sequence Detector instrument and Sequence Detector v.2.1.1 software (PE Applied Biosystems). PCR amplification conditions were as follows: 50°C, 2 min, followed by 95°C, 10 min, then 40 cycles at 95°C, 15 sec; and 40 cycles at 60°C, 1 min. PCR amplified cDNAs were detected by real time fluorescence on an ABI PRISM 9700 Sequence Detection System (Applied Biosystem). Quantitation of the target cDNAs in all samples was normalized to ribosomal 18s (18s; $Ct_{target} - Ct_{18s} = \Delta Ct$), and the effects of each compound on the target cDNA was expressed to the amount in the dimethyl sulfoxide (vehicle) control sample ($\Delta C_{tcompound} - \Delta Ct_{DMSO} = \Delta\Delta Ct$). Fold changes in target gene expression were determined by taking 2 to the power of the $\Delta\Delta Ct$ value ($2^{-\Delta\Delta Ct}$) as per user bulletin (PE Applied Biosystems, UK).

Statistical Analysis

Results were presented as the mean ± SD from three separate experiments. Statistical analysis was performed using Student's t test accepting $p < 0.05$ as significant.

Results and Discussion

Xenobiotic metabolism in mammals is a process mediated largely by the cytochrome P450 superfamily of enzymes. Induction of CYP has been shown to have considerable implications for drug therapy, namely autoinduction and drug-drug interactions (Simonsson et al., 2003;Worboys and Carlile, 2001). Therefore, it is important to understand the induction potential of a new chemical entity early in the drug discovery process.

Here, we described the usefulness and the implementation of a CYP induction assay based on previous studies which used long term cultured hepatocytes (Pichard-Garcia et al., 2002). The main advantages of this type of approach are that it is a high throughput assay which facilitates the investigation of CYP induction issues earlier in the drug discovery process and provides flexibility on the timing of the experiments.

The nifedipine oxidase (CYP3A4) and phenacetin deethylase (CYP1A2) activities of both the short-term cultured and long-term cultured hepatocytes were measured in intact cells prior to treatment with prototypical inducers for quality control purposes (Table 2). There was small inter-individual variability in the levels of phenacetin deethylase and nifedipine oxidase activities in both short and long term cultured hepatocytes reflecting the inherent variability within human donors (LeCluyse, 2001). The long term cultured hepatocytes of each individual showed a decrease in phenacetin deethylase and nifedipine oxidase activities compared to the corresponding short term cultured hepatocytes, except for individual number 2 who

showed same levels of nifedipine oxidase in both types of culture. However, the activity values obtained for both phenacetin deethylase and nifedipine oxidase in the long term cultured hepatocytes remained within normal historical ranges of short term cultured hepatocytes (LeCluyse, 2001; Madan et al., 2003). One of the advantages of using long term cultured hepatocytes is that the quality control activity measurement could be done by suppliers prior to shipment of the cells. This could potentially save time and resources to investigators if the human hepatocytes were not good for further studies.

Prototypical inducers of the major CYP isoforms were incubated for 24 hours in both short and long-term cultured hepatocytes isolated from the same donors (Figure 1). This method compensated for the inherent variability between human donors, and thus allowed the effect of the long-term culture conditions on CYP induction to be assessed directly. Our results showed that rifampicin, phenobarbital, clotrimazole, dexamethasone and omeprazole were able to induce CYP3A4 mRNA levels in both short and long-term cultured hepatocytes as reviewed by Luo (Luo et al., 2004) (Figure 2). Results showed differences in CYP3A4 mRNA fold changes between short and long term cultured hepatocytes for most of the drugs tested in this study, although these differences were not statistically significant (Figure 2A). An alternative way is to report data as percentage of positive control to get around of the fold change variability observed in response to the different inducers. Figure 2B showed that when data are expressed as percentage of positive control (RIF at 10 μM) induction values become more comparable between the two types of cultures and individuals. Pregnenalone 16 α-carbonitrile was used as a negative control. PCN is known to induce CYP3A1 in rat but does not induce CYP3A4 in humans (Kocarek et

al., 1995). As expected, PCN did not cause any induction of CYP3A4 mRNA in either the short term or long term cultured hepatocytes.

CYP1A1 and 1A2 mRNA levels were significantly increased in the presence of omeprazole (Figure 3A, 3B). This was consistent with previous reports in the literature using primary human hepatocytes (Lu and Li, 2001). The level of induction of CYP1A mRNA by omeprazole was not statistically different between the short term and long term cultured hepatocytes. None of the other drugs tested in this study induced CYP1A1 and CYP1A2 mRNA levels (data not shown).

Increased interest in the CYP2B6 enzyme has been stimulated by the discovery of polymorphic and ethnic differences in CYP2B6 expression (Lang et al., 2001), identification of additional substrates for CYP2B6, and evidence for cross-regulation with CYP3A4 expression (Makinen et al., 2002). As previously reported, several prototypical CYP3A4 inducers also induced the expression of CYP2B6 mRNA (Gervot et al., 1999;Goodwin et al., 2001;LeCluyse et al., 2000;Sahi et al., 2000). Rifampicin and phenobarbital were shown to be potent inducers of CYP2B6 mRNA in both short and long term cultures, with phenobarbital exhibiting a marked dose response effect (Figure 3C). Clotrimazole and dexamethasone also showed a dose response effect resulting in moderate induction of CYP2B6 mRNA in both culture types. Additionally, omeprazole was shown to be a potent inducer of CYP2B6, which to our knowledge has not been reported previously in the literature. The ability of omeprazole to potently induce CYP1A and CYP2B6 is perhaps not surprising based on the numerous reports of cross-talk between the different nuclear receptors.

CYP2C9 is also important in the metabolism of numerous clinically used drugs (Goldstein, 2001;Goldstein and de Morais, 1994;Lee et al., 2002;Miners and Birkett, 1998). As such, the induction of CYP2C9 mRNA was investigated in this study. Rifampicin, phenobarbital and dexamethasone were all shown to induce CYP2C9 mRNA in both culture types (Figure 3D). For phenobarbital and dexamethasone, the CYP2C9 mRNA fold change was equivalent in both the short and long term cultured hepatocytes. However, rifampicin showed a greater fold change in the short term cultured hepatocytes compared to the long term cultured hepatocytes at 25 μM. The magnitude of the fold change values of CYP2C9 mRNA following treatment with prototypical inducers was very small, ranging from approximately 1.5 to 3.5 fold. This is consistent with previous reports which suggest that the small fold change values are attributed to high basal CYP2C9 mRNA levels (Chen et al., 2004).

A variable induction response between hepatocytes from different individuals was observed in this study. This variation has previously been described and is believed to be due to a variety of factors including hepatocyte quality, together with health, diet, medication, polymorphisms, and basal mRNA expression of the donor (Kostrubsky et al., 1998;LeCluyse et al., 2000;Silva et al., 1998). A method to circumvent inter-individual variability is to report percentage induction relative to a standard inducer, with the standard inducer included in every experiment (Silva et al., 1998). Our results from this study showed that variability between individuals was greatly reduced by expressing data as percentage induction rather than fold induction. Therefore, this method would enable us to compare the induction potential of new chemical entities (NCEs) in hepatocytes from different individuals.

To demonstrate the utility of the long term cultured hepatocytes for induction screening of drug candidates, we investigated the induction potential of 5 MRL compounds in both the short and long term cultured hepatocytes. The induction liability of these MRL compounds was expressed as percentage of positive control as shown in figure 4. Both culture types allowed the test compounds to be ranked in the same order. For compounds that exhibited a dose response in the short term cultures, this was also observed in the long term cultured hepatocytes. Additionally, MRL-E dosed at 10 μM showed a decrease in CYP3A4 and 18S mRNA levels in both culture types, which was likely due to cell toxicity (data not shown). The same MRL compounds had also been tested in different short term cultured hepatocytes from different sources and similar percentage of positive control was reported (data not shown).

In summary, our results showed that induction of a range of CYP isoforms can be measured in primary human hepatocytes cultured on a 96 well plate with 30,000 cells/well only. The long term cultured hepatocytes have proven to be comparable to conventional culture systems with the addition of several advantages. Long term cultured hepatocytes can be preserved from previous isolations meaning that a fairly regular supply to the researcher can be maintained. The quality of long term cultured hepatocytes can also be assessed prior to use saving time and resources to investigators. This quality assessment, combined with the ability to culture hepatocytes in a 96 well format, and using TaqMan RT-PCR analysis, demonstrated long term cultured hepatocytes are an ideal tool for the assessment of induction potential of NCEs in a drug discovery environment.

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Figure 1

Experimental design as described in material and methods section.

Figure 2

CYP3A4 mRNA induction in short and long term cultured human hepatocytes following exposure to prototypical inducers for 24 hours. A) Results express as fold change versus control. B) Results express as percentage of positive control (10 µM Rifampicin). Results are the mean ± S.D. of three different donors. * = $P < 0.05$; ** = $P < 0.01$.

Figure 3

CYP mRNA expression levels in short and long term cultured human hepatocytes. A) CYP1A1; B) CYP1A2; C) CYP2B6 and D) CYP2C9. Results express as fold change versus control. Results are the mean ± S.D. of three different donors. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Figure 4

Induction of CYP3A4 mRNA levels in fresh and long term cultured human hepatocytes following exposure to MRL compounds at concentrations of 0.1, 1 and 10µM. Results are expressed as percentage of positive control (10µM RIF). Data are the mean ± S.D. of three different donors. * = $P < 0.05$; ** = $P < 0.01$.

Table 1: Human primer-probe sets and gene abbreviations

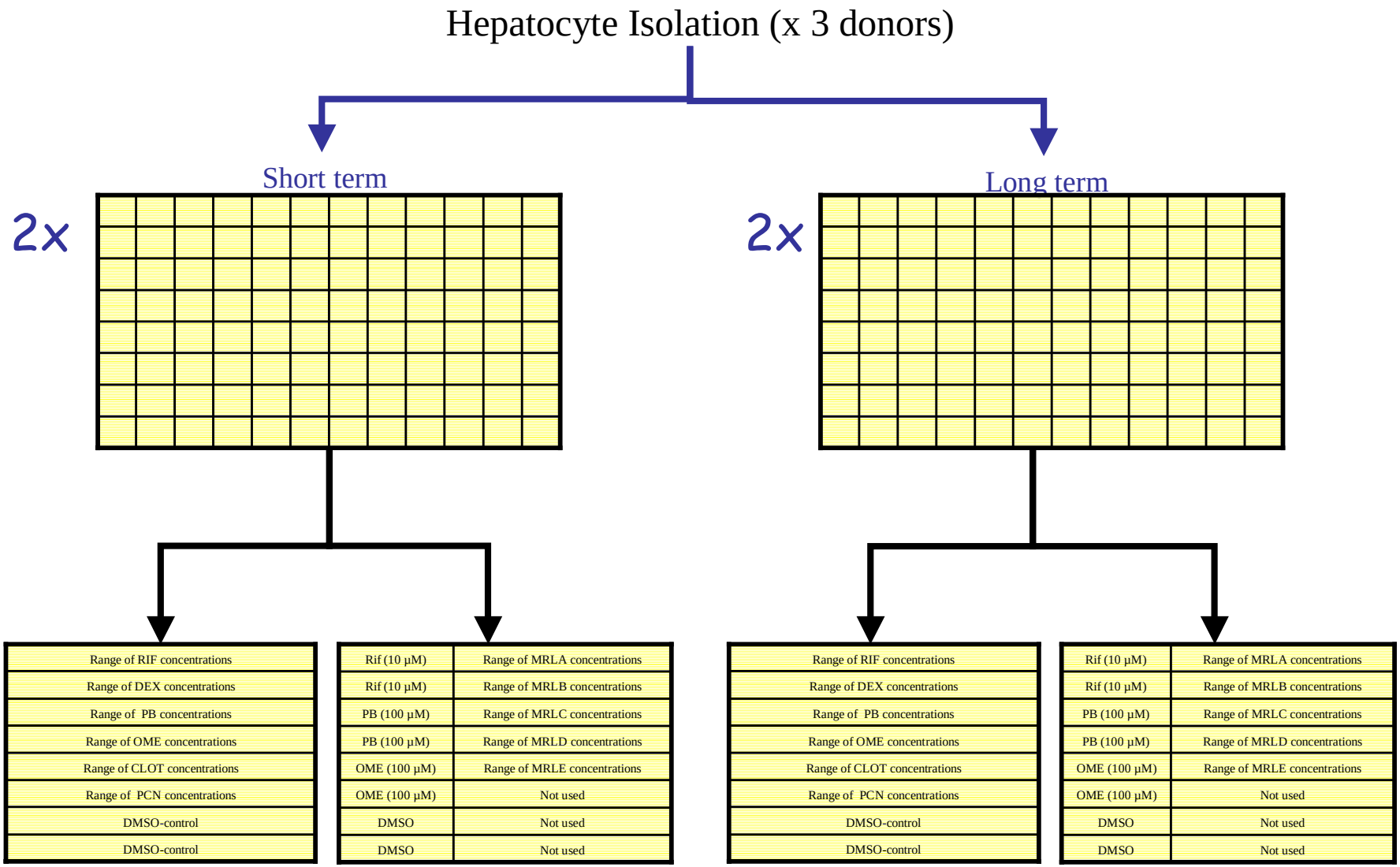
Gene	Accession Number	Forward Primer (5'→3')	Reverse Primer (5'→3')	Probe (5'FAM→3'TAMRA)
CYP1A1	K03191	GGCGTTGTGTCTTTGTAAACCA	AGGTAGGAACTCAGATGGGTTGAC	TGGCAGATCAACCATGACCAGAAGCTATG
CYP1A2	Z00036	AAATGCTGTGTCTTCGTAAACCAG	CGCTCAGGCCGGAACTC	CCAGAGCTGTGGGAGGACCCCTC
CYP2B6	M29874	GCCACCCTAACACCCATGAC	TGAGTAGGCCTCTTCTATCCATCTG	FAM-CACCAGGGCCCCGCCCTCT
CYP2C9	S46963	TGAAAGCTTGGAAAACACTGCA	GCATATCTCAGGGTTGTGCTTG	CGTCTCTGTCCCAGCTCCAAACAAGTCA
CYP3A4	M18907	GTGTGTTTCCAAGAGAAGTTACAAATTT	CCACTCGGTGCTTTTGTGTATC	CGAGGCGACTTTCTTTCATCCTTTTTACAGA

Table 2: Levels of nifedipine oxidase, phenacetin deethylase activity in short and long term cultured hepatocytes.

Individual	CYP3A		CYP1A	
	Short	Long	Short	Long
	nifedipine oxidase	nifedipine oxidase	phenacetin deethylase	phenacetin deethylase
n = 1	8.7	3.6	11.3	0.8
n = 2	10.8	9.6	5.4	0.7
n = 3	4.9	0.7	3.5	0.1

CYP3A and CYP1A activity values are expressed as nmol/hr/mg protein. The results are the mean of three determinations.

Figure 1



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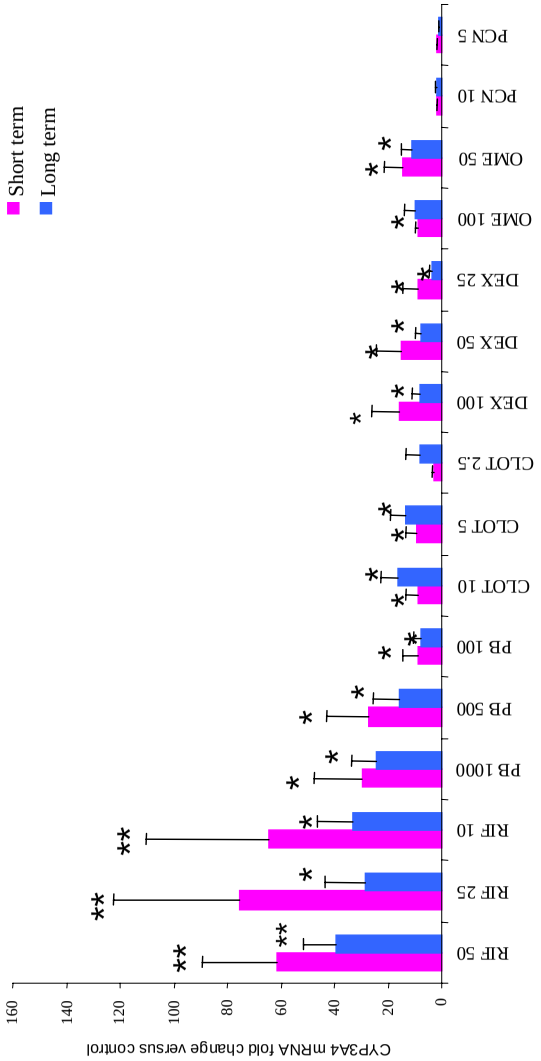

Figure 2
A

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Figure 2
B

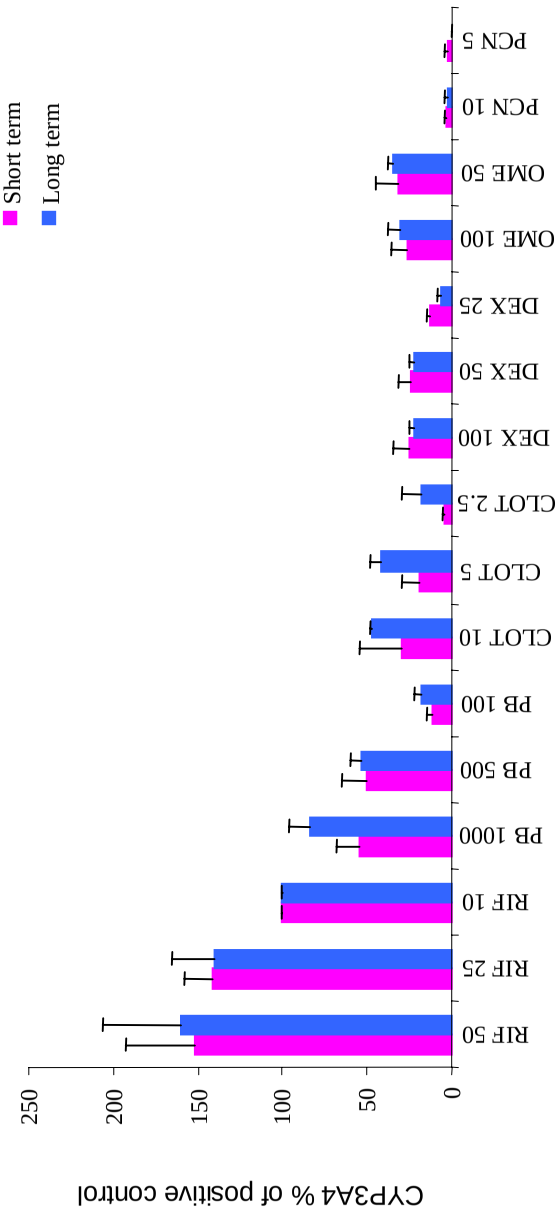


Figure 3
A

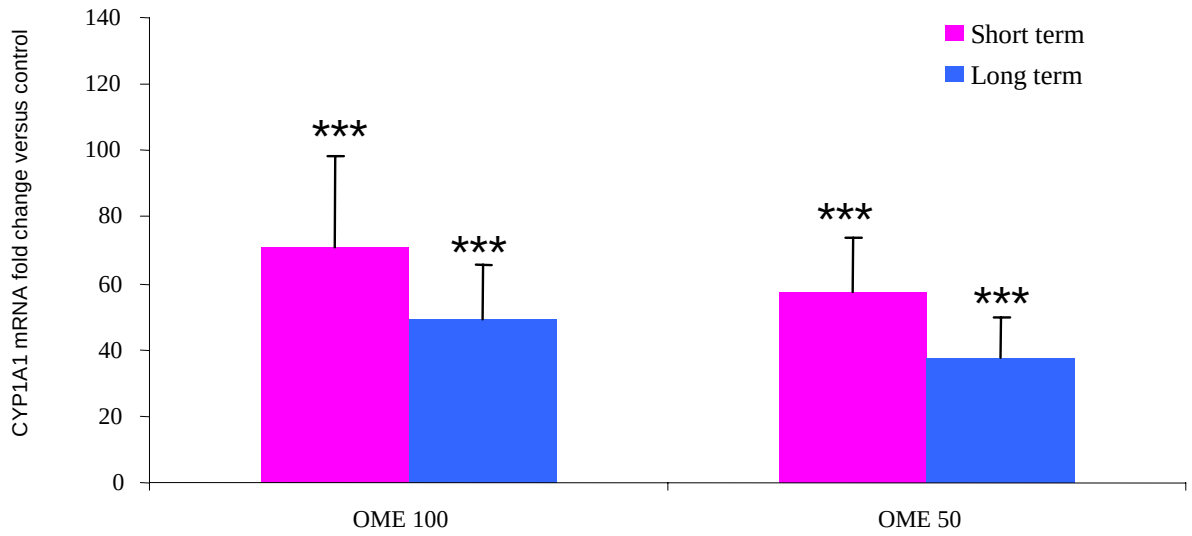


Figure 3B

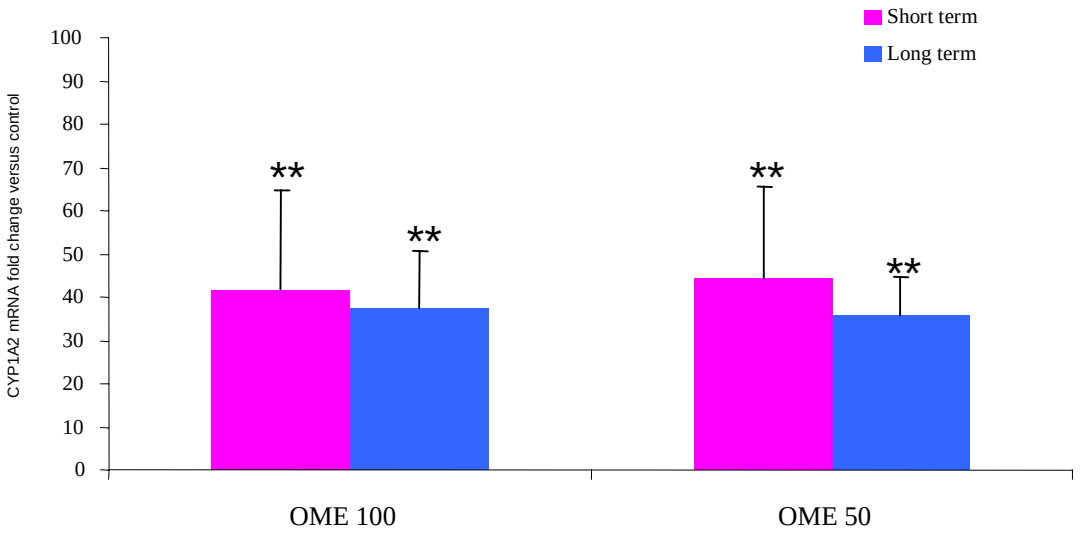
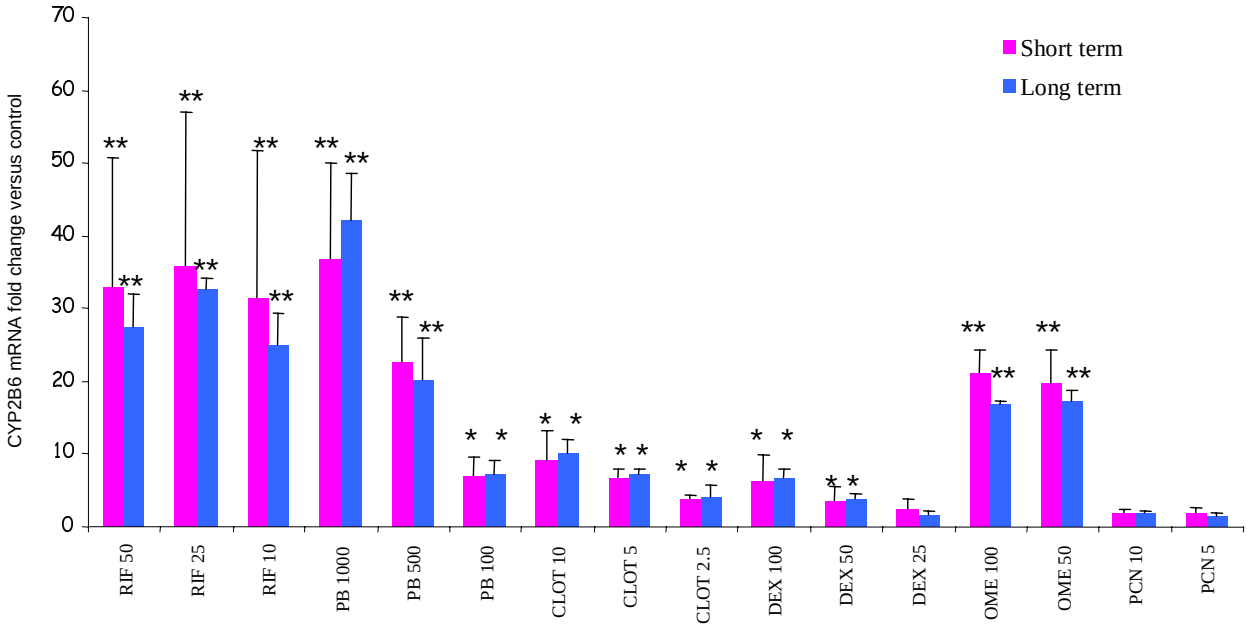


Figure 3C



DMD Fast Forward. Published on November 8, 2006 as DOI: 10.1124/dmd.106.009423
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Figure 3D

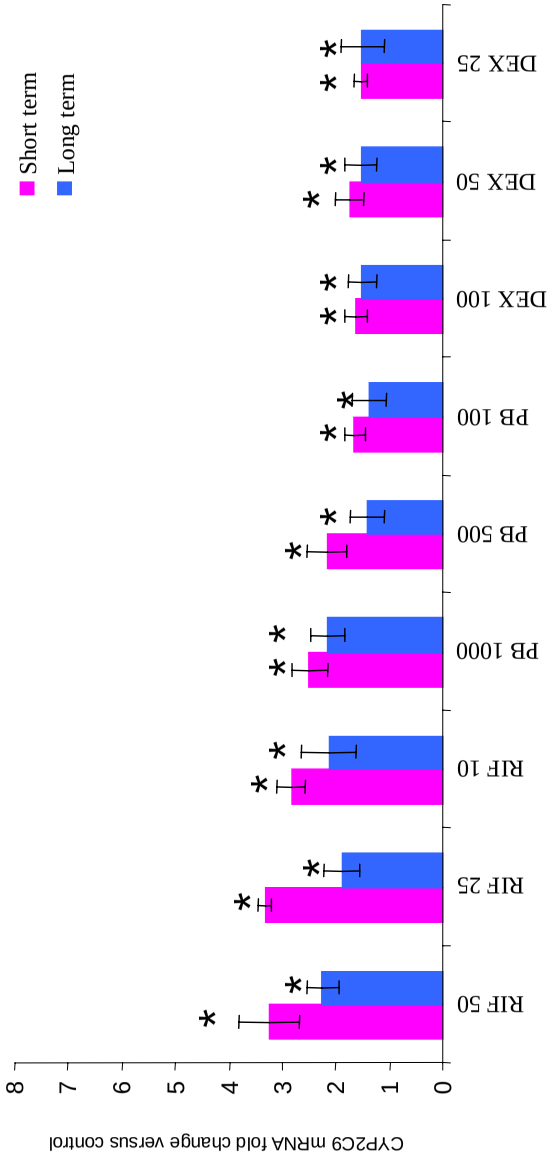

Figure 4