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Induction of cytochrome P450; Assessment in an immortalized human hepatocyte cell line (Fa2N4) using a novel higher throughput cocktail assay.

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Abbreviations. CYP, cytochrome P450; DDI, drug-drug interaction; NCE, new cemical entity; PXR, pregnane X receptor; AhR, aryl hydrocarbon receptor; CAR constitutive androstane receptor; MDR-1 , P-glycoprotein; RNA, ribosomal nucleic acid; DNA, deoxyribose nucleic acid PBS, phosphate buffered saline, KHB Krebs

buffer KHB; ANOVA, analysis of variance; RT-PCR, Reverse Transcription Polymerase Chain Reaction ; LC-MS, liquid chromatography-mass spectroscopy

Abstract

Over recent years the application of cocktail studies to measure biological markers, have become increasingly popular. The current study investigated a novel approach in assessing P450 enzyme induction in an immortalized cell line, using a 'cocktail' of five cytochrome P450 substrate probes compared with the traditional single probe approach. The findings reported herein support use of a cocktail approach to assess the induction of the major P450s; namely CYP3A4, 1A2, 2C9. Cytochrome P450s 2C19 and 2D6 could also be followed as part of the cocktail approach reported. Response to prototypical inducers did not differ to those observed in the presence of the specific probes alone. Consequently this approach requires significantly fewer sample numbers, if screening the induction potential of more than one cytochrome P450. Moreover, these studies highlight the utility of the immortalized cell line, Fa2N4 as a robust model system for induction studies. In conclusion, the current experimental set-up is an improvement on current approaches employed to assess cytochrome P450 induction, significantly increasing sample throughput.

Introduction

Drug-drug interactions (DDIs) are a particularly important type of adverse drug event that can result in the physiological levels of a drug climbing above or falling below this therapeutic window. A continual increase in plasma concentrations of a drug can ultimately result in toxicity, whilst a decrease, results in the failure in efficacy. In this regard, a new chemical entity's (NCEs) progression through the discovery/development process is rate-limited, depending upon the degree of undesirable properties/actions, which include in part interactions with members of the hepatic drug metabolising enzymes, in particular the cytochrome P450 family. Among the most important adverse interactions that NCEs must be assessed for is their propensity to act as perpetrators, in either the inhibition and/or induction of cytochrome P450 enzymes (see reviews by (Lin, 2006); (Lin and Lu, 1998)), the latter of which is the basis of the current series of studies.

The induction of cytochrome P450 enzymes, results in an increase in the steady-state concentrations of these enzymes following organ exposure to relevant stimuli. This is predominantly observed with hepatic cytochrome P450s; 3A4 and 1A2, and to a lesser extent 2C9 and 2B6 (Dickins, 2004). Over recent years, there has been a considerable increase in the knowledge surrounding the mechanism behind these processes. The cascade(s) involved in the eventual increase in the steady-state concentration has been shown to involve activation of several nuclear hormone receptors; the pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR) and the constitutive androstane receptor (CAR) (see reviews by (Wang and LeCluyse, 2003; Bock and Kohle, 2004))for more detail descriptions of these pathways). In brief, CAR and PXR, regulate distinct but overlapping sets of target genes, which include

certain phase I cytochrome P450 enzymes (e.g., CYP2B, CYP3A and CYP2C), phase II conjugation enzymes, such as UDP-glucuronosyltransferase UGT1A1 and sulfotransferase SULT2A, and phase III transporters, such as P-glycoprotein (MDR-1). The AhR receptor has been shown to regulate the expression of CYP1A (Whitlock, 1999).

In the current study, an immortalized human hepatic cell line (Fa2N4 cells) was used as a model system to assess enzyme induction by two established inducers; rifampicin (Waxman, 1999; Roymans et al., 2004) and omeprazole (Raucy, 2003; Roymans et al., 2004). A recent study by Mills and co-workers (Mills et al., 2004) has described the utility of Fa2N4 cells as an in vitro model to assess P450 induction, showing significant retention of activity of cytochrome P450 enzymes and hepatic transporters such as MDR1. More recently this cellular model system has been utilized to predict clinical induction caused by CYP 3A4 (Ripp et al., 2006). Here in we report a novel approach in assessing P450 enzyme induction using a 'cocktail' of substrate probes, allowing the quantification of up to five major P450s in a single assay, thus significantly increasing the throughput for these experiments. Throughput is further increased through the combined assessment of enzyme activity and mRNA in a single sample.

Methods

Chemicals

Midazolam, 1-OH midazolam, (S)-mephenytoin, 4-OH mephenytoin and 4-OH diclofenac were generated biosynthetically at Pfizer Inc (Sandwich, UK). Dextrorphan was purchased from Sigma/RBI (Natick, MA, USA), PBS Dulbeccos (Phosphate Buffered Saline without sodium bicarbonate was obtained from Gibco (Paisley, UK). Fa2N4 cells and MFE™ Support (serum free) and Plating media were obtained from MultiCell Technologies (Rhode Island, USA). Fetal calf serum (FCS, heat inactivated), penicillin (100 Units/ml) and streptomycin (100 µg/ml) were purchased from Invitrogen (Paisley, UK). Collagen I flasks and plates were purchased from BD Biosciences (Cambridge, UK). Organic solvents were obtained from Romil (Cambridge, UK). Promega (Southampton, UK) supplied nuclease free water and SV-96 RNA extraction kits. All other chemicals were purchased from Sigma-Aldrich (Poole, Dorset).

Cell Culture

Fa2N-4 cells were grown on Biocoat type I collagen coated flasks and maintained in MFE™ Support media (serum-free). Flasks were incubated at 37 °C, 5 % carbon dioxide, and 95 % relative humidity. Support media was replaced every 2 days and cells passaged at least once a week. For passaging, cells were trypsinized (1 ml per 25 cm^2 plate surface area) for 5min and subsequently harvested in MFE™ plating media (containing serum proteins) (1:3 v/v), centrifuged at 500 g for 5 min and the pellet reconstituted in fresh MFE™ plating media and added into Biocoat type I collagen flasks. After 4 hr incubation, MFE™ plating media was replaced with MFE™ support media. All experiments were performed using Fa2N4 cells at passages

between 10 and 20. No significant changes in basal activities in probe metabolism were observed between passages 10 to 20.

Enzyme Induction

For induction studies, Fa2N-4 cells were plated onto Biocoat type I collagen coated 24-well plates, at 0.5 x 10^6 cells/well in MFE™ plating media, supplemented with 100 Units/ml penicillin and 100 µg/ml streptomycin. After 4 hr, the plating media was replaced with MFE™ support media. At 48 hr post-seeding cells were supplemented with fresh MFE™ support media containing prototypical inducers; rifampicin and omeprazole. A range of inducers concentrations, were prepared from 25 mM stock solutions in DMSO. Aliquots were subsequently diluted in MFE™ media (1 µl/ml), giving a final concentration range of inducers to be studied as; 0, 0.5, 1, 5 10 and 25 µM. These solutions were supplemented at 0.5 ml into appropriate wells of the 24-well plates. The final organic content per well was equal to 0.1 %. This procedure was repeated using fresh inducer solutions every 24 hr, up to a total of 96 hr.

Analysis of enzyme activity.

Single probe studies; CYP3A4, 1A2 and 2C9 enzyme activities were determined by measuring the extent of metabolism of specific probes; tacrine (CYP1A2), diclofenac (CYP2C9), (S)-mephenytoin (CYP2C19), and midazolam (CYP3A4), to their respective metabolites; 1-hydroxy tacrine, 4-hydroxydiclofenac, 4-hydroxy mephenytoin and 1-hydroxy midazolam, by mass spectrometry (see analytical procedures described below). Midazolam was chosen as the CYP3A4 probe owing to its clinical relevance (Kato et al., 2005).

In brief, test inducer solutions were aspirated from each well, and the cells washed twice with phosphate buffered saline (PBS) pH 7.4. Each probe was prepared in Krebs buffer (KHB, pH 7.4) to give a final concentration of 25 μM. Cells were incubated with 350μL of each solution for 1hr at 37 °C (5 % CO_2), after which 300μL aliquots were collected and added to tubes containing 50 μL of ice-cold acetonitrile, to precipitate the proteins. These solutions were mixed and stored at -80 °C until analyzed.

Cocktail probe studies; the cocktail solution contained the probes substrates; tacrine (CYP1A2), diclofenac (CYP2C9), (S)-mephenytoin (CYP2C19), dextromethorphan (CYP2D6) and midazolam (CYP3A4), which were prepared collectively in KHB buffer, each at a final concentration of 25 μM. Test inducer solutions were aspirated from each well, and the cells washed twice with PBS. Cells were then incubated with the cocktail solution for 1 hr at 37 °C, (5 % CO_2). Aliquots (300 μL) were collected and added to tubes containing 50 μL of ice-cold acetonitrile, to precipitate the proteins. These solutions were mixed and stored at -80 °C until analyzed. Formation of dextrorphan was followed as a negative control, since CYP2D6 is not inducible.

Analytical Procedure

Enzyme activities were measured by LC/MS using a Sciex API4000 with Turbo IonSpray (Hertfordshire, UK), in conjunction with an Agilent 1100 LC binary pumpn (Cheshire, UK). Samples (80 μL), was injected onto a Phenomenex luna phenyl-hexyl column (50 x 4.6 mm) (Cheshire, UK), peaks were separated by reversed phase chromatography (0-1 min 100 %A 0 %B, 1-6 min 0 %A 100 %B, 6.1-8 min 100 %A, 0 %B) using the following mobile phases; (A) MeOH/ACN:H_2O (10:90) containing

2mM ammonium acetate and 0.025 % formic acid (v/v), (B) MeOH/ACN:H_2O (90:10) containing 2mM ammonium acetate and 0.025 % formic acid (v/v), at a flow rate of 1 mL/min. The current procedure allows separation of the probes compounds from their respective metabolites. These metabolites are subsequently quantified using authentic standards of each respective metabolite, prepared individually/or as part of a cocktail, depending on the enzyme activity analysis procedure used. Values were used to calculate enzyme activities as nanomoles of metabolite per million cells per minute of incubation.

RNA Isolation

For single probe studies, Fa2N4 cells were seeded into separate 24-well plates to allow assessment of mRNA induction. Following collection of samples for analysis of enzyme activities, the remaining probe mix was removed and the cells washed with sterile water. RNA was then extracted from these samples, using the Promega SV 96 Total RNA Isolation System. Lysis buffer containing ß-mercaptoethanol was then added to each well to disrupt cellular architecture and release DNA. Following 10 min incubation at 37 °C, (5 % CO_2), supernatant was titrated and collected into RNAse free tubes. Total

Purity of the RNA extracted was determined by determining the RNA:DNA ratio through absorbance intensity measurements at 260:280 nm. Ratios greater than 1.8 were considered to have negligible DNA contamination. Absorbance values at 260nm were then used to estimate the concentration of RNA in the sample, using the Beer-Lambert equation (1);

$$A = e\ b\ c \qquad (1)$$

where A =absorbance (no units); e = molar absorptivity or extinction coefficient (mol^{-1}.L cm^{-1}); b = path length of the sample (cm); and c = concentration of the compound in solution ($mol.L^{-1}$).

Agilent RNA 6000 Nano Assay kits (Cheshire, UK) were also employed to spot-check the robustness of the automated RNA extraction procedure and confirm the purity of RNA (as estimated by the 260:280nm ratio). The RNA 6000 Nano LabChip kit provides information on the quality and quantity of RNA samples. The assay kit is designed primarily to check the quality and determine the concentration of total RNA and mRNA samples. The method used followed that recommended by the suppliers. The frequency of extracted RNA with a 260:280 nm ratio of greater than 1.8 was >90 % using the current extraction procedure.

Assessment of changes in mRNA expression

For assessment of mRNA induction, samples were diluted to a final concentration of 10 ng/μL. mRNA expression was quantified for CYP3A4 only, using commercially available INVADER assay kits from Third Wave Technologies (Wisconsin, USA), in accordance with the procedure supplied.

Statistical Analysis

The formation rate data of the respective metabolites were initially log transformed and subsequently examined by ANOVA to measure the influence of inducer concentration on fold induction. Inspection of the residuals generated following ANOVA, indicated that log-transformation was required to stabilise the variance (this transformation was performed on practical grounds, as differences between means of

log (Response) become differences in fold change). Statistical differences between each inducer concentration and vehicle control were not calculated. However all figures included a line that represented a 2-fold increase in response compared with vehicle control, against which different inducer concentrations could be assessed. This approach is in line with criteria against which a compound might be classed as a potential inducer (Bjornsson et al., 2003). In addition the 95 % confidence intervals (CI) for each data set, was also included above and below the 2-fold change.

Results

The induction in catalytic activities of CYP1A2, 2C9 and 3A4 were studied in an immortalized human hepatocyte cell line: Fa2N4, following exposure to the known inducers rifampicin or omeprazole for 72 or 96 hr. These P450s were studied following supplementation of tacrine, diclofenac and midazolam either alone or as part of a cocktail set. The cocktail set also included (S)-mephenytoin and dextromethorphan as probe substrates for CYPs 2C19 and 2D6. Previous preliminary studies performed in house have examined the specificity of these five probes for metabolism by the cytochrome P450s under investigation and confirmed the lack of any interactions (data not shown). Assessment of dextromethorphan metabolism served as a negative control, as CYP2D6 is not inducible under the conditions of this study. This was confirmed in the cocktail probe study, where no changes in the constitutive CYP2D6 activity were observed, in the presence of either rifampicin or omeprazole. The average formation rate of dextrorphan was <2nmol/min/million cells following exposure of either inducer between 0 and 25 μM. (data not shown).

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Cytochrome P450 3A4. Midazolam was incubated with Fa2N4 cells either alone or as part of a cocktail set. Figure 1A highlights the changes in CYP3A4 enzyme activity (nmoles/min/10^6 cells), as measured by 1-OH midazolam formation following incubation with midazolam only (single probe), after exposure to rifampicin for either 72 or 96 hr. A significant induction in CYP3A4 activity was observed as a function of increasing rifampicin concentration, following 72 and 96 hr exposure (ANOVA; $p<0.05$ in each case). Figures 1B and C show the formation rate data, following log transformation. The solid line represents a 2-fold increase from vehicle control, whilst the dashed lines represent the 95 % CI associated with the experimental data set. Rifampicin exposure for 72 hr at 0.5 and 1 μM (Figure 1B) resulted in a 2-fold induction of CYP3A4 activity, and a >2-fold induction was observed with 5, 10 and 25 μM rifampicin. Similar responses were seen following rifampicin exposure for 96 hr (Figure 1C). However, no significant differences were observed between the levels of CYP3A4 fold-induction following 72 or 96 hr exposure, at any rifampicin concentration studied.

Assessment of CYP3A4 induction using midazolam as part of a cocktail probe set yielded similar results to those observed using the single probe (Figures 2A-C). CYP3A4 activity increased as a function of increasing rifampicin concentration following 72 and 96 hr exposure (ANOVA; $p<0.05$ in each case). Formation rates were lower than those seen in the single probe study. However, these differences were not found to be statistically significant. Rifampicin exposure for 72 hr at 0.5 and 1 μM (Figure 2B) resulted in a 2-fold induction of CYP3A4 activity, and a >2-fold induction was observed with 5, 10 and 25 μM rifampicin, consistent with fold changes observed in the single probe experiment. . Rifampicin exposure for 96 hr

also resulted in a 2-fold induction of CYP3A4 activity at 0.5 and 1 μM (Figure 2B), and a >2-fold induction at 5, 10 and 25 μM rifampicin, again consistent with fold changes observed in the single probe experiment. Again, no significant differences were observed between the levels of CYP3A4 fold-induction following 72 or 96 hr exposure, at any rifampicin dose studied. Moreover, no significant differences were observed between single and cocktail studies.

RNA was isolated using the commercially available kits from Promega. Isolated RNA was free from contamination with DNA, as determined by the OD260:280 ratio. Only samples with ratios >1.8 were used in these studies. The robustness of the isolated mRNA was further confirmed using Agilent RNA 6000 Nano Assay kits.

Induction of CYP3A4 mRNA was determined using commercially available kits from Third Wave technologies. RNA was isolated from cells previously exposed to midazolam, either alone (single probe) or as part of the cocktail probe set. In the case of the single probe study, induction of CYP3A4 mRNA increased as a function of rifampicin concentration following exposure for either 72 or 96 hr (Figure 3A) (ANOVA $p<0.05$ in each case). Log transformation of the data showed that a 2-fold induction of CYP3A4 mRNA with rifampicin at 0.5 μM, and > 2-fold induction at concentrations from 1-25 μM, following exposure for 72 hr (Figure 3B). In contrast, 96 hr exposure resulted in >2-fold induction of CYP3A4 mRNA, at all rifampicin concentrations studied (Figure 3C). The fold-increases measured in the cocktail probe study (Figure 4A-C), followed the same pattern as those in the single probe study, with no significant differences between the responses in the two approaches, single versus cocktail studies.

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Cytochrome P450 1A2. Figure 5A highlights the changes in CYP1A2 enzyme activity (nmoles/min/10^6 cells), after exposure to the recognized inducer omeprazole, as measured by 1-OH tacrine formation (single probe). A significant induction in CYP1A2 activity was observed as a function of increasing omeprazole dose following 72 and 96 hr exposure (ANOVA; $p<0.05$ in each case). Figure 5B shows the log-transformed data following omeprazole exposure for 72 hr. A 2-fold induction of CYP1A2 2-fold induction was measured between 1-10 μM, and a >2-fold induction at 25 μM omeprazole. Following 96 hr omeprazole exposure (Figure 5C), a 2-fold induction was measured at 1 μM, however a >2-fold induction measured at 5-25 μM omeprazole. Responses at 96 hr were higher than those seen at 72 hr exposure, although this was only significant at 25 μM omeprazole ($P<0.05$).

Using the cocktail probe approach, a similar induction in CYP1A2 activity was observed, as a function of increasing omeprazole dose following 72 and 96 hr exposure (ANOVA; $p<0.05$ in each case) (Figure 6A). No induction was observed at 72 hr exposure with omeprazole at 0.5-5 μM. (Figure 6B). However, a 2-fold induction was observed at 10μM and a >2-fold induction at 25 μM omeprazole. Exposure of omeprazole for 96 hr resulted in a 2-fold induction at 5 μM and >2-fold at 10 and 25 μM (Figure 6C). Overall no significant differences were observed between responses observed at 72 and 96 hr omeprazole exposure. Comparisons between single and cocktail probe studies showed no differences between each of the approaches, with the exception of omperazole exposure for 96 hr at 25 μM, where the response were found to be higher when supplementing tacrine alone.

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Cytochrome P450 2C9. Figure 7A highlights the changes in CYP2C9 enzyme activity (nmoles/min/10^6 cells), after exposure to the recognized inducer rifampicin, as measured by 4-OH diclofenac formation (single probe). A significant induction in CYP2C9 activity was observed as a function of increasing rifampicin dose, following 72 and 96 hr exposure (ANOVA; $p<0.05$ in each case). Figure 7B and C show the log-transformed activity data following rifampicin exposure for 72 and 96 hr respectively. A 2-fold induction was measured at 0.5-25 μM at both exposure times.

Using the cocktail probe approach, a significant induction in CYP2C9 activity was also observed as a function of increasing rifampicin dose, following 72 and 96 hr exposure (ANOVA; $p<0.05$ in each case) (Figure 8A). Figures 8B and C show the log-transformed activity data following rifampicin exposure for 72 and 96 hr respectively. A 2-fold induction was observed with 5-25 μM rifampicin following 72hr incubation. In comparison 2-fold induction was only observed at 25 μM rifampicin following 96 hr incubation. However, no significant differences between the responses using single versus cocktail approaches were observed.

Cytochrome P450 2C19. In the current series of experiments no induction of CYP2C19 was observed using either the cocktail or single probe approach (data not shown). The average formation rate of 4-OH mephenytoin was <1nmol/min/million cells following rifampicin exposure between 0 and 25 μM.

Discussion

The current study compared the robustness of a cocktail probe approach to investigate the induction of the major drug metabolizing cytochrome P450 enzymes, and

CYP3A4 mRNA levels, with the routinely employed single probe approach. The specificity of these probes towards their targeted P450s has previously been reported (Hakala et al., 2005; Mohutsky et al., 2005; Turpeinen et al., 2005). Studies were performed in an immortalized human hepatocyte cell line: Fa2N4 (Mills et al., 2004), which has been reported to exhibit phenotypic properties, commonly expressed in hepatocytes, including inducibility of CYP1A2, CYP2C9, CYP3A4, UGT1A, and MDR1 mRNA in response to known inducers. These observations were in agreement with studies performed in cultured primary human hepatocytes (Madan et al., 2003), confirming their suitability as a substitute *in vitro* model to fresh human hepatocytes and other reported hepatic cell model systems (Gomez-Lechon et al., 2003; Trubetskoy et al., 2005; Kafert-Kasting et al., 2006). Moreover, these cells are readily available, and can be cultured as experimental needs demand, in contrast to fresh human hepatocytes, whose use is reliant upon the availability of a suitable liver tissue donor.

The induction of cytochrome P450 enzymes, following hepatic exposure to NCEs, results in an increase in the amount of protein. This is predominantly observed with CYPs 3A4 and 1A2, and to a lesser extent 2C9 and 2B6 (Dickins, 2004). The mechanism involved in the up-regulation of these proteins been shown to involve activation of nuclear hormone receptors; PXR AhR and/or CAR (Wang and LeCluyse, 2003; Bock and Kohle, 2004). The effect of activation of these nuclear receptors can influence disposition of many xenobiotics and endogenous substances. For example, a drug that activates CAR or PXR (Wei et al., 2002) may induce changes in the metabolism or transcellular transport of one or more co-administered drugs, reducing their therapeutic efficacy e.g. oral contraceptives containing

ethinylestradiol. Conversely, activation of PXR may lead to drug-induced toxicity, as seen with acetaminophen, (Guo et al., 2004). Clearly, early identification of any undesirable properties is an important feature in the drug discovery process.

Mills and co-workers (Mills et al., 2004) reported that Fa2N4 cells provide a suitable model to assess induction of cytochrome P450 enzyme activities and mRNA transcripts. In their studies two separate assays were performed to collect material to measure these markers. Owing to a dramatic increase in the number of NCEs in the field of drug discovery, there has been a drive by the pharmaceutical industry to improve efficiency, and screening techniques. Traditional assays generate large numbers of samples, requiring cumbersome, labour-intensive and cost-ineffective analytical procedures. To this end, the application of cocktail studies to measure biological markers, have become increasingly popular. With respect to drug metabolism and in particular *in vitro* DDIs, numerous cocktail procedures have been reported, allowing simultaneous determination of a NCE on the activity of the major drug metabolizing enzymes (Dierks et al., 2001; Zhou et al., 2004; Hakala et al., 2005; Kim et al., 2005; Mohutsky et al., 2005; Turpeinen et al., 2005). The majority of these studies report higher throughput *in vitro* cytochrome P450 techniques for evaluating the potency of putative inhibitors (Dierks et al., 2001; Zhou et al., 2004; Hakala et al., 2005; Kim et al., 2005; Mohutsky et al., 2005; Turpeinen et al., 2005). These assays have been shown to employ a variety of analytical techniques and differing numbers of substrates. A recent review by Zhou and co-workers (Zhou et al., 2004), described drug cocktail studies having employed between two and six-probe substrates simultaneously. More recently procedures measuring between seven

(Dierks et al., 2001) and nine probe molecules simultaneously (Kim et al., 2005), have been reported.

Within the context of the current study, few cocktail approaches have examined the cytochrome P450 inductive potential of NCEs *in vitro*. Mohutsky and co-workers, (Mohutsky et al., 2005) reported a cassette strategy to measure induction of CYPs 1A2, 2C9 and 3A4 enzyme activities in primary hepatocytes. They found that induction in the presence of prototypical inducers were similar to those measured using the single probe substrate approach. The current study, also investigated the induction of CYPs 1A2, 2C9 and 3A4. However, the cocktail probe set also included probes for CYPs 2C19 and 2D6, the latter of which served as a negative control as this CYP isoform is not inducible. It is important to note that induction is not limited to Phase I drug metabolising enzymes, but that other Phase II enzymes could also be induced, such as UGT enzymes (Soars et al., 2004). However, the use of well known prototypical inducers of cytochrome P450 enzymes supports the use of the probes used in these studies, namely; tacrine (CYP1A2), diclofenac (CYP2C9), (S)-mephenytoin (CYP2C19), dextromethorphan (CYP2D6) and midazolam (CYP3A4), despite some potential for Phase II metabolism of their metabolites.

Observations in the current study, confirm the robustness of the Fa2N4 cell line as a model to assess P450 induction. Cytochrome P450 3A4 exhibited the largest response following exposure to the prototypical inducer, rifampicin, followed by CYP1A2 (omeprazole) and CYP2C9 (rifampicin). Furthermore, the cocktail approach described, can be employed to determine the effects of a NCE on the activity of the major drug metabolizing CYPs 3A4, 1A2 and 2C9. The induction

responses to the prototypical inducers did not differ to those measured using single probes. This confirmed that the probe substrates when present as a cocktail did not interact adversely in anyway to affect any activities. The induction pattern (CYP3A4 >CYP1A2 >CYP2C9) was in agreement with previous studies (Soars et al., 2004), having shown that induction via the PXR pathway is more pronounced than that via CAR or AhR pathways (LeCluyse, 2001).

Whilst, both CYP3A4 and 2C9 are induced via the PXR pathway, differences in the sequences of their respective DNA promoter regions have been shown to explain the observed lower induction response of CYP2C9 to rifampicin, compared with CYP 3A4 (Goodwin et al., 1999; Gerbal-Chaloin et al., 2001). Differences in the architecture of the promoter regions of CYP2C19 have also been reported, explaining its reduced response to induction by rifampicin (Gerbal-Chaloin et al., 2001). However, the current study failed to identify induction of CYP2C19 with rifampicin at any concentration studied. Although, induction of CYP2C19 using *in vitro* models systems have been reported previously (Wang et al., 2004), induction is significantly lower than that of CYPs 3A4 and 1A2. The absence of CYP2C19 induction may simply be indicative of the current cell-model. Few if any clinical DDIs have been reported as a consequence of CYP2C19 induction. However its induction should not be dismissed, despite the ability to measure basal activities.

Whilst Fa2N4 cells exhibit inductive responses to prototypical inducers, the dynamic ranges over which these responses are observed are also important. Fold-changes in induction were identical in both the single and cocktail-probe approaches, further supporting the utility of this approach to measure induction of cytochrome P450

enzymes. It has been recommended that the minimum induction for CYP3A4 and 1A2 should be 2-fold (http://www.fda.gov/cder/drug/druginteractions/guidance.htm). Using the cocktail approach, the enzyme activities of CYP3A4, 1A2 and 2C9 were induced by at least 2-fold. In each case the level of induction was higher following incubation with inducers for 96 hr compared with 72 hr, albeit not significant, suggesting the shorter incubation time would suffice. Even shorter incubation times may provide a suitable fold-increase in the activities of these P450 enzymes. However, this was not investigated here. Interestingly, the current experimental model, showed a 2-fold-induction of CYP2C9, in contrast to recent studies, where no significant induction of CYP2C9 was observed (Madan et al., 2003; Mohutsky et al., 2005). This may reside in the differences in models used; immortalized compared with primary hepatocytes, and/or the experimental conditions, for example the length of time inducers were incubated with the cells, and the CYP specific probes used.

Although enzyme activity represents the gold-standard measure of *in vitro* induction, greater emphasis is being paid towards assessment in changes in cytochrome P450 mRNA expression. The current study reports that material collected from a single experimental well could be used to assess both enzyme activities and mRNA, helping minimize confounding inter-well influences, if having to run separate experiments. Owing to recent advances in technologies and techniques, such as RT-PCR, promoter gene assays, and gene chips, growing emphasis is being paid towards measurement of mRNA induction. Although cocktail approaches can help significantly reduce sample numbers to be analyzed, procedures to assess mRNA provide another tool whereby the pharmaceutical industry can significantly increase throughput. Changes in mRNA levels of cytochrome P450s can provide an early screen of the inductive potential of

compounds and related chemical series, with shorter analysis time than analytical techniques such as LC-MS. Moreover, there is a greater dynamic range across which changes in CYP induction can be studied. Moreover, measurement of changes in mRNA together with enzyme activities together can provide significantly more information than with either alone. In this regard, the inclusion of mRNA assessment would reduce false negatives, as might been seen with potent P450 inhibitors, including mechanism based inhibitors, which would significantly reduce P450 metabolism of probe molecules, but would not necessarily effect transcriptional up-regulation of the cytochrome P450 mRNA.

In summary the observations made in the current study, support the application of Fa2N4 cells as an *in vitro* model system to assess cytochrome P450 induction. Moreover the findings support use of a cocktail approach to assess the induction of CYP3A4, 1A2, 2C9. Response to prototypical inducers did not differ to those observed in the presence of the specific probes alone. As such this cassette approach reduces the sample numbers if screening the induction of more than one cytochrome P450. Furthermore, the current approach allows for simultaneous collection of samples to measure changes in enzyme activities and the mRNA expression. In conclusion, the current experimental set-up is an improvement on previous approaches employed to assess cytochrome P450 induction.

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Figure Legends

Figure 1. Effect of rifampicin (0-25μM) exposure for 72 and 96 hr on the induction of CYP3A4 in Fa2N4 cells, as measured by formation of 1-OH midazolam (nmoles/min/million cells) using single probe approach (A). Fold increases in 1-OH midazolam formation relative to vehicle control are also reported after 72 hr (B) and 96 hr (C) exposure. Solid line represents 2-fold difference from vehicle control, dashed lines represent 95 % confidence interval around this 2-fold change.

Figure 2. Effect of rifampicin (0-25μM) exposure for 72 and 96 hr on the induction of CYP3A4 in Fa2N4 cells, as measured by formation of 1-OH midazolam (nmoles/min/million cells) using cocktail probe approach (A). Fold increases in 1-OH midazolam formation relative to vehicle control are also reported after 72 hr (B) and 96 hr (C) exposure. Solid line represents 2-fold difference from vehicle control, dashed lines represent 95 % confidence interval around this 2-fold change.

Figure 3. Effect of rifampicin (0-25μM) exposure for 72 and 96 hr on the induction of CYP3A4 in Fa2N4 cells, as measured by increase in mRNA production using single probe approach (A). Fold increases in 1-OH midazolam formation relative to vehicle control are also reported after 72 hr (B) and 96 hr (C) exposure. Solid line represents 2-fold difference from vehicle control, dashed lines represent 95 % confidence interval around this 2-fold change.

Figure 4. Effect of rifampicin (0-25μM) exposure for 72 and 96 hr on the induction of CYP3A4 in Fa2N4 cells, as measured by increase in mRNA production using cocktail probe approach (A). Fold increases in 1-OH midazolam formation relative to

vehicle control are also reported after 72 hr (B) and 96 hr (C) exposure. Solid line represents 2-fold difference from vehicle control, dashed lines represent 95 % confidence interval around this 2-fold change.

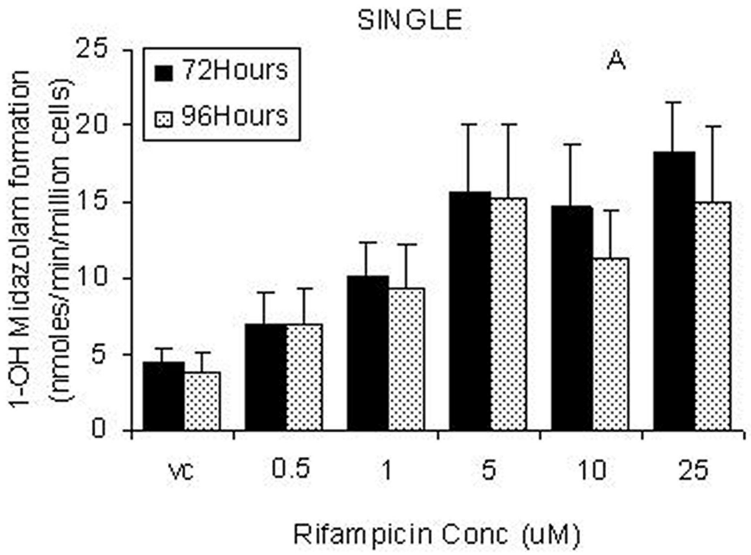
Figure 5. Effect of omeprazole (0-25μM) exposure for 72 and 96 hr on the induction of CYP1A2 in Fa2N4 cells, as measured by formation of 1-OH tacrine (nmoles/min/million cells) using single probe approach (A). Fold increases in 1-OH tacrine formation relative to vehicle control are also reported after 72 hr (B) and 96 hr (C) exposure. Solid line represents 2-fold difference from vehicle control, dashed lines represent 95 % confidence interval around this 2-fold change.

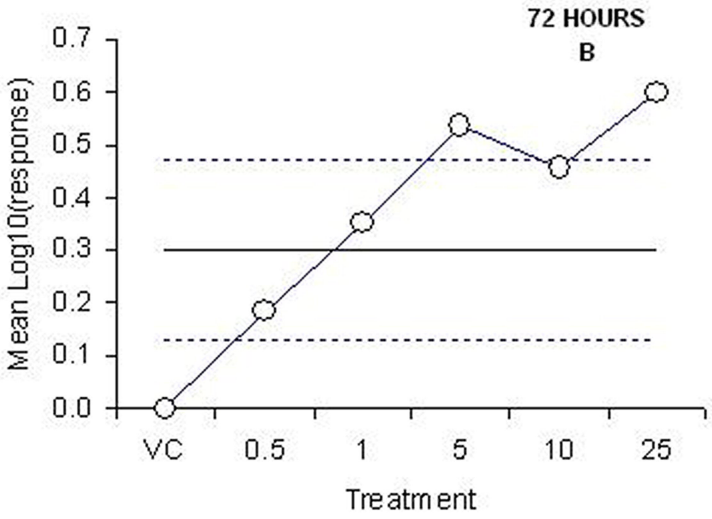
Figure 6. Effect of omeprazole (0-25μM) exposure for 72 and 96 hr on the induction of CYP1A2 in Fa2N4 cells, as measured by formation of 1-OH tacrine (nmoles/min/million cells) using cocktail probe approach (A). Fold increases in 1-OH tacrine formation relative to vehicle control are also reported after 72 hr (B) and 96 hr (C) exposure. Solid line represents 2-fold difference from vehicle control, dashed lines represent 95 % confidence interval around this 2-fold change.

Figure 7. Effect of rifampicin (0-25μM) exposure for 72 and 96 hr on the induction of CYP2C9 in Fa2N4 cells, as measured by formation of 4-OH diclofenac (nmoles/min/million cells) using single probe approach (A). Fold increases in 4-OH diclofenac formation relative to vehicle control are also reported after 72 hr (B) and 96 hr (C) exposure. Solid line represents 2-fold difference from vehicle control, dashed lines represent 95 % confidence interval around this 2-fold change.

Figure 8. Effect of rifampicin (0-25μM) exposure for 72 and 96 hr on the induction of CYP2C9 in Fa2N4 cells, as measured by formation of 4-OH diclofenac (nmoles/min/million cells) using cocktail probe approach (A). Fold increases in 4-OH diclofenac formation relative to vehicle control are also reported after 72 hr (B) and 96 hr (C) exposure. Solid line represents 2-fold difference from vehicle control, dashed lines represent 95 % confidence interval around this 2-fold change.

Figure 1A-C


SINGLE
A
72Hours
96Hours
1-OH Midazolam formation (nmoles/min/million cells)
0
5
10
15
20
25
vc
0.5
1
5
10
25
Rifampicin Conc (uM)


72 HOURS
B
Mean Log10(response)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
VC
0.5
1
5
10
25
Treatment

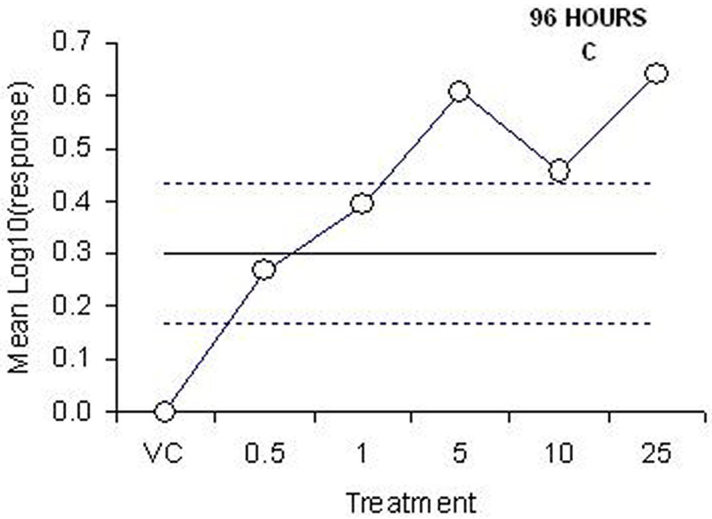
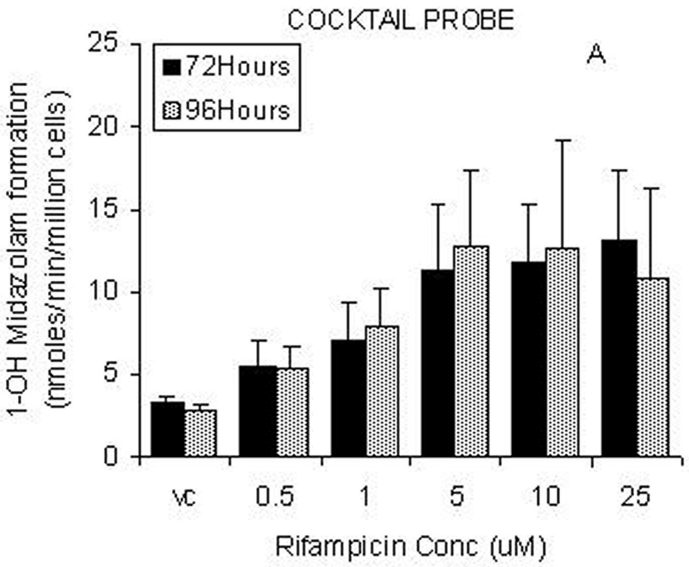
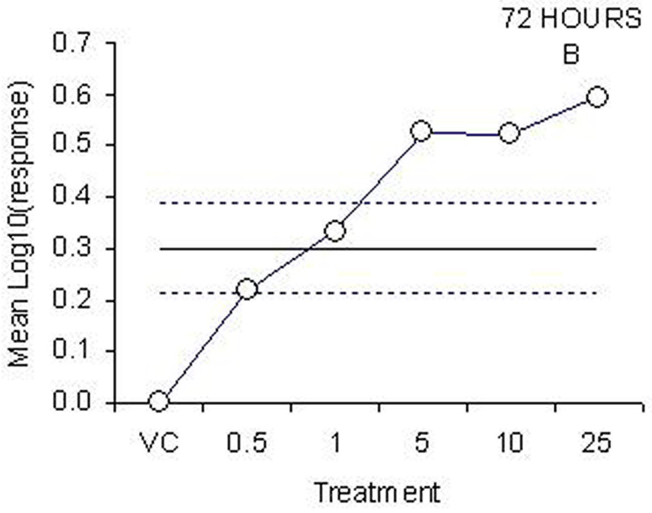

96 HOURS
C
Mean Log10(response)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
VC
0.5
1
5
10
25
Treatment

Figure 2A-C


COCKTAIL PROBE
A
72Hours
96Hours
1-OH Midazolam formation (nmoles/min/million cells)
25
20
15
10
5
0
vc
0.5
1
5
10
25
Rifampicin Conc (uM)


72 HOURS
B
Mean Log10(response)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
VC
0.5
1
5
10
25
Treatment

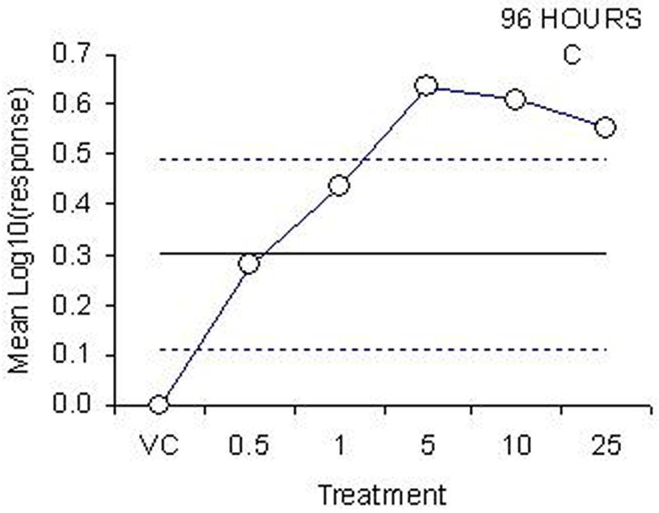
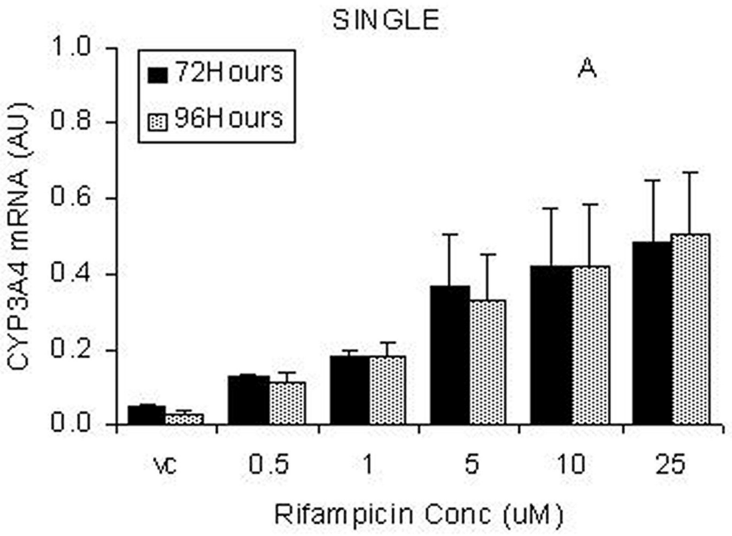
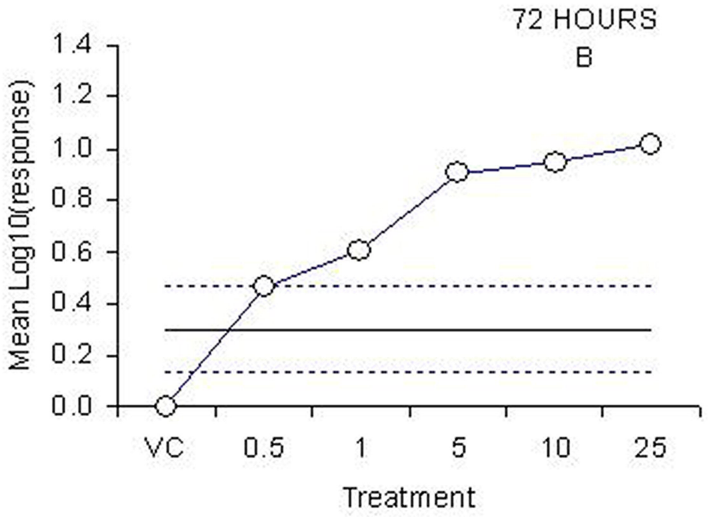

96 HOURS
C
Mean Log10(response)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
VC
0.5
1
5
10
25
Treatment

Figure 3A-C


SINGLE
A
72Hours
96Hours
CYP3A4 mRNA (AU)
1.0
0.8
0.6
0.4
0.2
0.0
VC
0.5
1
5
10
25
Rifampicin Conc (uM)


72 HOURS
B
Mean Log10(response)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
VC
0.5
1
5
10
25
Treatment

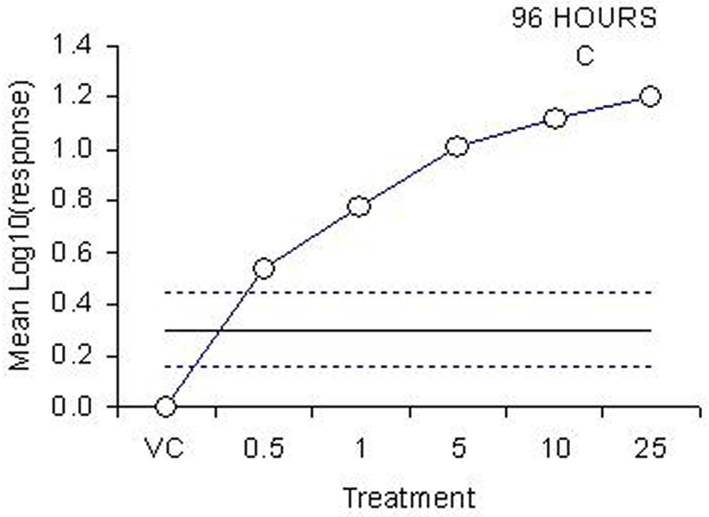
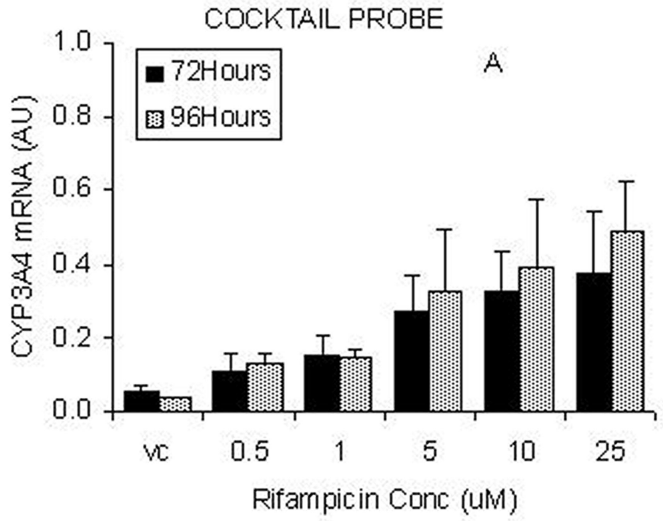
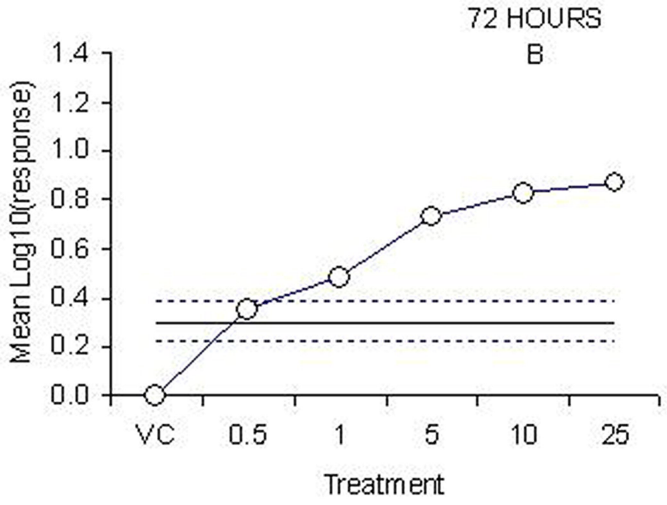

96 HOURS
C
Mean Log10(response)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
VC
0.5
1
5
10
25
Treatment

Figure 4A-C


COCKTAIL PROBE
A
72Hours
96Hours
CYP3A4 mRNA (AU)
1.0
0.8
0.6
0.4
0.2
0.0
vc
0.5
1
5
10
25
Rifampicin Conc (uM)


72 HOURS
B
Mean Log10(response)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
VC
0.5
1
5
10
25
Treatment

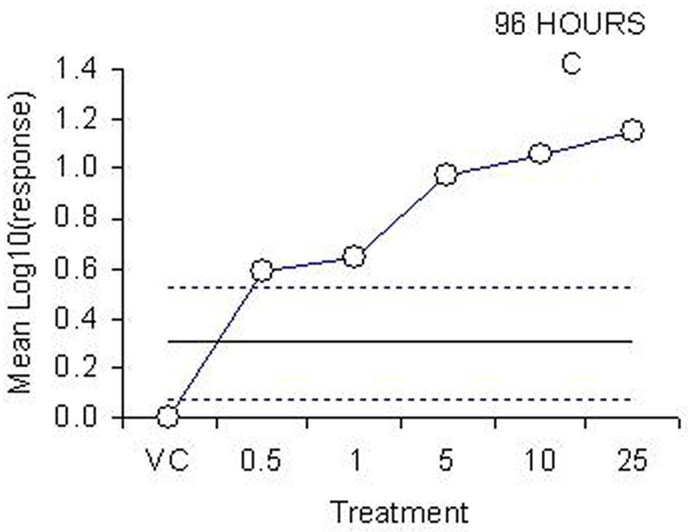
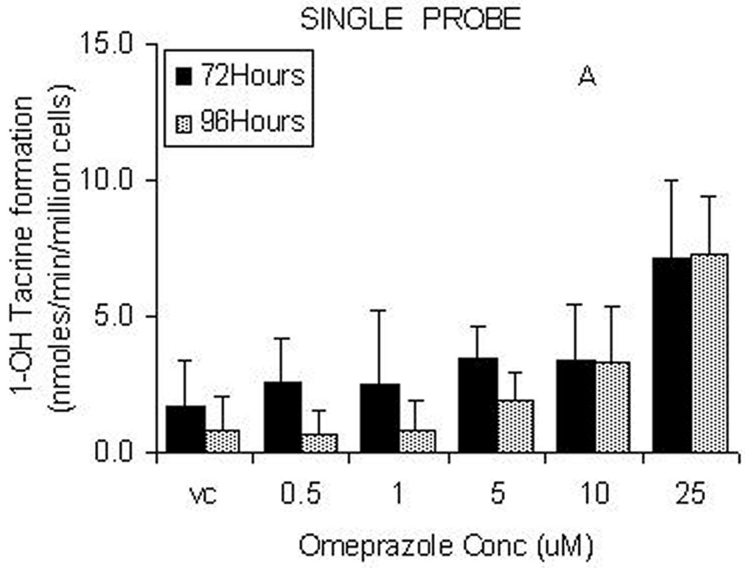
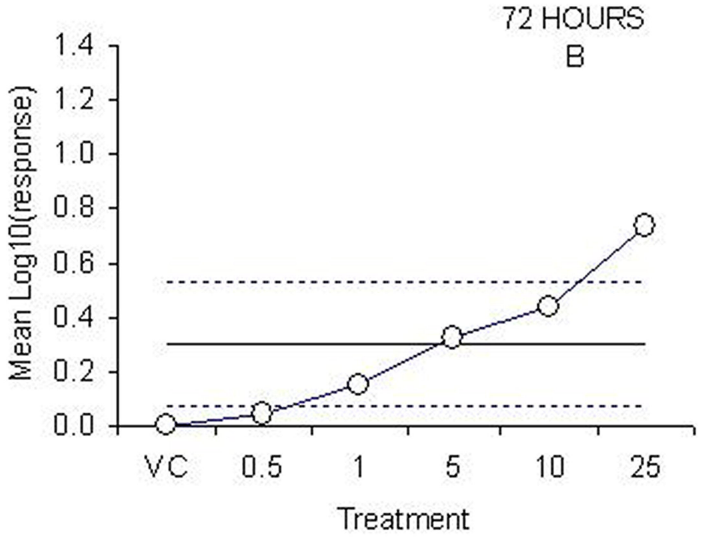

96 HOURS
C
Mean Log10(response)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
VC
0.5
1
5
10
25
Treatment

Figure 5A-C


SINGLE PROBE
A
72Hours
96Hours
1-OH Tacrine formation (nmoles/min/million cells)
15.0
10.0
5.0
0.0
vc
0.5
1
5
10
25
Omeprazole Conc (uM)


72 HOURS
B
Mean Log10(response)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
VC
0.5
1
5
10
25
Treatment

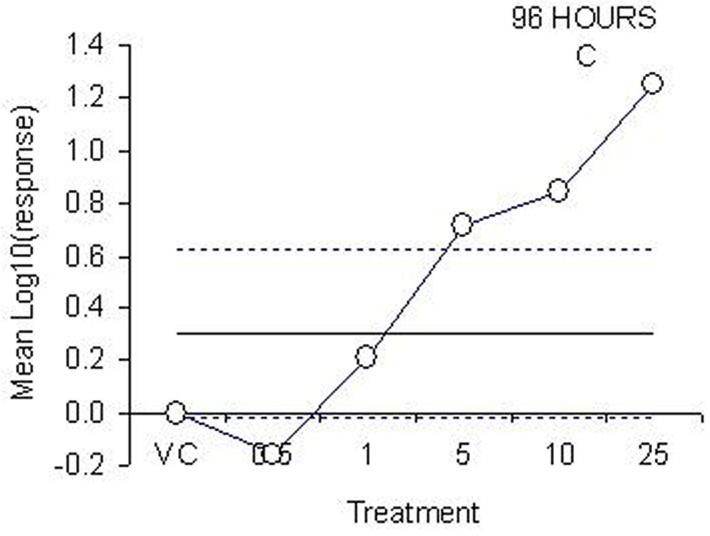
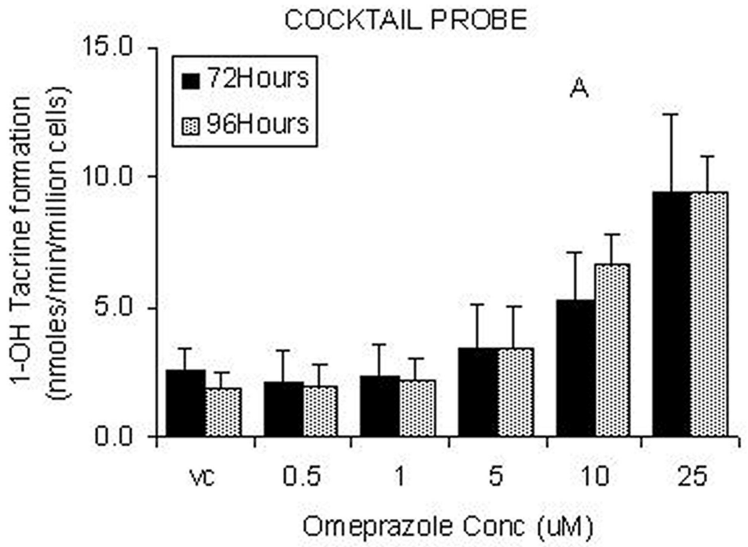
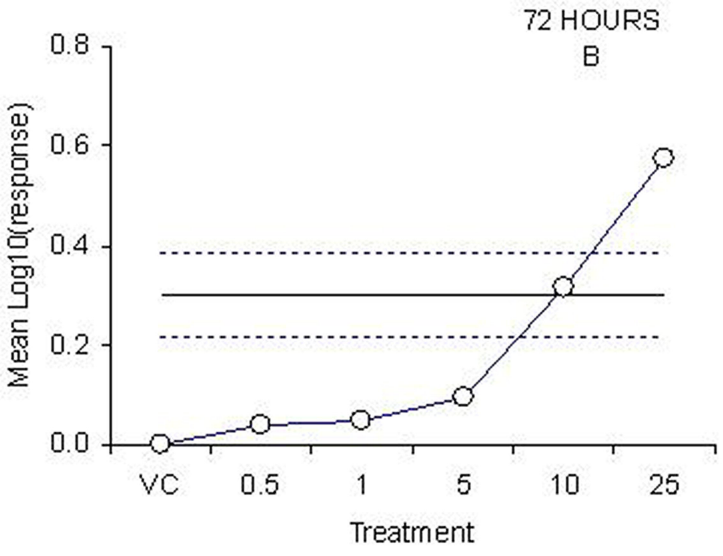

96 HOURS
C
Mean Log10(response)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
VC
0.5
1
5
10
25
Treatment

Figure 6A-C


COCKTAIL PROBE
A
72Hours
96Hours
1-OH Tacrine formation (nmoles/min/million cells)
15.0
10.0
5.0
0.0
VC
0.5
1
5
10
25
Omeprazole Conc (uM)


72 HOURS
B
Mean Log10(response)
0.8
0.6
0.4
0.2
0.0
VC
0.5
1
5
10
25
Treatment

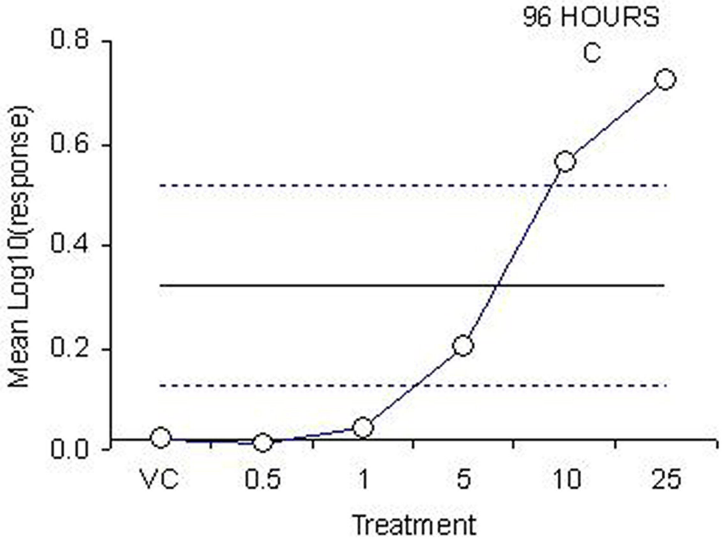
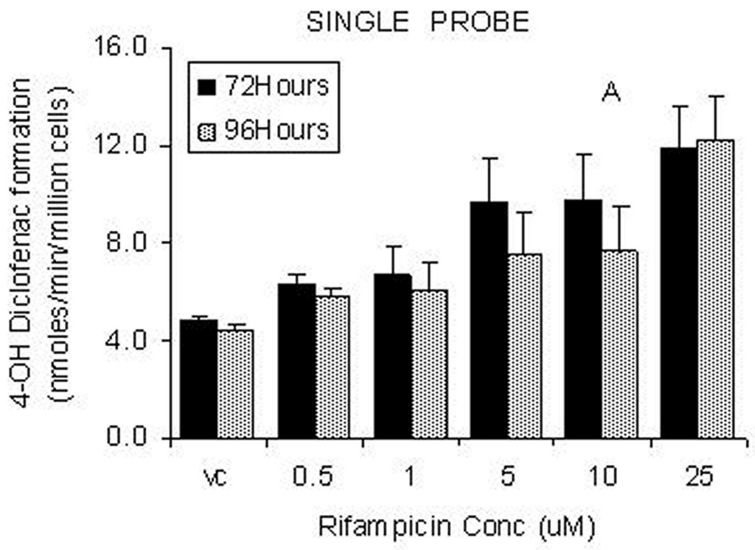
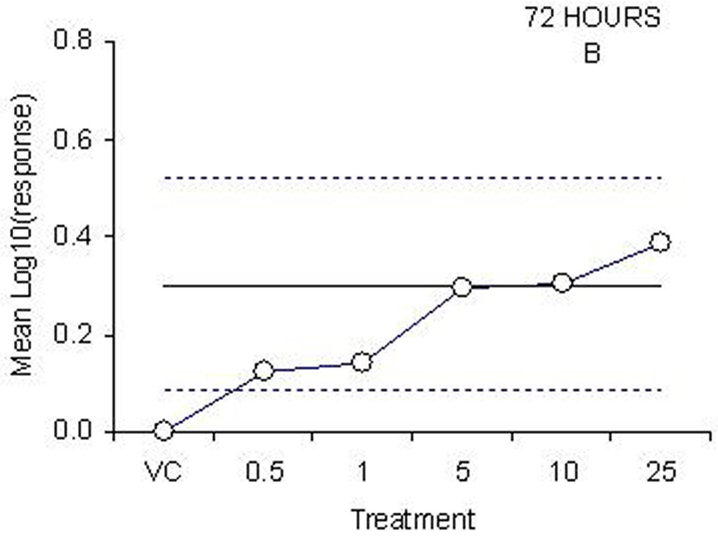

96 HOURS
C
Mean Log10(response)
0.8
0.6
0.4
0.2
0.0
VC
0.5
1
5
10
25
Treatment

Figure 7A-C


SINGLE PROBE
A
72Hours
96Hours
4-OH Diclofenac formation (nmoles/min/million cells)
16.0
12.0
8.0
4.0
0.0
VC
0.5
1
5
10
25
Rifampicin Conc (uM)


72 HOURS
B
Mean Log10(response)
0.8
0.6
0.4
0.2
0.0
VC
0.5
1
5
10
25
Treatment

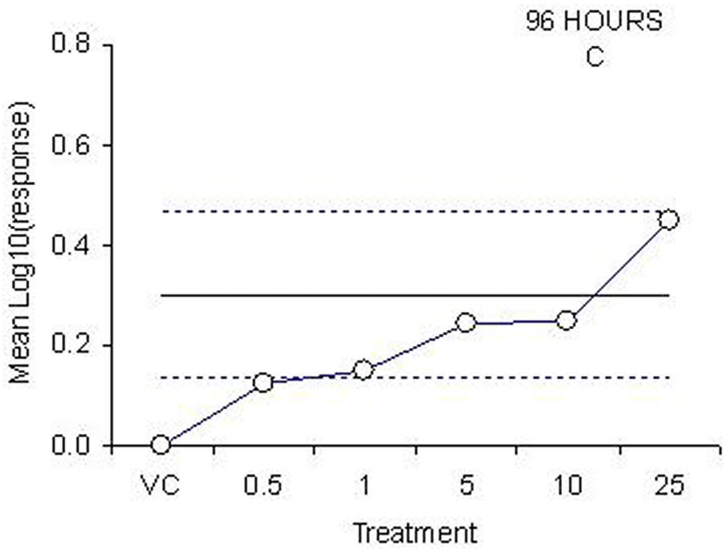
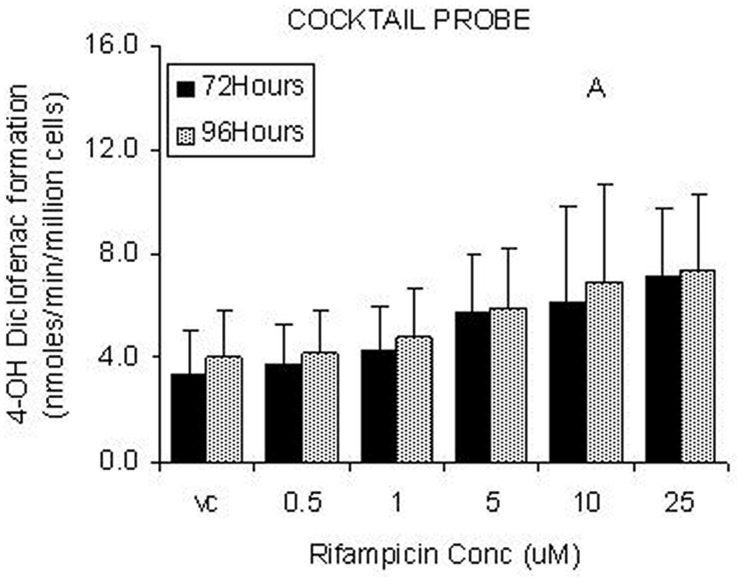
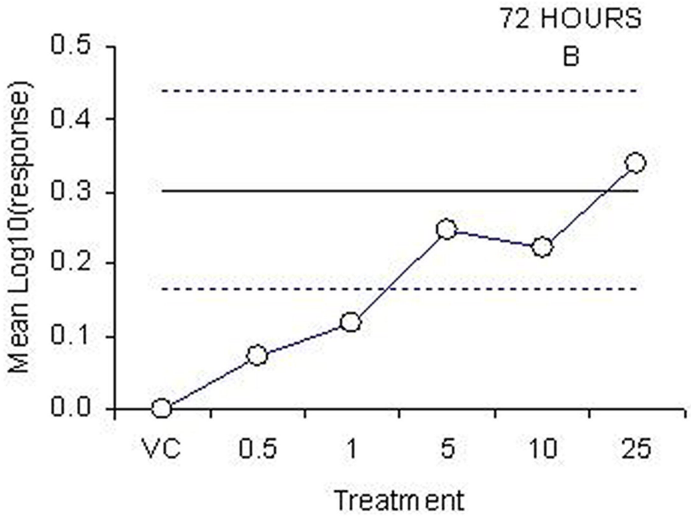
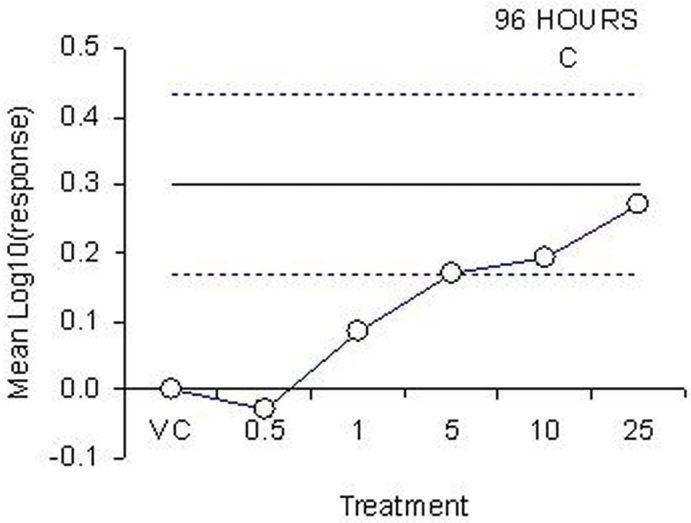

96 HOURS
C
Mean Log10(response)
0.8
0.6
0.4
0.2
0.0
VC
0.5
1
5
10
25
Treatment

Figure 8A-C


COCKTAIL PROBE
A
72Hours
96Hours
4-OH Diclofenac formation (nmoles/min/million cells)
16.0
12.0
8.0
4.0
0.0
vc
0.5
1
5
10
25
Rifampicin Conc (uM)


72 HOURS
B
Mean Log10(response)
0.5
0.4
0.3
0.2
0.1
0.0
VC
0.5
1
5
10
25
Treatment


96 HOURS
C
Mean Log10(response)
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
VC
0.5
1
5
10
25
Treatment