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**CYTOCHROME P450 GENE INDUCTION IN RATS *EX VIVO* ASSESSED BY
QUANTITATIVE REAL-TIME REVERSE-TRANSCRIPTION POLYMERASE
CHAIN REACTION (TAQMAN[®])**

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Running Title Page:

- a) **Running title:** Rat Taqman[®] Cytochrome P450 Gene Induction
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Abbreviations used are: CAR, constitutively activated / androstane receptor; C_{max}, maximum plasma concentration; EROD, 7-ethoxyresorufin O-dealkylation; 6-FAM, 6-carboxyfluorescein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Mrp2, multidrug resistance-associated protein 2; PXR, pregnane X receptor; qRT-PCR, quantitative real-time reverse transcriptase-polymerase chain reaction; TAMRA, 6-carboxy-tetramethyl-rhodamine; TDI, time-dependent inhibitor; test 6 β -oh, testosterone 6 β -hydroxylation; UGT, UDP-glucuronosyltransferase.

Abstract:

Drug-induced changes in expression of cytochrome P450 (CYP) genes are a significant issue in the pre-clinical development of pharmaceuticals. For example, pre-clinically CYP induction can impact safety studies by reducing the systemic exposure of a compound undergoing toxicological evaluation, thus limiting the exposure that can be safely investigated in patients. Consequently, the induction potential of candidate drugs has been studied as part of the drug development process, typically utilising protein and/or catalytic end points. However, measuring changes in the levels of mRNA using TaqMan[®] technology offers the opportunity to investigate this issue with the advantages of better dynamic range and specific enzyme identification. Here, we describe the TaqMan[®] application to study *ex vivo* the P450 gene induction in the rat. Initially, livers from rats dosed with the prototypic CYP inducers β -naphthoflavone (BNF), phenobarbital (PB), dexamethasone (DEX) and clofibric acid (CLO) were analysed for mRNA levels of CYP1A1, 1A2, 2B1, 2B2, 2E1, 3A2, 3A23 and 4A1 and compared to control animals. The maximum fold induction of mRNA varied; 2500-fold for CYP1A1 with BNF, 680-fold for CYP2B1 with PB, 59-fold for CYP3A23 with DEX and 16-fold for CYP4A1 with CLO. This method was then applied to estimate the inductive potential of putative drug candidates undergoing rodent toxicological evaluation. We present a summary of these data which demonstrates the sensitivity and specificity of the TaqMan[®] assay to distinguish between inducers and non-inducers and which offers a highly specific alternative to the quantification of drug effects on CYP expression using immunodetection and substrate metabolism.

Introduction

The cytochromes P450 (CYPs) are a ubiquitous superfamily of haem-containing mono-oxygenase enzymes, which play a fundamental role in the metabolism of a variety of chemically diverse compounds, including endogenous chemicals and pharmaceutical agents (Nelson et al., 1996; Nelson et al., 1999; P450 nomenclature on <http://drnelson.utmem.edu/CytochromeP450.html> and Parkinson A, 2001). Consequently, these enzymes are involved in many pharmacokinetic drug-drug interactions (DDIs), where multiple drug therapy results in the interference of one drug with the metabolism of another (Guengerich FP, 1997). Drug interactions mediated by CYP induction is a well-established mechanism of pharmacokinetic DDIs and can result either in a marked increase in production of a toxic metabolite (Lin and Lu, 1998) or can reduce the efficacy of other co-administered medications due to an increase in clearance (Park et al., 1996). A more comprehensive listing of drugs that are inducers of CYP enzymes can be found in Dave Flockhart CYP drug interaction tables (<http://medicine.iupui.edu/flockhart/>) and reported by Michalets (1998).

Estimating the potential of a candidate drug to be an inducer of CYP expression is an important consideration for the early stages of discovery and development of new chemical entities (NCEs). In particular, toxicokinetic findings associated with a candidate drug inducing its own metabolism (auto-induction) may include sub-proportional increases in exposure (C_{max} , AUC) with increasing doses and reduced exposure after repeat administration (Worboys et al., 2001). However, if the enzyme(s) induced are not responsible for the clearance of the candidate drug, these parameters will not be affected, but may result in altered kinetics of other drugs. In both cases, these treatment-related effects may be associated with liver weight increases and histological findings of hepatomegaly through

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induction of cellular hypertrophy and hyperplasia of the liver (Staudinger et al., 2001). It has also been reported that, CYP induction in the rat may also be associated with thyroid toxicity (Hood A et al., 1999).

Currently, quantification of CYP induction in preclinical species can be tested as part of toxicological/safety evaluation, prior to the candidate drug being selected for clinical development. This is achieved primarily by measuring changes in their enzyme activity by catalytic end points or by quantification of gross changes in CYP protein levels using specific antibodies suitable for western blot analysis (Parkinson A, 1996). However, since the major mechanism of CYP induction is via increased rates of transcription, this testing can be addressed by studying changes in specific CYP gene expression by measuring messenger RNA (mRNA) levels using quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) (Gibson et al., 1996; Godfrey et al., 2005).

Here we report the sensitivity, specificity, wide dynamic range, reproducibility, accuracy and application of an automated qRT-PCR protocol (TaqMan[®]) for the measurement of mRNA levels of CYP genes in total RNA extracted from rat liver. To evaluate this method, we assessed the inducibility of CYP1A, CYP2B, CYP3A and CYP4A following administration of the prototypical CYP inducers β -naphthoflavone, phenobarbital, dexamethasone and clofibric acid, respectively, to rodents. CYP2E1 mRNA levels were also analysed, although it is recognised that this enzyme is regulated by mRNA stabilisation, increased mRNA translatability and decreased protein degradation. Once evaluated, we assessed the potential of a significant number of putative drug candidates (n=71) to cause induction of the major rat CYPs (CYP1A, 2B, 2E, 3A and 4A) and this data along with the positive controls are reported here.

Materials and Methods

Materials

β -naphthoflavone, sodium phenobarbitone, dexamethasone and clofibrac acid were obtained from Sigma-Aldrich Co. Ltd (Poole, UK). The RNeasy[®] 96 total RNA Isolation Kit, RLT lysis buffer, Hotstar Taq Mastermix and MinElute PCR purification kit were obtained from Qiagen Ltd (Crawley, UK). DNase I (RNase free-2U/ μ L) and DEPC-treated water were obtained from Ambion (Cambs, UK). Ribogreen[®] RNA Quantitation Kit was purchased from Molecular Probes Inc. (Oregon, USA). Oligo (dT)₁₂₋₁₈ primer (0.5 μ g/ μ L) kit, dNTP mix (10 mM 2' deoxynucleoside 5'-triphosphate), the Superscript[™] II Reverse Transcriptase (RT) (200 units/ μ L) kit (containing DTT: 0.1 M dithiothreitol and 5 x first strand buffer) and 4% E-Gel[®] were purchased from Invitrogen Ltd. (Paisley, UK). Oligonucleotide fluorogenic probes, TaqMan[®] Universal PCR Mastermix and 96 well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Warrington, UK). Oligonucleotide primers were purchased from Sigma-Genosys Ltd (Cambs, UK) with rat genomic DNA (0.2 μ g/ μ L) being obtained from Bioline (London, UK). RNA polymerase promoter primers, T7 and SP6, were purchased from Promega (Southampton, UK). All other reagents used in this study were of molecular biology or analytical grade and purchased from commercial sources.

Animals

Male Sprague Dawley rats (200–250g) were obtained from Charles River (UK) and this strain of rat was chosen because of the considerable knowledge within GlaxoSmithKline of the strain's general pathology and response to a wide variety of drugs. Rats were housed at 19 –

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23 °C with a 12 hour light-dark cycle and had free access to food and water. Animals were allowed to acclimatise to their environment for at least 5 days prior to drug administration.

Administration of CYP Inducers

To evaluate the TaqMan[®] protocol outlined in this report, rats were administered prototypical CYP inducers by daily intraperitoneal injections of: β -naphthoflavone (BNF; CYP1A inducer at 80 mg/kg in corn oil); phenobarbital (PB; CYP2B inducer at 80 mg/kg in saline); dexamethasone (DEX; CYP3A inducer at 150 mg/kg in corn oil) or clofibrac acid (CLO; CYP4A inducer at 200 mg/kg in corn oil) for three consecutive days. Three rats were used in each group with control animals being administered with vehicle only (a separate group for corn oil and saline controls). Animals were killed 24 hours after their last dose and the livers immediately excised. Liver samples were placed in foil bags and snap-frozen in liquid nitrogen and stored at *ca* -80°C prior to extraction of total RNA.

Drug candidate studies

Once evaluated, the TaqMan[®] protocol was used to investigate the induction potential of a number of putative drug candidates (n=71). Livers were typically collected from 4-7 day rat toxicology studies, as described above, following administration of a vehicle control and three drug concentrations (n= 3 male and/or female Sprague Dawley rats/ dose group).

Extraction of Total RNA from Liver

Liver homogenates (7% (w/v)) were prepared in RLT lysis buffer using a polytron homogeniser (20 x 1000 rpm, < 30 sec bursts at room temperature) from a representative portion of liver (approximately 1g tissue). Although a fraction of this quantity was required

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for the TaqMan[®] protocol, it ensured a homogeneous sample of liver was analysed. The homogenates were further diluted with RLT lysis buffer to give a final homogenate preparation of 0.28% (w/v). An equal volume of ethanol was then added to the 0.28% (w/v) homogenate using a Zymark rapidplate robot. Total RNA was extracted from quadruple samples of the liver homogenate (600 µl of homogenate/ethanol mix per extraction column) using a RNeasy[®] 96 kit on a Qiagen 3000 robot with vacuum, according to the manufacture's protocol. The RNA was eluted from each column with two aliquots of 140 µL RNase-free water, each aliquot being spun at *ca* 5600 g, for 4 min at room temperature.

DNase Treatment

Total RNA aliquots (approximately 1 µg) were treated with a DNase bulk mix, which consisted of RNase-free DNase I (0.5 Units), 10x DNase I buffer (10 µL) and DEPC-treated water (0.75 µL). The DNase-treated plate was incubated in a thermocycler at 37°C for 10 min, followed by 75°C for 5 min. This eliminated any contaminating genomic DNA and allowed gene(s) to be quantified using a rat genomic DNA standard curve (described later). Any remaining total RNA left untreated was stored at *ca.* -80°C.

Quantitation of Total RNA

The quantity of total RNA (DNase-treated) was determined by measurement of a fluorescent RNA-binding probe, RiboGreen[®] according to the manufacture's RNA quantitation kit instructions (Molecular Probes, Eugene, USA) (Jones et al., 1998). The RiboGreen[®] assay was prepared on a Qiagen 3000 robot, using a ribosomal RNA standard curve (final concentration ranging from 15.62 to 1000 ng/mL). 100 µL of the fluorescent RNA-binding

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probe (diluted 1 in 200) was added to both the RNA isolates and RNA standards (5 μ L RNA or RNA standard in 95 μ L TE buffer). The fluorescence in each well was measured using a Polarstar plate reader (BMG Labtechnologies Ltd., Germany) set at excitation 480 nm and emission 520 nm. The concentration of the total RNA isolates was interpolated from the standard curve.

No-amplification controls

A no-amplification control (NAC) plate was set up to evaluate the efficiency of the DNase process and to test for the degree of any contaminating genomic DNA in the RNA sample. Aliquots of DNase-treated total RNA (5 μ L) were not subjected to cDNA synthesis (were not reverse transcribed) but added directly to a TaqMan[®] reaction mix and quantified by real-time qRT-PCR (TaqMan[®]) as described later.

cDNA Synthesis

First-strand cDNA synthesis was performed on the remaining DNase-treated total RNA which was reverse-transcribed using Oligo (dT)₁₂₋₁₈ primer (0.5 μ g), heated to 70°C for 10 min with immediate cooling on ice and Superscript[™] II bulk mix (200 Units of Superscript[™] II RNase H⁻ reverse transcriptase, dNTP mix, DTT and 5x first strand buffer), heated to 42°C for 50 min followed by 70°C for 15 min, according to the manufacture's instructions. In addition, duplicate no template control samples were run in identical conditions as those described above, except the RNA sample was substituted for DEPC-treated water. All resulting cDNA was subjected to PCR amplification using TaqMan[®] technology.

Principles of TaqMan[®] Technology (theory)

Relative differences in mRNA expression (i.e. target gene expression) were assessed based on different PCR cycling threshold (Ct) values, which were determined by the PE Biosystems ABI 7900 sequencer software (SDS Version 2.0). A difference of one Ct value is equivalent to two fold difference in gene expression (an exponential relationship). Quantitation of the initial number of copies of mRNA of the target gene detected was calculated from the experimental Ct value by interpolation from the standard curve generated using known amounts of rat genomic DNA (gDNA).

Primers and Fluorogenic Probe Design

The primers and probes were designed to a definitive gene sequence in a single exon to allow the amplification of a gDNA standard curve (contrary to Gibson et al., 1996) and as close as possible to the 3'-coding region of the target gene sequence obtained from GenBank (<http://www.ncbi.nlm.nih.gov/GenBank>). Regions that satisfied this criteria were then put into Primer Express and primer and probe sequences were designed according to the parameters incorporated in the Primer Express software (Versions 1 and 2, PE Applied Biosystems). Optimal primers and probes were 20-80% GC rich, between 9-40 bases in length, primer T_m values were 58-60°C (<2°C difference between forward and reverse primer), with probes ideally having a T_m 10°C higher than the primer T_m. The probe selected was close to the 3' end of the forward primer, had more bases of Cs than Gs and with <4 contiguous Gs in the strand (as recommended by the manufacturer & described by Livak et al., 1995). Probes with a G at the 5' end were avoided as this has been shown by the manufacturer to exert a quenching effect on the reporter fluorochrome. Finally, the amplicons (typically 70 –100 base pairs long) were homology-searched to ensure that they were specific for the target mRNA

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transcript using an NCBI BLAST search. The final TaqMan[®] probe/primer sequences designed to be specific for rat CYP1A1, 1A2, 2B1, 2B2, 2E1, 3A2, 3A23, 4A1 and the house keeping gene, glyceraldehyde-3-phosphate (GAPDH), are summarised with their sequences and corresponding accession number of the target genes in Table 1. The probe/primer set designed for GAPDH, while amplifying GAPDH in the rat, was also capable of amplifying mouse and human GAPDH due to its conserved nature across species.

Specificity of Primer Set

Determination of the specificity of the primer set was initiated by producing a PCR product with genomic DNA. The PCR 50 μ L reaction mix contained: 10 μ L of genomic DNA (final quantity 100 ng, stock 10ng/ μ L), 2 μ L of 100 μ M forward primer and 2 μ L of 100 μ M reverse primer (final concentration 4 μ M of each primer), 25 μ L Hotstar Taq Mastermix (Qiagen) and 11 μ L DEPC-treated water. After sealing the plate it was spun at *ca* 5600 g, for 1 min at room temperature. The PCR product was produced by thermocycling conditions being initiated with an enzyme activation step of 95°C for 10 min, followed by 35 PCR cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min.

Purification of the PCR product (10 μ L) was performed using a MiniElute PCR purification kit (Qiagen) according to the manufacturer's protocol. The DNA was eluted with 10 μ L PCR-grade water by centrifugation at *ca* 5600 g for 1 min at room temperature.

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Once purified the PCR product was loaded in to wells of a 4% E-Gel[®] (5 μ L PCR product in 15 μ L PCR-grade water per well) and run at 75 volts for approximately 30 min in parallel with a 10 base pair (bp) DNA ladder (2 μ L of 10 bp of DNA in 18 μ L of PCR-grade water). The molecular weight of the amplified product (seen as a single band with a transilluminator) was identified using the DNA ladder.

The quantity of total DNA was determined by measurement of a fluorescent DNA-binding probe, PicoGreen[®] according to the manufacture's quantitation kit instructions (Molecular Probes, Eugene, USA). The DNA sample was diluted 1 in 10 (1 μ L of PCR product in 9 μ L DEPC-treated water) prior to commencing DNA quantitation (refer to RiboGreen[®] assay for details, replacing the RiboGreen[®] probe and RNA samples/standards with PicoGreen[®] probe and DNA equivalents, respectively).

In order to prime the DNA synthesised for DNA sequencing, the small segment of DNA product was amplified (by approximately 39 bp) by repeating the PCR reaction (minus the genomic DNA) with the addition of RNA polymerase promoter primers, T7 and SP6. The final DNA product was sequenced (GSK sequencing group, Harlow) to give a definitive amplicon gene sequence.

Transcription detection and quantification by Real-Time RT-PCR (TaqMan[®])

Quantitative analysis of specific mRNA expression was performed by real-time qRT-PCR, by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7900 Sequence Detection System (TaqMan[®]) (Perkin Elmer-Applied Biosystems, Foster City, CA, USA). The TaqMan[®] 25 μ L reaction mix contained: 0.5 μ L of 5 μ M probe (final concentration 100 nM), 1 μ L of 10 μ M forward primer and 1 μ L of 10 μ M reverse primer (final concentration 400 nM of each primer), 12.5 μ L TaqMan[®] Universal

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Mastermix, 5 μ L DEPC-treated water and 5 μ L cDNA sample (typically 50 ng total RNA). Assay controls were incorporated onto the same TaqMan[®] plate, namely no-template controls (NTCs) to test for the contamination of any assay reagents. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 50°C for 2 min with an enzyme activation step of 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 seconds, and anneal/extension at 60°C for 1 minute.

In order to accurately reflect the quantity of each target PCR product (and gDNA standard), principal parameters were manually adjusted on the TaqMan[®] instrument to obtain the optimal measurement. The background fluorescence was set between Ct values 3 and 15, with threshold levels on the amplification plots typically adjusted to 0.1. It is imperative that the threshold is set correctly, in order to capture the Ct value in the exponential phase of the PCR reaction.

Standard curve

A rat genomic DNA (gDNA) standard curve (ranging from 2.5×10^5 to 2.5 single stranded copies per 5 μ L) was assayed in duplicate on the same plate as the cDNA samples, under identical conditions as those described above. The standard curve calculation was based on the assumption that the PCR reaction was exhibiting 100% amplification efficiency (i.e. the PCR products will double with each cycle during exponential amplification) (Leutenegger et al., 2001). The slope of the gDNA standard curve plot was used to estimate PCR efficiency (E) according to the equation: $E = 10^{-1/\text{slope}}$ (calculation for real-time PCR efficiency is detailed on <http://www.wzw.tum.de/gene-quantification/efficiency.html> and described by Rasmussen, 2001). An ideal PCR reaction would exhibit a standard curve slope of -3.32, with a PCR efficiency of $E = 2$.

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Quantitation of gene expression

The specific gene expression was quantitatively detected for the following genes: CYP1A1, 1A2, 2B1, 2B2, 2E1, 3A2 (males only, as it is primarily a male specific gene), 3A23 and 4A1 and the housekeeping gene GAPDH. Data has been reported as fold induction of control.

Results

RNA quantity

Total RNA was extracted from liver samples taken from control and treated rats. The average quantity of total RNA eluted, after loading the equivalent of 0.84 mg of liver onto each extraction column, was approximately 4 μ g of total RNA, at a concentration of approximately 14 μ g/mL. The % CV for quadruple RNA extractions of the same liver was approximately 8 %. Subsequent qRT-PCR reactions had approximately 50 ng total RNA added per reaction, thus the quantity of total RNA isolated is sufficient to investigate the expression levels of up to 80 distinct genes.

Probe/primer efficiency

The PCR efficiencies for the TaqMan[®] probe/primer sets used in this study ranged from 1.82 to 1.99 (close to the ideal value of 2) and therefore exhibited an acceptable real-time PCR efficiency of > 91 % (data not shown).

Assay controls

Appropriate controls, such as the NAC plate, demonstrated that there was negligible contamination of the isolated total RNA by genomic DNA, with Ct values being ≥ 39 (little to no signal detected). Likewise, NTC samples demonstrated that there was no contamination of any assay reagents, given that no signal was detected (Ct value of 40).

Reproducibility

The choice of an appropriate number of replicates for TaqMan[®] studies is an important issue. In our experience, quadruple sampling provided the ideal number of replicates for any given

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sample. Less than this and you potentially risk an unacceptable high error of measurement, whereas excessive replication may increase the cost and reduce the number of samples that can be analysed on a single plate.

Basal gene expression

The basal gene expression levels for CYP1A1, 1A2, 2B1, 2B2, 2E1, 3A2, 3A23, 4A1 and GAPDH, were determined in livers from vehicle control male Sprague Dawley rats involved in 4-7 day toxicology studies (n = 78 livers). The mean number of copies detected for each CYP gene (expressed as mRNA detected/ng of total RNA) is shown in Table 2. A broad range of expression levels were observed between the different CYP genes and a typical basal expression profile was $CYP2E1 \geq CYP3A2 > CYP1A2 > CYP4A1 > CYP3A23 > CYP2B2 \geq GAPDH > CYP2B1 > CYP1A1$, with over four orders of magnitude difference between the lowest and highest abundant CYP gene. Specifically, CYP1A1 and CYP2B1 had considerably low levels of expression (< 4 copies of mRNA detected/ng total RNA) compared to CYP2E1 and CYP3A2, the most highly expressed genes detected (*ca* 6,000 copies of mRNA detected/ng total RNA).

House keeping gene

The mRNA expression of the house keeping gene, GAPDH, was used as a quality control for the sample and was not used to normalise against. It was relatively uniform throughout all the samples analysed, confirming the integrity of the RNA used in the assays. Fold change was *ca* < 30 % from controls for all the prototypical CYP inducer samples.

Prototypical CYP inducers

The ability of prototypical inducers to induce CYP gene expression was examined in *ex vivo* rat liver and was the basis for validating the TaqMan[®] technique. Data for the fold induction of control following treatment with the classical inducers, BNF, PB, DEX and CLO are shown in Table 3.

BNF (80 mg/kg/day) specifically induced CYP1A1 and CYP1A2 mRNA by 2500- and 23-fold, respectively, with all other CYP genes determined being relatively unaffected following 3 days of treatment. The difference in magnitude of fold induction between CYP1A1 and CYP1A2 is largely due to their relative constitutive expressions, with CYP1A1 being extremely low compared to a high expression of CYP1A2 but also because of CYP1A1 being the major inducible CYP1A enzyme.

In PB (80 mg/kg/day) treated animals, CYP2B1 was induced to a greater extent than CYP2B2 gene expression with increases of approximately 680- and 22-fold, respectively, relative to vehicle control animals. Also, increases in the amount of mRNA for CYP3A2 (3-fold) and CYP3A23 (5-fold) were detected in rat liver in response to PB treatment. This is fairly predictable given that the molecular mechanism of phenobarbital-type induction has been reported to partly overlap with that mediated by the pregnane X receptor, PXR, which regulates CYP3A induction (Moore et al., 2000; Wei et al., 2002).

As expected from the literature (Huss and Kasper, 1998), the two rat CYP3A genes were differentially responsive to induction by the glucocorticoid CYP3A inducer, DEX (150 mg/kg/day). Specifically, there was a marked increase in the CYP3A23 mRNA (the major glucocorticoid-responsive rat CYP3A gene), which corresponded to a 59-fold induction of control. Whereas, CYP3A2 classified as the “male-specific” gene and represents the predominant CYP3A form in control liver, was less responsive to DEX (7-fold induction of

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control). CYP2B mRNA expression was also induced by DEX, with CYP2B2 being induced to a greater degree by DEX than PB (mean fold induction of control was 49 and 50 for CYP2B1 and CYP2B2, respectively). This up-regulation of CYP2B is to be expected given the cross-talk that is reported between the intracellular “orphan” nuclear receptors, PXR and the constitutively activated/androstane receptor, CAR, which mediates the induction of CYP2B by xenobiotics (Honkakoski et al., 2003; Waxman, 1999).

Administration of the peroxisome proliferator, clofibric acid (200 mg/kg) up-regulated mRNA levels of CYP4A1 by 16-fold compared to the vehicle-treated control group. There was also evidence of clofibric acid having an effect on other CYP enzymes. In particular, CYP2B2 increases were comparable to those seen with PB and are in agreement with other reported studies (22-fold induction of control) (Bars 1993; Pan et al., 2002).

Induction potential of putative drug candidates/NCEs

Following completion of the TaqMan[®] assay evaluation, the methodology was subsequently used to analyse livers from compounds undergoing toxicological evaluation in the rat. As a result, the induction potential of a significant number of in-house NCEs (n = 71) was evaluated. The prototypical inducers response was considered as the maximal possible induction.

It was apparent from the data that a number of NCEs had little or no inductive effect on the gene expression of CYP1A and 3A and the effects were considered to fall within the “background noise” of the constitutive expression of these genes. Consequently, a reportable inductive effect of a specific gene was initially defined as being one-which exceeded a somewhat arbitrary preset threshold. Given the low basal expression of CYP1A1, 2B1 and 2B2 and the capacity for large fold changes with prototypic inducers (Table 3) a threshold of

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≥ 5 fold induction of control was defined as an up-regulation of these genes, with the remaining CYP genes having a threshold of ≥ 2 fold induction of control as being a notable response.

Comparison of mRNA expression to catalytic activity

The fold induction of CYP gene expression was compared, where possible, to data generated using conventional marker enzyme activities. These catalytic activities were run using established assays such as 7-ethoxyresorufin O-dealkylation (EROD), a CYP1A-dependent catalytic activity and testosterone 6 β -hydroxylation (test 6 β -oh), a marker of CYP3A-dependent catalytic activity (methods based on Burke et al., 1985 and Funae & Imaoka, 1987, respectively). To simplify this comparison the fold change in gene expression determined for CYP1A1 and CYP1A2 were pooled, as the EROD assay does not discriminate between the two different CYP1A enzymes. This strategy was similarly applied to CYP3A2 and CYP3A23. Data for the fold induction of CYP1A1/1A2 mRNA and CYP3A2/3A23 mRNA with the corresponding catalytic tie-ups are shown in Figs. 1 and 2, respectively. Corresponding catalytic induction data for the same compounds were grouped and defined as < 2 -fold (solid circles), 2 - 4-fold (triangles) and > 4 -fold (squares) induction of the corresponding control group.

In general, the fold change of CYP gene expression measured by TaqMan[®] was greater than the changes detected at the protein level. But interestingly, a number of NCEs that caused a marked increase in a specific CYP mRNA did not demonstrate a corresponding marked increase in catalytic activity. For example, one compound tested (marked with an arrow in Fig. 1) caused an up-regulation of CYP1A1/1A2 mRNA (*ca* 300-fold), which contrasted with a lack of notable effect on EROD activity (< 2 -fold). Likewise, several NCEs

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(marked with arrows in Fig. 2) that caused 10 – 40-fold increases in CYP3A2/3A23 mRNA levels, showed \leq 4-fold increases in test 6 β -oh activity.

This irregularity was later explained when these drug candidates were identified as time-dependent CYP inhibitors (TDIs) and exhibited a loss of enzyme activity (data not shown). Therefore, when studied catalytically any potential inductive effect of these drug candidates may be masked.

Discussion

The examination of hepatic mRNA levels of specific CYP gene expressions using TaqMan[®] technology has a number of advantages over conventional methods for assessing the potential of a drug to cause CYP induction. The key advantages of this method, that make it both precise and reproducible (Gibson et al., 1996; Bustin, 2000; Ginzinger, 2002), are features such as it is a completely homogenous assay, with a specific target gene being detected. It is further enhanced by the detection system being fully automated with a 96-well sample format, which ultimately leads to an accelerated throughput. The TaqMan[®] method is also exquisitely sensitive, being able to amplify small amounts of mRNA, in contrast to commonly used methodologies which typically require relatively large amounts of total RNA, are unsuitable for high throughput and usually only semi-quantitative in nature.

The TaqMan[®] technology also has the advantage of quantifying changes in gene expression over a wide dynamic range, due in part to its ability to measure low levels of gene expression. For example, CYP1A1 is constitutively expressed at extremely low levels in the rat liver and is poorly detected (if at all) using conventional western blots. Also, CYP3A levels in the female rat quantified by catalytic activity are typically low. However, using qRT-PCR in this study, the basal expression of CYP1A1, CYP3A2 and CYP3A23 mRNA were readily measured.

To investigate the applicability of qRT-PCR to measure CYP induction, the effects of prototypical inducers on the major rat CYP genes were measured to evaluate the TaqMan[®] protocol. The study clearly demonstrated that there is a marked increase in the mRNA levels of the induced CYP enzyme following administration of the corresponding prototypic inducer. The maximum fold induction for individual CYP enzymes varied widely, ranging from approximately 7-fold for CYP3A2 to 2500-fold for CYP1A1. Note that the large

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differences in the potential magnitude of induction across the P450 genes results in cases where, increases in expression may be significant for one gene (e.g., 20-fold for 3A23 or 16-fold for CYP4A) but meaningless for another (e.g., 20-fold is minor for 1A1). These results were similar to those reported by Goodsaid et al (2003). There was also evidence of induction of both CYP2B by DEX and CYP3A by PB. This interplay or cross-talk between the “orphan” nuclear hormone receptors suggesting they can activate each other’s target genes, is now well documented (Honkakoski et al., 2003; Handschin and Meyer 2003).

During the evaluation of the TaqMan[®] protocol another important consideration was whether to normalise to a "house-keeping" gene. Historically, quantitative gene expression assays were often referenced to an internal control, such as the ubiquitously expressed house-keeping gene, GAPDH, since it is present in all nucleated cell types as a key enzyme in glycolysis. However, recent publications have shown that house-keeping genes such as GAPDH are not ideal as an internal reference, in view of the fact that their expression levels can be up- or down-regulated in response to experimental conditions and increased drug exposure (Schmittgen and Zakrajsek, 2000). In this study, to account for the differences in RNA loading between the samples, the data was normalised to accurately quantitated total RNA and not the house-keeping gene. However, GAPDH mRNA levels were measured in all samples to provide a marker of mRNA integrity.

Subsequently, *ex-vivo* measurements of CYPs in livers from rats were taken to estimate the inductive potential of putative drug candidates undergoing toxicological evaluation. To evaluate the potency of the NCEs ability to up-regulate CYP gene expression, the prototypical inducers response was considered the maximal potency indicator. It was also assumed that a change in mRNA levels would be indicative of an eventual change in CYP enzyme levels. Generally, there was good agreement between the up-regulation of CYP

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mRNA levels as determined by TaqMan[®] and previously generated CYP enzyme activities, although not in a directly proportional manner. However, some NCEs appeared to have the potential to induce CYP gene expression, but did in fact have little inductive effect when measured by conventional catalytic methods. A number of these candidate drugs were later established to cause a time dependent loss in CYP enzyme activity. The few remaining NCEs that demonstrated increases in mRNA levels without a functional change and that could not be explained by the effects of time dependent inhibition, were often identified as inducing other CYP enzymes by both TaqMan[®] and catalytic analysis. Thus, in the absence of any supporting TaqMan[®] data we may under-predict the inductive drive these compounds have on the liver. Although inhibition and induction may be considered to have cancelled one another out many other genes and proteins are induced by, for example, PXR ligands that wouldn't be subject to ameliorating inhibitory effects by the compound.

Having established the TaqMan[®] methodology to examine the ability of a drug to modulate CYP gene expression using rat *ex vivo* livers, it can be adapted to measure mRNA changes in other tissue (Medhurst et al., 2000), species (Pan et al., 2000), *in vitro* systems and other gene targets. Many studies have been published employing cultured hepatocytes as a model system to evaluate the induction of CYP enzymes of rat (Burczynski et al., 2001), mouse (Pan et al., 2000) and human (Bowen et al., 2000; Perez 2003; Rodriguez-Antona et al., 2000), with more limited articles using cultured precision-cut rat liver slices (Pan et al., 2002; Meredith et al., 2003). Although, attention is primarily placed on CYP-mediated drug interactions, the importance of other mechanisms as a cause of DDIs is slowly being recognised. In particular, non-oxidative pathways such as the response of UDP-glucuronosyltransferases (UGTs) to inducers (Soars et al., 2004) and also the efflux

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transporter proteins, e.g. P-glycoprotein (Lin and Yamazaki, 2003) and the multidrug resistance-associated protein 2, Mrp2 (Johnson and Klaassen, 2002).

We have proved that TaqMan[®] is a powerful tool in measuring changes in the expression of CYP genes in rats *ex vivo* and is a technique that can be utilised to study the potential of a drug to induce drug metabolising enzymes as part of their safety evaluation (Worboys and Carlile, 2001). Thus evaluating drug mediated induction at an early stage of development can aid the selection of a drug candidate over structurally similar backups and facilitate in interpreting the toxicokinetics and toxicology as well as indicating a potential risk of clinical DDIs.

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References

Bars RG, Bell DR and Elcombe CR (1993) Induction of cytochrome P450 and peroxisomal enzymes by clofibrac acid in vivo and in vitro. *Biochem Pharmacol* **45(10)**: 2045-53.

Bowen WP, Carey JE, Miah A, McMurray HF, Munday PW, James RS, Coleman RA and Brown AM (2000) Measurement of cytochrome P450 gene induction in human hepatocytes using quantitative real-time reverse transcriptase-polymerase chain reaction. *Drug Metab Dispos* **28 (7)**: 781-788.

Burczynski ME, McMillian M, Parker JB, Bryant S, Leone A, Grant ER, Thorne JM, Zhong Z, Zivin RA and Johnson MD (2001) Cytochrome P450 induction in rat hepatocytes assessed by quantitative real-time reverse-transcription polymerase chain reaction and the RNA invasive cleavage assay. *Drug Metab Dispos* **29 (9)**: 1243-1250.

Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT (1985) Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P450. *Biochem Pharmacol*, **34**: 3337-3345.

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* **25**: 169-193.

Funae Y and Imaoka S (1987) Purification and characterization of liver microsomal cytochrome P450 from untreated male rats. *Biochim Biophys Acta*, **926**: 349-358.

Gibson UE, Heid CA and Williams PM (1996) A novel method for real-time quantitative RT-PCR. *Genome Research* **6**: 995-1001.

Ginzinger DG (2002) Gene quantification using real-time quantitation PCR: An emerging technology hits the mainstream. *Exp Hematology* **30**: 503-512.

Godfrey TE and Kelly LA (2005) Development of quantitative reverse transcriptase PCR assays for measuring gene expression. *Methods in Mol Biol* **291**: 423-445.

Goodsaid FM, Palamanda JR, Montgomery D, Mandakas G, Gu C, Li Z, You X, Morton L, Smith R, Chu I, Soares T, Alton K, Kishnani NS and Rosenblum IY (2003) Assessment of temporal biochemical and gene transcription changes in rat liver cytochrome P450: Utility of real-time quantitative RT-PCR. *Pharm Res* **20 (9)**: 1373-1380.

Guengerich, FP (1997) Role of Cytochrome P450 Enzymes in Drug-Drug Interactions. Drug-Drug Interactions: Scientific and Regulatory Perspectives in *Advances in Pharmacology* **43**: 7-35, Academic Press.

Handschin C and Meyer UA (2003) Induction of drug metabolism: The role of nuclear receptors. *Pharmacol Rev* **55(4)**: 649-673.

Honkakoski P, Sueyoshi T and Negishi M (2003) Drug-activated nuclear receptors CAR and PXR. *Ann Med*, **35**: 172-182.

Hood A, Hashmi R and Klaassen CD (1999) Effects of Microsomal Enzyme Inducers on Thyroid-Follicular Cell Proliferation, Hyperplasia, and Hypertrophy. *Toxicol App Pharm* **160**: 163–170.

Huss JM and Kasper CB (1998) Nuclear receptor involvement in the regulation of rat cytochrome P450 3A23 expression. *J Biol Chem* **273 (26)**: 16155-16162.

Jones LJ, Yue ST, Cheung CY, and Singer VL (1998) RNA quantitation by fluorescence-based solution assay: Ribogreen[®] reagent characterization. *Anal Biochem* **265**: 368-374.

Johnson DR and Klaassen CD (2002) Regulation of rat multidrug resistance protein 2 by classes of prototypical microsomal enzyme inducers that activate distinct transcription pathways. *Toxicol Sci* **67**: 182-189.

Leutenegger CM, Higgins J, Matthews T, Tarantal AF, Luciw PA, Pedersen NC and North TW (2001) Real-time TaqMan[®] PCR as a specific and more sensitive alternative to the branched-chain DNA assay for quantitation of simian immunodeficiency virus RNA. *AIDS Research and Human Retroviruses* **17(3)**: 243-251.

Lin JH and Lu AYH (1998) Inhibition and induction of cytochrome P450 and the clinical implications. *Clin Pharmacok*, **35**: 361-390.

DMD #8185

Lin JH and Yamazaki M (2003) Role of P-glycoprotein in pharmacokinetics - Clinical implications. *Clin Pharmacok* **42 (1)**: 59-98.

Livak KJ, Flood SJA, Marmaro J, Giusti W and Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl* **4**: 357-362.

Medhurst AD, Harrison DC, Read SJ, Campbell CA, Robbins MJ and Pangalos MN (2000) The use of TaqMan[®] RT-PCR assays for semiquantitative analysis of gene expression in CNS tissues and disease models. *J Neuroscience Methods* **98**: 9-20.

Meredith C, Scott MP, Renwick AB, Price RJ and Lake BG (2003) Studies on the induction of rat hepatic CYP1A, CYP2B, CYP3A and CYP4A subfamily form mRNAs *in vivo* and *in vitro* using precision-cut rat liver slices. *Xenobiotica* **33 (5)**: 511-527.

Michalets EL (1998) Update: Clinically significant cytochrome P450 drug interactions. *Pharmacotherapy* **18 (1)**: 84-112.

Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL and Kliewer SA (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* **275**: 15122-15127.

DMD #8185

Nelson DR Koymans L Kamataki T Stegeman JJ Feyereisen R Waxman DJ Waterman MR Gotoh O Coon MJ Estabrook RW Gunsalus IC Nebert DW (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6(1)**: 1-42.

Nelson DR (1999) Cytochrome P450 and the individuality of species. *Arch Biochem Biophys* **369**: 1-10.

Pan J, Xiang Q and Ball S (2000) Use of a novel real-time quantitative reverse transcription-polymerase chain reaction method to study the effects of cytokines on cytochrome P450 mRNA expression in mouse liver. *Drug Metab Dispos* **28 (6)**: 709-713.

Pan J, Xiang Q, Renwick AB, Price RJ, Ball SE, Kao J, Scatina JA and Lake BG (2002) Use a quantitative real-time reverse transcription-polymerase chain reaction method to study the induction of CYP1A, CYP2B and CYP4A forms in precision-cut rat liver slices. *Xenobiotica* **32 (9)**: 739-747.

Park BK, Kitteringham NR, Pirmohamed M and Tucker GT (1996) Relevance of induction of human drug-metabolizing enzymes: Pharmacological and toxicological implications. *Br J Clin Pharmacol* **41**: 477-491.

Parkinson A (2001) Biotransformation of xenobiotics. Casarett and Doull's Toxicology: *Basic Sciences of Poisons*. 6th Ed. New York: McGraw-Hill Medical Publishing Division, 133-224.

DMD #8185

Perez G, Tabares B, Jover R, Gomez-Lechon MJ, Castell JV (2003) Semi-automatic quantitative RT-PCR to measure CYP induction by drugs in human hepatocytes. *Toxicology in Vitro* **17(5-6)**: 643-649.

Rasmussen R (2001) Quantification on the LightCycler. In Meuer S, Wittwer C and Nakagawara K (eds) *Rapid Cycle Real-time PCR, Methods and Applications*. Springer Press, Heidelberg, 21-34.

Rodriguez-Antona C, Jover R, Gomez-Lechon M-J and Castell JV (2000) Quantitative RT-PCR measurement of human cytochrome P450s: Application to drug induction studies. *Arch Biochem Biophys* **376 (1)**: 109-116.

Schmittgen TD and Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time quantitative RT-PCR. *J Biochem Biophys Methods* **46**: 69-81.

Soars MG, Petullo DM, Eckstein JA, Kasper SC and Wrighton SA (2004) An assessment of UDP-glucuronosyltransferase induction using primary human hepatocytes. *Drug Metab Dispos* **32 (1)**: 140-148.

Staudinger J, Liu Y, Madan A, Habeebu S and Klaassen CD (2001) Coordinate Regulation of Xenobiotic and Bile Acid Homeostasis by Pregnane X Receptor. *Drug Metab Dispos* **29 (11)**: 1467-1472.

DMD #8185

Waxman D (1999) Minireview: P450 gene induction by structurally diverse xenochemicals: Central role of nuclear receptors CAR, PXR and PPAR. *Arch Biochem Biophys* **369** (1), 11-23.

Wei P, Zhang J, Dowhan DH, Han Y and Moore DD (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics J* **2**: 117-126.

Worboys PD and Carlile DJ (2001) Implications and consequences of enzyme induction on preclinical and clinical drug development. *Xenobiotica* **31(8/9)**: 539-556.

Figures

Figure 1. Fold Induction of CYP1A1/1A2 mRNA as determined by TaqMan[®] for seventy one NCEs and CYP1A catalytic activity.

Figure 1 footnote:

All data points represent fold induction of CYP1A1/1A2 mRNA as determined by TaqMan[®]. Comparable catalytic CYP1A data (EROD) for the same NCE, representing < 2 fold induction are shown as solid circles (●); 2 – 4 fold induction as triangles (▲) and > 4 fold induction as a square (■). NCEs with no comparable catalytic data are shown as clear circles (○). The maximum response by the prototypical CYP1A inducer, BNF (80 mg/kg/day) is shown by a star (★). The compound with known time-dependent inhibition (TDI) properties is indicated by an arrow.

Figure 2. Fold Induction of CYP3A2/3A23 mRNA as determined by TaqMan[®] for seventy one NCEs and CYP3A catalytic activity.

Figure 2 footnote:

All data points represent fold induction of CYP3A2/3A23 mRNA as determined by TaqMan[®]. Comparable catalytic CYP3A data (6β-hydroxy testosterone) for the same NCE, representing < 2 fold induction are shown as solid circles (●); 2 – 4 fold induction as triangles (▲) and > 4 fold induction as a square (■). NCEs with no comparable catalytic data are shown as clear circles (○). The maximum response by the prototypical CYP3A inducer,

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DEX (150 mg/kg/day) is shown by a star (★). Compounds with known time-dependent inhibition (TDI) properties are indicated by arrows.

Table 1. TaqMan® fluorogenic probes with forward and reverse primers

Gene	Accession No. (genbank*)	Oligo	Sequence (5'—3')	Amplicon (bp)	Exon
CYP1A1	M26129 *	* Probe	TTCTCACTCAGGTGTTTGTCCAGAGTGCC	91	7
		FP	CCAAACGAGTTCCGGCCT		
		* RP	TGCCCAAACCAAAGAGAATGA		
CYP1A2	K02422*	Probe	CAATGACAACACGGCCATCGACAAG	81	3
		FP	CGCCCAGAGCGGTTTCTTA		
		* RP	TCCAAGCCGAAGAGCATC		
CYP2B1	J00719*	Probe	CCATACACTGATGCAGTTATCCATGAGATTCAGA	93	7
		FP	AACCCTTGATGACCGCAGTAAA		
		* RP	TGTGGTACTCCAATAGGGACAAGATC		
CYP2B2	J00720-728*	Probe	CCATACACTGATGCAGTCATCCACGAGATTC	84	7
		FP	CCATCCCTTGATGATCGTACCA		
		* RP	AATTGGGGCAAGATCTGCAAA		
CYP2E1	J02627*	* Probe	ATAGCAGACAGGAGCAGAAACAATTCCATGC	90	9
		FP	AAAGCGTGTGTGTGTTGGAGAA		
		* RP	AGAGACTTCAGGTAAAATGCTGCA		
CYP3A2	M13646*	Probe	CATTTATGGATCTTTCTAAGTGTCTATACGAAGTACCAC	99	13
		FP	GCTCTTGATGCATGGTTAAAGATTTG		
		* RP	ATCACAGACCTTGCCAACCTCCTT		
CYP3A23	X96721*	* Probe	TCTCTTGCCTCAAGTATTTTACCAAAATGTCT	80	6
		FP	ATGTTCCCTGTCATCGAACAGTATG		
		* RP	TTCACAGGGACAGGTTTGCCT		
CYP4A1	M14972*	Probe	AAGGTCCCCATCCCCTTACCACGA	71	12
		FP	TTGAGCTACTGCCAGATCCCAC		
		* RP	CCCATTTTTGGACTTCAGCACA		
GAPDH	AF106860	Probe	ACCACAGTCCATGCCATCACTGCCA	90	1
		FP	CAAGGTCATCCATGACAACCTTG		
		* RP	GGGCCATCCACAGTCTTCTG		

FP – forward primer; RP – reverse primer; *sequence designed to the reverse complement.

Table 2. Basal gene expression levels in control male Sprague Dawley rat liver by qRT-PCR (TaqMan[®])

Gene	Mean copies of mRNA detected/ng total RNA	± S.E.M.
CYP1A1	0.56	0.066
CYP1A2	1,000	67
CYP2B1	3.6	0.79
CYP2B2	20	2.3
CYP2E1	5,900	550
CYP3A2	5,700	510
CYP3A23	180	16
CYP4A1	320	34
GAPDH	19	1.2

Mean ± S.E.M. of 78 control male Sprague Dawley rat livers, expressed to 2 significant figures.

Table 3. Fold induction of control rat CYP genes following administration of the classical CYP inducers BNF, PB, DEX and CLO.

Gene	Mean Fold Induction of Control \pm S.E.M.			
	BNF (80 mg/kg/day)	PB (80 mg/kg/day)	DEX (150 mg/kg/day)	CLO (200 mg/kg/day)
CYP1A1	2500 \pm 520	0.83 \pm 0.66	1.7 \pm 0.62	0.78 \pm 0.22
CYP1A2	23 \pm 4.3	0.70 \pm 0.11	0.18 \pm 0.018	0.54 \pm 0.12
CYP2B1	1.3 \pm 0.12	680 \pm 110	49 \pm 13	3.1 \pm 0.82
CYP2B2	0.82 \pm 0.23	22 \pm 2.3	50 \pm 2.6	22 \pm 3.7
CYP2E1	0.91 \pm 0.12	0.60 \pm 0.24	0.64 \pm 0.052	1.5 \pm 0.13
CYP3A2	1.9 \pm 0.28	2.4 \pm 0.98	7.4 \pm 0.26	2.7 \pm 0.33
CYP3A23	1.0 \pm 0.23	4.9 \pm 0.33	59 \pm 12	1.2 \pm 0.15
CYP4A1	1.2 \pm 0.37	0.44 \pm 0.14	0.78 \pm 0.11	16 \pm 2.4

Mean \pm S.E.M. of 3 male Sprague Dawley rat livers, expressed to 2 significant fig

Figure 1

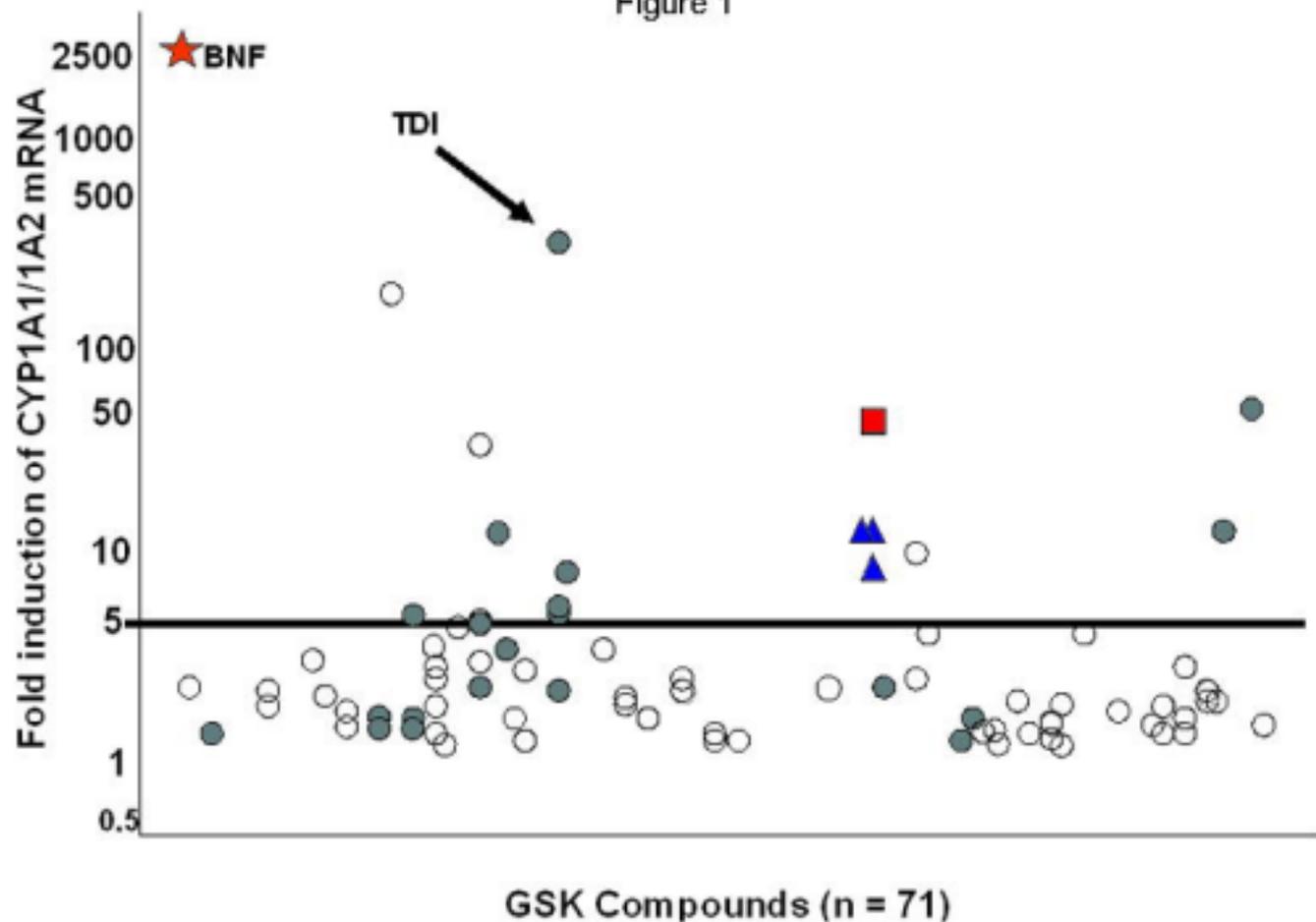


Figure 2

