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Quantitative analysis of the Ah receptor/cytochrome P450 CYP1B1/CYP1A1 signalling pathway

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Abstract

Cytochrome P450 (CYP) drug metabolising enzymes CYP1A1 and CYP1B1 are regulated through the ligand-activated aryl hydrocarbon (Ah) receptor. Differential expression of CYP1A1 and CYP1B1 mRNA and protein has previously been reported in human tissues with the presence of the message often extrapolated to indicate the presence of protein. The aim of this study was to clarify these potentially misleading findings, by analysing components of the Ah receptor pathway (CYP1B1, CYP1A1, Ah receptor and ARNT) using a combination of quantitative real-time RT–PCR and immunoblotting. Three human cell lines (MOG-G-CCM, MCF7 and HEPG2) known to differentially express CYP1A1 and CYP1B1 mRNA and protein were exposed to the Ah receptor agonist 3-MC, and basal and inducible levels of CYP1A1, CYP1B1, Ah receptor and ARNT were determined. The key finding of this study was the demonstration of equivalent levels of CYP1B1 mRNA in both the treated and untreated MOG-G-CCM cell lines, with expression of the corresponding CYP1B1 protein only after exposure to an Ah receptor agonist. This finding suggests that a post-transcriptional mechanism is involved in the regulation of CYP1B1. In addition, the expression pattern of CYP1B1 mRNA and protein in the MOG-G-CCM cells highlights this cell line as a potential model for studying CYP1B1 expression in human tissue.

Keywords: Cytochrome P450; CYP1B1; Aryl hydrocarbon receptor; Differential expression; Quantitative real-time PCR

1. Introduction

Polycyclic aromatic hydrocarbons induce a battery of xenobiotic metabolising enzymes, including the CYP1A1 and CYP1B1, these enzymes function to metabolise and excrete incoming xenobiotics. Potent inducers of the CYP1 family include polycyclic aromatic hydrocarbons, such as β-naphthoflavone or halogenated aromatic hydrocarbons, e.g. 2,3,7,8,-tetrachorodibenzo-*p*-dioxin (TCDD), these inducers invoke their gene regulation effects *via* a specific receptor protein, termed the Ah receptor [1–3]. The Ah receptor is a ubiquitous cytosolic protein that responds to planar aromatic ligands by forming an activated complex with two molecules of the molecular chaperone hsp90, and the X-associated protein 2 [4,5]. Upon ligand binding the Ah receptor undergoes a conformational change and translocates to the nucleus. Once nuclear translocation has occurred, hsp90 is released from the Ah receptor, either at the translocation stage or upon dimerisation with another bHLH partner, such as aryl hydrocarbon nuclear translocator (ARNT). This heterodimeric Ah receptor/ARNT complex binds to xenobiotic-responsive elements located upstream of the *CYP1A1* and *CYP1B1* genes and promotes their transcription. These CYP enzymes are involved in the metabolism of a range of xenobiotics. Indeed these enzymes play a central role in the activation of a variety of environmental carcinogens and mutagens [1,6,7].

Following exposure to environmental carcinogens, such as polycyclic aromatic hydrocarbons, CYP1A1 mRNA and protein have been detected in both the liver and in a number of extra-hepatic tissues [7,8]. This P450 is highly inducible following exposure to chemical carcinogens, and several groups, including ourselves, have demonstrated enhanced

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Abbreviations: Ah, aryl hydrocarbon; ARNT, aryl hydrocarbon nuclear translocator; CV, coefficient of variation; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; FAM, 6-carboxy-fluoroscein; hsp90, heat shock protein 90; 3-MC, 3-methylcholantherene; TAMRA, 6-carboxy-tetramethyl-rhodamine; TCDD, 2,3,7,8,-tetrachorodibenzo-*p*-dioxin.

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expression of a member of the CYP1 family mRNA and protein in a number of solid tumours, including renal cell carcinoma [9,10]. Although CYP1A1 and CYP1B1 are under the regulatory control of the Ah receptor, there is evidence for differential regulation of the genes encoding these two enzymes [11,12]. Moreover, CYP1A1 and CYP1B1 do not show the same tissue distribution and they are not always expressed in concert either in human tissues or cultured cells. CYP1B1 is an important tumour-related enzyme, which has been shown to be over-expressed in tumours, with expression of the protein restricted to tumour cells [13,14]. Although CYP1B1 mRNA has previously been shown in a number of normal human tissues, including the liver, the presence of the corresponding protein in these tissues has not been detected [14,15].

A variety of issues concerning the regulation of CYP1B1 expression remain unanswered. Studies of human tumour derived cell lines have demonstrated distinct cell-type expression of basal and induced CYP1A1 and CYP1B1 using a combination of Northern blot analysis, qualitative and semi-quantitative PCR, and Western blotting. Several investigators have shown low basal levels of CYP1A1 mRNA in HEPG2 cells, which is highly induced following exposure to the Ah receptor agonist TCDD (both mRNA and protein) [16,17]. Conflicting evidence in the literature exists with regards to the presence of constitutive and induced levels of CYP1B1 mRNA in HEPG2 cells; CYP1B1 mRNA is generally not found to be expressed either constitutively or following induction with an Ah agonist in this cell line [16,18]. Indeed, Kress and Greenlee have shown that CYP1B1 mRNA was not detectable by Northern blot analysis in HEPG2 cells either before or after exposure to TCDD, however, nuclear run-off assays confirmed the presence of an intact *CYP1B1* gene in this cell line [16]. In contrast to these observations, several laboratories have recently demonstrated the presence CYP1B1 mRNA in HEPG2 cells by qualitative RT–PCR, which increases following exposure to Ah agonists, such as TCDD [17,19], or dimethyl-benz[*a*]anthracene (DMBA) [20].

In the renal cancer cell line (ACHN), CYP1B1 shows weak constitutive expression, and both CYP1B1 mRNA and protein are detected after exposure to dioxin but CYP1A1 mRNA is not present even following exposure to TCDD [16,21]. Following exposure to TCDD in the breast carcinoma MCF7 cell line, CYP1A1 and CYP1B1 immunoreactive protein and mRNA have been identified. However, in the uninduced MCF7 cells constitutive CYP1B1 mRNA has been identified but CYP1A1 mRNA was not detected by semi-quantitative RT–PCR [22]. Given that CYP1B1 is a tumour-related enzyme and an important target for chemotherapeutic intervention in cancer research, it is essential to have a fuller understanding of the regulatory mechanism governing the differential expression of CYP1B1. Thus, it is necessary to have a model of CYP1B1 expression comparable to the situation found in human tissues. In this study, we highlight the MOG-G-CCM cell line as one such model.

2. Materials and methods

2.1. Cell lines

All cell lines used in this study (MCF7, HEPG2 and MOG-G-CCM) were purchased from European Collection of Cell Cultures (ECACC).

The HEPG2, MCF7 and MOG-G-CCM cell lines were used for both qualitative and quantitative mRNA analyses of the Ah receptor and the CYP enzymes regulated by this ligand-activated receptor. All cell lines were grown under the recommended optimal growth conditions (ECACC guidelines [23]) as follows; HEPG2 and MCF7 cell lines were grown in DMEM supplemented with 2 mM glutamine plus 10% (v/v) foetal calf serum (FCS). MOG-G-CCM cells were cultured in Ham's F10:DMEM (1:1) supplemented with 2 mM glutamine and 10% FCS. In this study, the cell lines were cultivated in streptomycin/penicillin free conditions, and maintained as monolayer, or multilayer (HEPG2 cells grow as clumps and rarely as single monolayer growth), cultures in 15 mL of the appropriate media in 75 cm^2 plastic tissue culture flasks. The cultures were incubated at 37° in a humidified atmosphere of 5% CO_2/95% air. Cultured cells were routinely passaged every 7 days at a seeding density of 1×10^4 to 5×10^4 cells/cm^2 with media changes every 48–72 hr. Cells at 70–80% confluence were used in all experiments.

2.2. P450 induction in cultured cells

An initial series of experiments was undertaken to determine the response of the cell lines to the Ah receptor agonist 3-methylcholantherene (3-MC) (Sigma). The cells in this investigation were plated out at equal densities for both the DMSO control and the 3-MC treatment regimens. When cells reached 70–80% confluence 3 days after passage, they were exposed to 25 μM of 3-MC (DMSO at a final concentration of 0.1% v/v) for 24 hr. Control cells were exposed to DMSO (0.1%) for 24 hr and treated in the same manner as those exposed to 3-MC.

Following this initial investigation, the MOG-G-CCM, HEPG2 and MCF7 cell lines were then used to investigate the exposure of the cells to 25 μM 3-MC at the following time points: 0, 6, 12, 24 and 48 hr, control cells received 0.1% v/v DMSO. Cells exposed to either 3-MC or DMSO were collected at each time interval.

2.3. RNA isolation

Following exposure of the cells to 3-MC or DMSO, the cells were washed with 2×10 mL of ice-cold PBS, and then manually scraped into 10 mL of ice-cold PBS, before being centrifuged at 1000 *g* for 5 min to pellet the cells (ALC: PK121 Multi-Speed Centrifuge, Thomson Scientific). Total RNA was isolated using RNAzolB according to the manufacturer's instructions (Biogenesis Ltd). DNase

treatment is not routinely used in our laboratory. The RNA was resuspended in diethyl pyrocarbonate (Sigma) treated water and quantified spectrophotometrically at 260 and 280 nm, respectively. All the RNA samples had a 260 nm:280 nm ratio between 1.9 and 2.0 and the integrity of the RNA sample was confirmed by agarose gel electrophoresis which showed the presence of intact 18S and 28S ribosomal RNA bands (data not presented).

2.4. cDNA synthesis

Synthesis of cDNA was performed using a reverse transcriptase system (Promega), with the following reaction conditions as recommended by Promega: 1 μg of RNA, 1× reverse transcriptase buffer (10 mM Tris–HCl, pH 8.8, 50 mM KCl and 0.1% Triton X-100), 5 mM $MgCl_2$, 1 mM dNTP, 0.5 U RNasin, 15 U avian myeloblastosis reverse transcriptase and 0.5 μg oligo dT_{15} primer in a final volume of 20 μL. Synthesis of cDNA was performed at 42° for 60 min and the reverse transcriptase reaction was stopped by heating to 99° for 5 min followed by chilling on ice. Adequate cDNA was prepared for all experiments performed in this study and stored at −75° until required.

2.5. Qualitative RT–PCR

Qualitative RT–PCR was performed as previously described [9,21]. Both negative and positive controls were included in each PCR reaction. The negative control was double autoclaved water in place of the template cDNA. Human adult liver (cDNA), which we have previously shown to express CYP1A1 and CYP1A2 [24], was used as the positive control for CYP1A1, CYP1A2 and Ah receptor, while the positive control for CYP1B1 was a plasmid containing a full-length human CYP1B1 cDNA that was a gift from Dr. W.F. Greenlee, University of Massachusetts, USA. The PCR products (10 μL) were separated by gel electrophoresis, using a 1.5% agarose gel, stained with ethidium bromide (Sigma) and visualised by trans-illumination with ultra-violet light. The gels were then photographed with Polaroid type 665 black and white film. The presence of a particular amplicon in a sample was demonstrated by the visualisation of a single product on the gel of the expected molecular size.

2.6. Quantitative RT–PCR

Real-time quantitative RT–PCR analyses for CYP1A1, CYP1B1, Ah receptor and ARNT mRNAs were performed using the ABI 7700 Sequence Detection System (PE Applied Biosystems Inc.). Unlabelled primers and labelled probes for the real-time quantitative RT–PCR were designed to meet specific criteria for optimal design set by Primer Express software version 1 (PE Applied Biosystems Inc.). Synthesis of the oligonucleotides was performed by PE Applied Biosystems. The 5′- and 3′-end nucleotides of each probe were labelled with a reporter (FAM) and a quencher dye (TAMRA), respectively. The sequences of the PCR primer pairs and fluorogenic probes are detailed in Table 1. Primers and the fluorogenic probe for the endogenous control ribosomal RNA (rRNA) labelled with VIC™ were purchased from PE Applied Biosystems; sequence information for the ribosomal RNA primer/probe set is neither provided nor available from the supplier.

The fluorescent signal of each product of interest generated was normalised to an internal reference ROX (ΔRn) and the sequence detection software (version 1.7) sets the threshold cycle C_t, which is used to quantify the input target number. The relative expression level of the gene of interest was computed with respect to ribosomal RNA

Table 1
Sequences of primer and fluorogenic probes for quantitative RT–PCR

Primer	Sequence	Amplicon size
Ah receptor		115 bp
Forward	^{4522}tcttactctgccgcccaaac^{4541}	
Reverse	^{4637}ccgcaatcccagcaactc^{4620}	
Probe[a]	$^{FAM\,4571}$tgcaacctctacctcctgggttcaagt$^{4600\,TAMRA}$	
ARNT		77 bp
Forward	^{862}gtggcagtagctctgtggacc^{882}	
Reverse	^{939}agccaagtccattcctgcat^{920}	
Probe[a]	$^{FAM\,888}$tctgtgaataggctgagctttgtgaggaaca$^{918\,TAMRA}$	
CYP1A1		69 bp
Forward	^{1605}caaatgcagctgcgctctt^{1623}	
Reverse	^{1674}cccaaccagaccaggtagaca^{1654}	
Probe[a]	$^{FAM\,1627}$tgcttgagagccctgaggcctagactc$^{1653\,TAMRA}$	
CYP1B1		75 bp
Forward	^{1441}gagggaccgtctgccttgtat^{1461}	
Reverse	^{1497}gcgcatggcttcataaagga^{1516}	
Probe[a]	$^{FAM\,1465}$tgaccagcccaacctgccctatgtc$^{1489\,TAMRA}$	

[a] Fluorogenic probe 5′-end nucleotide labelled with reporter dye: FAM = 6-carboxy-fluoroscein, 3′-end nucleotide labelled with quencher dye: TAMRA = 6-carboxy-tetramethyl-rhodamine.

(an endogenous control), to account for any variance in the quality of mRNA and the amount of input cDNA (ABI PRISM 7700 Sequence Detection System Users Manual).

A standard curve for both the endogenous gene (18S ribosomal RNA) and the target gene (CYP1A1, CYP1B1, Ah receptor and ARNT) was generated to determine the amount of the transcripts in the individual experimental samples. The latter was constructed with serial dilutions of appropriate known positive control samples; for CYP1B1 the full-length plasmid CYP1B1 cDNA (1 fg–10 ng), for CYP1A1 serial dilutions (1 pg–500 ng) of cDNA from HEPG2 cell line exposed to 3-MC and serial dilutions of normal liver for ribosomal RNA, Ah receptor and ARNT (1 pg–100 ng) (ABI PRISM 7700 Sequence Detection System Users Manual).

The amount of target gene was divided by the amount of reference gene (i.e rRNA) to obtain a normalised target value. To better understand the effect of 3-MC on levels of gene expression, the data were presented as a fraction of the DMSO control. PCR was performed with the TAQMAN Universal PCR Master Mix (PE Applied Biosystems Inc.) using 100 ng of cDNA, and the following probe concentrations which had previously been optimised (according to TAQMAN Universal PCR Master Mix Protocol): 175 nmol/L for ribosomal RNA, Ah receptor, ARNT or 200 nmol/L for CYP1A1 and CYP1B1; the optimal primer concentration for both the forward and reverse primers in all reactions was 300 nmol/L in a final 50 μL reaction mixture. Controls containing no template DNA (negative control) were included in the assay for each primer set used. After a 2-min incubation stage at 50° to allow for UNG (Amperase uracil-*N*-glycosylase) cleavage (Amperase UNG treatment prevents the re-amplification of carry-over PCR products). AmpliTaq Gold was activated by incubation for 10 min at 95° followed by 40 cycles of the following two step procedure: 15 s at 95° and 60° for 1 min (ABI PRISM 7700 Sequence Detection System Users Manual: user bulletin 2).

2.7. Data analysis: coefficient of variation (CV)

In each run of the assay cDNA for ribosomal RNA, Ah receptor, ARNT, CYP1A1 and CYP1B1 were analysed in triplicate. Mean and standard deviation were calculated for each of the samples, which were then further analysed to determine the CV. Based on our experience of quantitative real-time RT–PCR and following communication with the manufacturers (Applied Biosystems), a CV of 10% was accepted as the maximum variation allowed under the conditions used, any sample out with this value was discarded and repeated.

2.8. Preparation of microsomes

Following exposure of the cells to 3-MC or DMSO, the cells were washed with 2 × 10 mL of ice-cold PBS, and then manually scraped into 10 mL 0.01 M Tris–HCl containing 0.25 M sucrose and 15% glycerol. The cells were homogenised using a Polytron PT3000 Homogeniser (Kinematica AG). The homogenate was centrifuged at 15,000 *g* for 20 min at 4° using a Centrikon T-124 centrifuge (Kontron Instruments). The resultant supernatants were then centrifuged at 180,000 *g* (44,000 rpm) for 1 hr at 4° using a Pegasus AP65 centrifuge (Thomson Scientific). The pellet obtained after centrifugation was resuspended in 0.1 M Tris–HCl, containing 15% glycerol and 1 mM EDTA. The resultant microsomes were stored at −75° prior to use. Protein concentrations for each sample of microsomes were determined using the Bradford method [26].

2.9. Antibodies

In this study a monoclonal antibody (MAb) raised against a 15 amino acid peptide located in the C-terminal third of the human CYP1B1 protein was used to determine CYP1B1 immunoreactivity. This antibody specifically recognised CYP1B1 and did not recognise either CYP1A1 or CYP1A2 [14]. CYP1A1 immunoreactivity was determined by a rabbit polyclonal antibody raised against a synthetic peptide to the C-terminal region of human CYP1A1 ^{503}CEHFQMQLRS^{513} (Auvation). The limits of antigen detection were 0.025 pmol for CYP1B1 [14] and 0.05 pmol for CYP1A1 (unpublished observation) over a range of antigen concentrations (0.005–10 pmol).

2.10. Immunoblotting

Microsomal proteins (30 μg of protein loaded per lane for each cell line) were electrophoretically separated at constant current in a 10% polyacrylamide gel using a Hoefer SE600 electrophoresis system (Hoefer Pharmacia Biotech Inc.) and then transferred at constant current for 18 hr to nitrocellulose (Hybond ECL, Amersham Life Sciences) by electroblotting using a Hoefer TE42 blotting system (Hoefer Pharmacia Biotech Inc.). Non-specific binding sites were blocked by incubation of the nitrocellulose membrane for 60 min at room temperature in wash buffer consisting of 2% non-fat milk (Marvel, Premier Beverages) in 10 mM phosphate buffered saline containing 0.05% Tween 20 (Sigma). The nitrocellulose was then sequentially incubated with either the CYP1B1 MAb (1/100) or the CYP1A1 polyclonal antibody (1/5000) and either goat anti-mouse or goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (1/2000; Bio-Rad). After each antibody application, the membrane was washed for five 10-min periods. Horseradish peroxidase was then demonstrated using an enhanced chemiluminescent technique (Amersham life Sciences), which was performed as previously described [13,27]. X-ray film was exposed for a time determined to give the optimum ‘signal-to-noise’ ratio (1–5 min). Microsomes prepared from human lymphoblastoid cells,

that contained either expressed human CYP1A1 or CYP1B1, were used as positive controls and were obtained from Gentest Corp.

3. Results

In this study the qualitative and quantitative RT–PCR results are discussed in context. The presence of β-actin (an endogenous control used to assess the efficiency of reverse transcriptase) was detected in all the cell lines used in this study (Fig. 1C). All samples were subject to PCR for the individual P450s and the Ah receptor. The approach we took to ensure the absence of genomic contamination was encompassed in the design of the Ah receptor primers which were designed (using the seqnet 'Prime program': http://www.dl.ac.uk/seqnet/home/html) to span the 772 bp intron G [28], this allowed a direct visual assessment of both the cDNA product (262 bp) and any potential genomic contamination (1034 bp) (Table 2). The presence of the Ah receptor cDNA product was visualised as a band at 262 bp following gel electrophoresis in the MCF7, HEPG2 and MOG-G-CCM cell lines. No evidence of genomic contamination was detected in any of the samples investigated (Fig. 1D).

Real-time quantitative RT–PCR provides a mechanism to accurately and reproducibly quantify the differential expression of all the transcripts investigated in the time course experiments [29]. The standard curves constructed gave excellent linearity with correlation coefficients or $R^2 = 0.92–0.99$. Sample variation was assessed by determining the CV for all samples, which was generally below 5% with a maximum permitted variation of 10%. All mean values presented in tables and figures are normalised against the endogenous control rRNA.

3.1. Quantitative analysis of mRNA expression

3.1.1. Ah receptor mRNA expression

Although qualitative RT–PCR identified Ah receptor mRNA in the MCF7, HEPG2 and MOG-G-CCM cell lines at a basal level and following exposure to 3-MC, no assessment of the level and amount of the Ah receptor could be made either between treatment or cell line (Fig. 1D). In contrast, quantitative real-time RT–PCR showed marked differential expression of the Ah receptor between the cell lines with Ah receptor expression at least 100-fold higher in the MOG-G-CCM cell line compared to either the MCF7 or HEPG2 cell line (Table 3, Fig. 2C). Expressing Ah receptor mRNA as a fraction of the DMSO control, little change in mRNA was observed in the 3-MC-treated MCF7 and HEPG2 cells compared to the DMSO control. The MOG-G-CCM cell line demonstrated

Table 2
Primer sequences for qualitative RT–PCR

Primer	Sequence	Amplicon size	Reference
Ah receptor		262 bp (1034 bp genomic product)	Current study
Forward	^{1117}ggacagaaaaagaaagggaaag^{1138}		
Reverse	^{1378}cggcacaataaagcatatcag^{1358}		
CYP1A1		394 bp	[25]
Forward	^{967}tggatgagaacgccaatgtc^{986}		
Reverse	^{1358}tgggttgacccatagcttct^{1339}		
CYP1A2		416 bp	[25]
Forward	^{626}acagcacttcccgagagta^{645}		
Reverse	^{1041}tctggatctcctctgtatc^{1022}		
CYP1B1		680 bp	[24]
Forward	^{1311}ccactatcactgacatcttc^{1330}		
Reverse	^{1990}gcctcttgcttcttattg^{1973}		
β-Actin		661 bp	Stratagene
Forward	^{1038}tgacgggtcacccacactgtgcccatcta^{1067}		
Reverse	^{1876}ctagaagcattgcggtggacgatggaggg^{1905}		

Table 3
Expression of Ah receptor mRNA (picogram) normalised against rRNA

Time (hr)	MCF7 (DMSO)	MCF7 (3-MC)	HEPG2 (DMSO)	HEPG2 (3-MC)	MOG-G-CCM (DMSO)	MOG-G-CCM (3-MC)
0	24.18	24.18	10.04	10.04	287.57	287.57
6	38.34	47.14	8.44	8.83	577.10	556.89
12	21.66	8.06	8.19	14.74	258.95	184.13
24	7.71	7.01	9.45	8.69	96.75	330.09
48	4.29	6.103	3.08	2.44	128.18	183.60

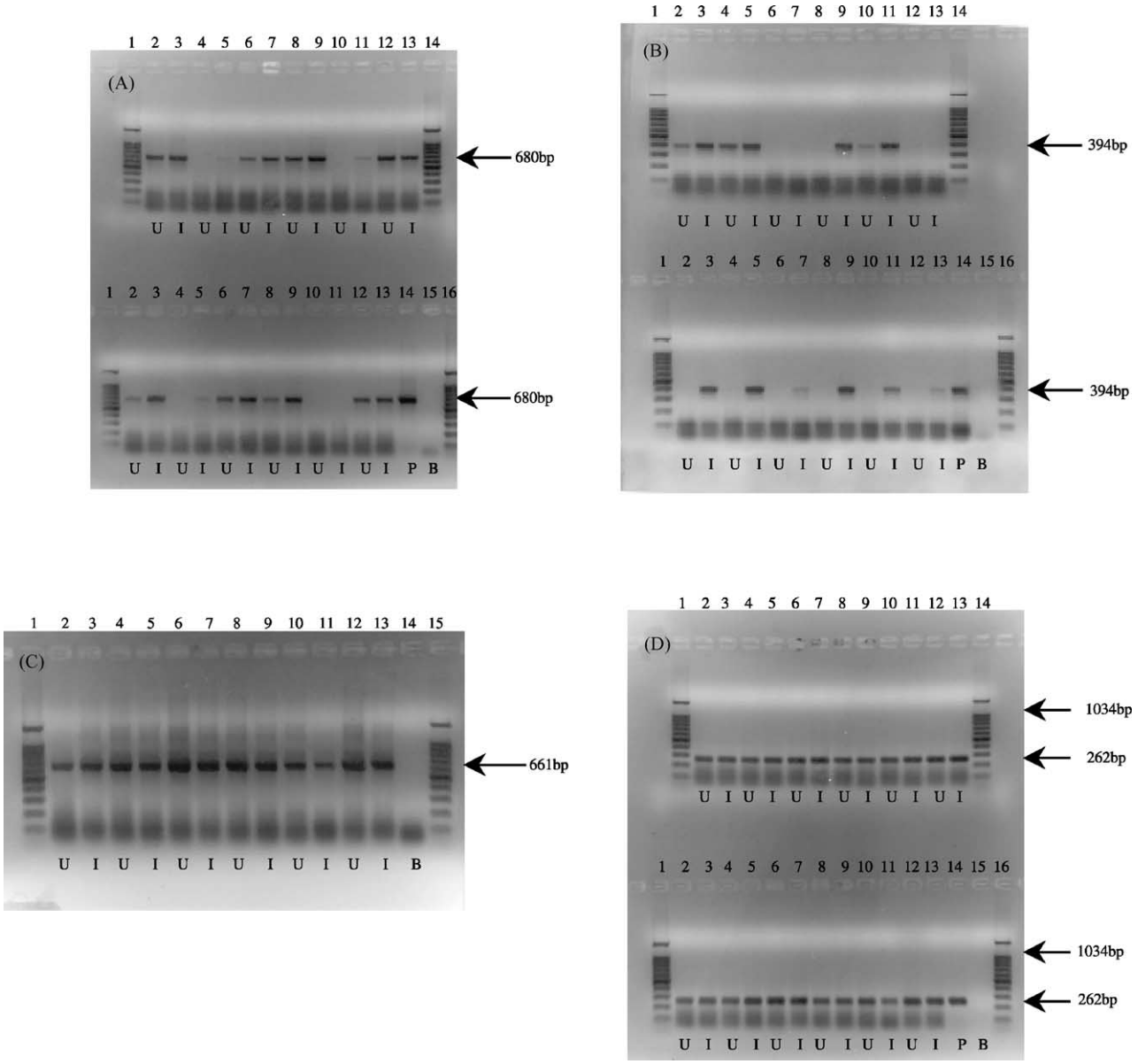

Fig. 1. Qualitative RT–PCR figures. Abbreviations: U = DMSO control, I = 3-MC-treated cells, P = positive control lane, B = negative control. (A) Qualitative CYP1B1 RT–PCR. Top panel: lanes 1 and 14: 100 bp molecular weight markers, lanes 2–7: 6-hr time point, lanes 8–13: 12-hr time point. Lanes 2 and 8 DMSO: control MCF7 cells, lanes 3 and 9: 3-MC-treated MCF7 cells, lanes 4 and 10: DMSO control HEPG2 cells, lanes 5 and 11: 3-MC-treated HEPG2 cells, lanes 6 and 12: DMSO control MOG-G-CCM cells, lanes 7 and 13: 3-MC-treated MOG-G-CCM cells. Bottom panel: lanes 1 and 16: 100 bp molecular weight markers, lanes 2–7: 24-hr time point, lanes 8–13: 48-hr time point. Lanes 2 and 8 DMSO: control MCF7 cells, lanes 3 and 9: 3-MC-treated MCF7 cells, lanes 4 and 10: DMSO control HEPG2 cells, lanes 5 and 11: 3-MC-treated HEPG2 cells, lanes 6 and 12: DMSO control MOG-G-CCM cells, lanes 7 and 13: 3-MC-treated MOG-G-CCM cells. Lane 14: positive control (plasmid cDNA), lane 15: negative control (water). (B) Qualitative CYP1A1 RT–PCR. Top panel: lanes 1 and 14: 100 bp molecular weight markers, lanes 2–7: 6-hr time point, lanes 8–13: 12-hr time point. Lanes 2 and 8: DMSO control HEPG2 cells, lanes 3 and 9: 3-MC-treated HEPG2 cells, lanes 4 and 10: DMSO control MCF7 cells, lanes 5 and 11: 3-MC-treated MCF7 cells, lanes 6 and 12: DMSO control MOG-G-CCM cells, lanes 7 and 13: 3-MC-treated MOG-G-CCM cells. Bottom panel: lanes 1 and 16: 100 bp molecular weight markers, lanes 2–7: 24-hr time point, lanes 8–13: 48-hr time point. Lanes 2 and 8: DMSO control HEPG2 cells, lanes 3 and 9: 3-MC-treated HEPG2 cells, lanes 4 and 10: DMSO control MCF7 cells, lanes 5 and 11: 3-MC-treated MCF7 cells, lanes 6 and 12: DMSO control MOG-G-CCM cells, lanes 7 and 13: 3-MC-treated MOG-G-CCM cells. Lane 14: positive control (human liver cDNA), lane 15: negative control (water). (C) β-Actin RT–PCR of 24- and 48-hr time points. Lanes 1 and 15: 100 bp molecular weight markers, lanes 2–7: 24-hr time point, lanes 8–13: 48-hr time point. Lane 14: negative control (dH_2O), lanes 2 and 8: HEPG2 cells DMSO control, lanes 3 and 9: 3-MC-treated HEPG2 cells, lanes 4 and 10: DMSO control MCF7 cells, lanes 5 and 11: 3-MC-treated MCF7 cells, lanes 6 and 12: DMSO control MOG-G-CCM cells, lanes 7 and 13: 3-MC-treated MOG-G-CCM cells. (D) Ah receptor RT–PCR. Top panel: 6- and 12-hr time points. Bottom panel: 24- and 48-hr time points. Top panel: lanes 1 and 14: 100 bp molecular weight markers, lanes 2–7: 6-hr time point, lanes 8–13: 12-hr time point. Lanes 2 and 8: DMSO control HEPG2 cells, lanes 3 and 9: 3-MC-treated HEPG2 cells, lanes 4 and 10: DMSO control MCF7 cells, lanes 5 and 11: 3-MC-treated MCF7 cells, lanes 6 and 12: DMSO control MOG-G-CCM cells, lanes 7 and 13: 3-MC-treated MOG-G-CCM cells. Bottom panel: lanes 1 and 16: 100 bp molecular weight markers, lanes 2–7: 24-hr time point, lanes 8–13: 48-hr time point. Lanes 2 and 8: DMSO control HEPG2 cells, lanes 3 and 9: 3-MC-treated HEPG2 cells, lanes 4 and 10: DMSO control MCF7 cells, lanes 5 and 11: 3-MC-treated MCF7 cells, lanes 6 and 12: DMSO control MOG-G-CCM cells, lanes 7 and 13: 3-MC-treated MOG-G-CCM cells. Lane 14: positive control (human liver cDNA), lane 15: negative control (water).

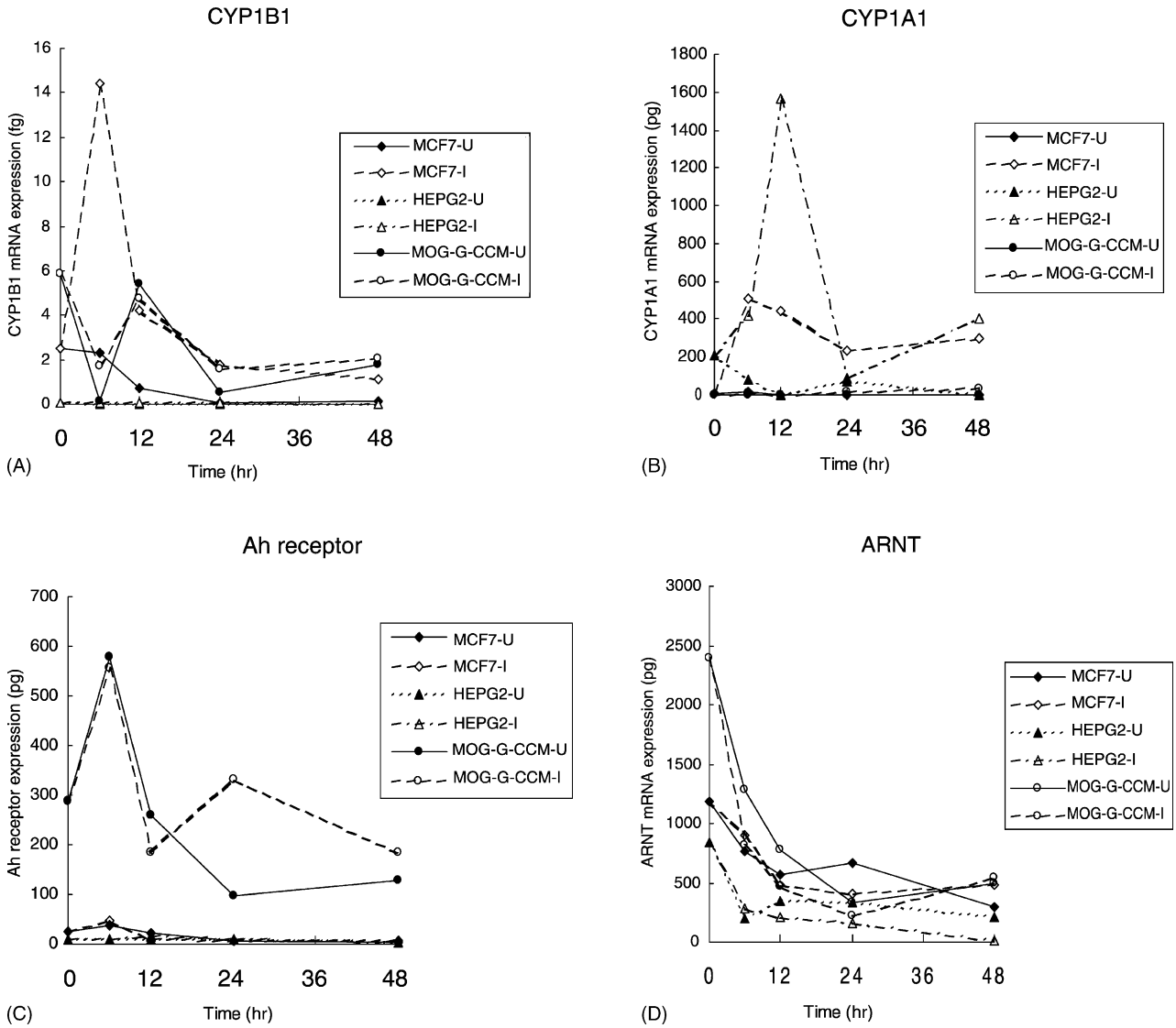

Fig. 2. Quantitative mRNA expression of components of the Ah receptor signalling pathway in three cell lines (MCF7, HEPG2 and MOG-G-CCM) exposed to either DMSO or 3-MC over 48-hr time period. Abbreviations: U = DMSO control, I = 3-MC-treated cells. Expression of CYP1B1 (A), CYP1A1 (B), Ah receptor (C) and ARNT (D) mRNA. Expression normalised against rRNA for all samples.

a small increase (3.4-fold) in Ah receptor expression at the 24-hr time point.

3.1.2. ARNT

Real-time quantitative RT–PCR demonstrated ARNT mRNA expression in the three cell lines examined at both the basal level and following exposure to 3-MC. Slightly higher levels of ARNT mRNA expression were observed in the MCF7 and MOG-G-CCM cell lines compared to the HEPG2 cell line (Table 4, Fig. 2D). No difference between ARNT expression was observed between the DMSO control and 3-MC-treated cell in any of the cell lines investigated (Fig. 3D).

3.1.3. CYP1A1

Qualitative RT–PCR demonstrated both basal and inducible CYP1A1 mRNA in the MCF7 and HEPG2 cells (Fig. 1C). CYP1A1 mRNA was detected in the MOG-G-CCM cell line following exposure to 3-MC for 24 and 48 hr (Fig. 1B).

In contrast to the qualitative RT–PCR, quantitative real-time RT–PCR demonstrated measurable basal levels of CYP1A1 mRNA in all three cell lines (Table 5). HEPG2 cell line demonstrated higher basal CYP1A1 mRNA expression than either the MCF7 or MOG-G-CCM cells, respectively (Fig. 2B). CYP1A1 expression in all three cell lines increased following exposure to 3-MC (Table 5). The strongest level of CYP1A1 mRNA expression following exposure to 3-MC was observed in the HEPG2 cell line 12 hr after exposure to 3-MC (Fig. 2B), a greater than 400-fold increase in CYP1A1 mRNA was observed in the cells exposed to 3-MC compared to DMSO (Fig. 3B). The MCF7 cell line demonstrated the greatest increase in the level of CYP1A1 mRNA expression following exposure to 3-MC compared to DMSO control (Fig. 3B). Although the MOG-G-CCM cell line demonstrated

Table 4
Expression of ARNT mRNA (picogram) normalised against rRNA

Time (hr)	MCF7 (DMSO)	MCF7 (3-MC)	HEPG2 (DMSO)	HEPG2 (3-MC)	MOG-G-CCM (DMSO)	MOG-G-CCM (3-MC)
0	1189.46	1189.46	838.86	838.86	2393.34	2393.338
6	762.34	899.74	200.41	281.13	1281.81	812.58
12	563.76	485.25	348.90	209.71	775.60	467.66
24	662.51	405.04	332.54	160.54	334.35	227.67
48	295.82	485.75	205.65	12.98	495.94	542.20

Table 5
Expression of CYP1A1 mRNA (picogram) normalised against rRNA

Time (hr)	MCF7 (DMSO)	MCF7 (3-MC)	HEPG2 (DMSO)	HEPG2 (3-MC)	MOG-G-CCM (DMSO)	MOG-G-CCM (3-MC)
0	4.87	4.87	207.25	207.25	1.87	1.87
6	15.60	511.58	83.30	416.92	0.40	9.65
12	1.11	440.50	3.51	1567.21	0.77	2.41
24	0.33	230.82	71.59	89.29	0.60	18.75
48	0.07	295.93	0.47	402.33	0.18	31.79

CYP1A1 mRNA expression at the zero time point, it was considerably lower (2.6- and 112-fold) than the basal expression in the MCF7 and HEPG2 cell lines, respectively (Table 5). Following 3-MC treatment in the MOG-G-CCM cell line, a slight increase in CYP1A1 mRNA expression was observed compared to DMSO control (Table 5, Fig. 3D).

3.1.4. CYP1B1

Qualitative RT–PCR demonstrated the presence of CYP1B1 mRNA in the MCF7 and MOG-G-CCM cells, which was visualised as a band at the correct size (680 bp) by agarose gel electrophoresis (Fig. 1A). No visible difference in intensity of the CYP1B1 band was observed either in the treated or untreated MCF7 and MOG-G-CCM cell lines. In the HEPG2 cell line, no detectable basal CYP1B1 mRNA expression was observed by qualitative RT–PCR; however, following exposure to 3-MC, CYP1B1 was observed at 12 and 24 hr (Fig. 1A). Comparison with real-time quantitative RT–PCR demonstrated measurable basal levels of CYP1B1 mRNA in all three cell lines (Table 6). The MCF7 and MOG-G-CCM cell lines expressed higher basal levels of CYP1B1 mRNA than the HEPG2 cell line (Table 6). In the MCF7 cell line the greatest level of CYP1B1 expression was observed following exposure to 3-MC for 6 hr (Fig. 2A), however, when expressed as a fraction of the DMSO control, the increase in CYP1B1 mRNA expression was greatest at 24 hr (Fig. 3A). No difference in CYP1B1 expression in the MOG-G-CCM cell line was observed when expressed as a fraction of the DMSO control (Fig. 3A).

3.1.5. Comparison of CYP1A1 and CYP1B1 mRNA expression

This study illustrated higher relative levels of CYP1A1 mRNA compared to CYP1B1 mRNA detected (Fig. 2) in all three cell lines at both the basal and induced levels (Fig. 2); CYP1A1 transcripts were expressed at picogram compared to femtogram levels for CYP1B1. In the MCF7 cells, CYP1A1 and CYP1B1 mRNA expression in the 3-MC-treated cells expressed as a fraction of the DMSO control showed maximal CYP1A1 expression at 48 hr with the greatest increase in CYP1B1 mRNA expression observed at 24 hr (Fig. 3).

The HEPG2 cell line exhibited the highest CYP1A1 and lowest CYP1B1 basal and induced levels of mRNA expression of the three cell lines examined (Table 6, Fig. 2). Measurable levels of both transcripts were observed by quantitative real-time RT–PCR at the zero time point. In contrast to the findings observed for the HEPG2 cell line, the MOG-G-CCM cells demonstrated the lowest absolute level of CYP1A1 of any of the three cell lines but displayed similar levels of CYP1B1 mRNA to that observed for the MCF7 cell line (Fig. 2).

3.1.6. Comparison of Ah receptor and P450 expression

Expression of the Ah receptor was greatest in the MOG-G-CCM cell line, this level of expression corresponded

Table 6
Expression of CYP1B1 mRNA (femtogram) normalised against rRNA

Time (hr)	MCF7 (DMSO)	MCF7 (3-MC)	HEPG2 (DMSO)	HEPG2 (3-MC)	MOG-G-CCM (DMSO)	MOG-G-CCM (3-MC)
0	2.49	2.49	0.09	0.09	5.83	5.83
6	2.32	14.39	0.03	0.04	0.136	1.74
12	0.76	4.23	0.00	0.009	5.43	4.76
24	0.06	1.80	0.00	0.04	0.50	1.61
48	0.10	1.15	0.00	0.006	1.75	2.04

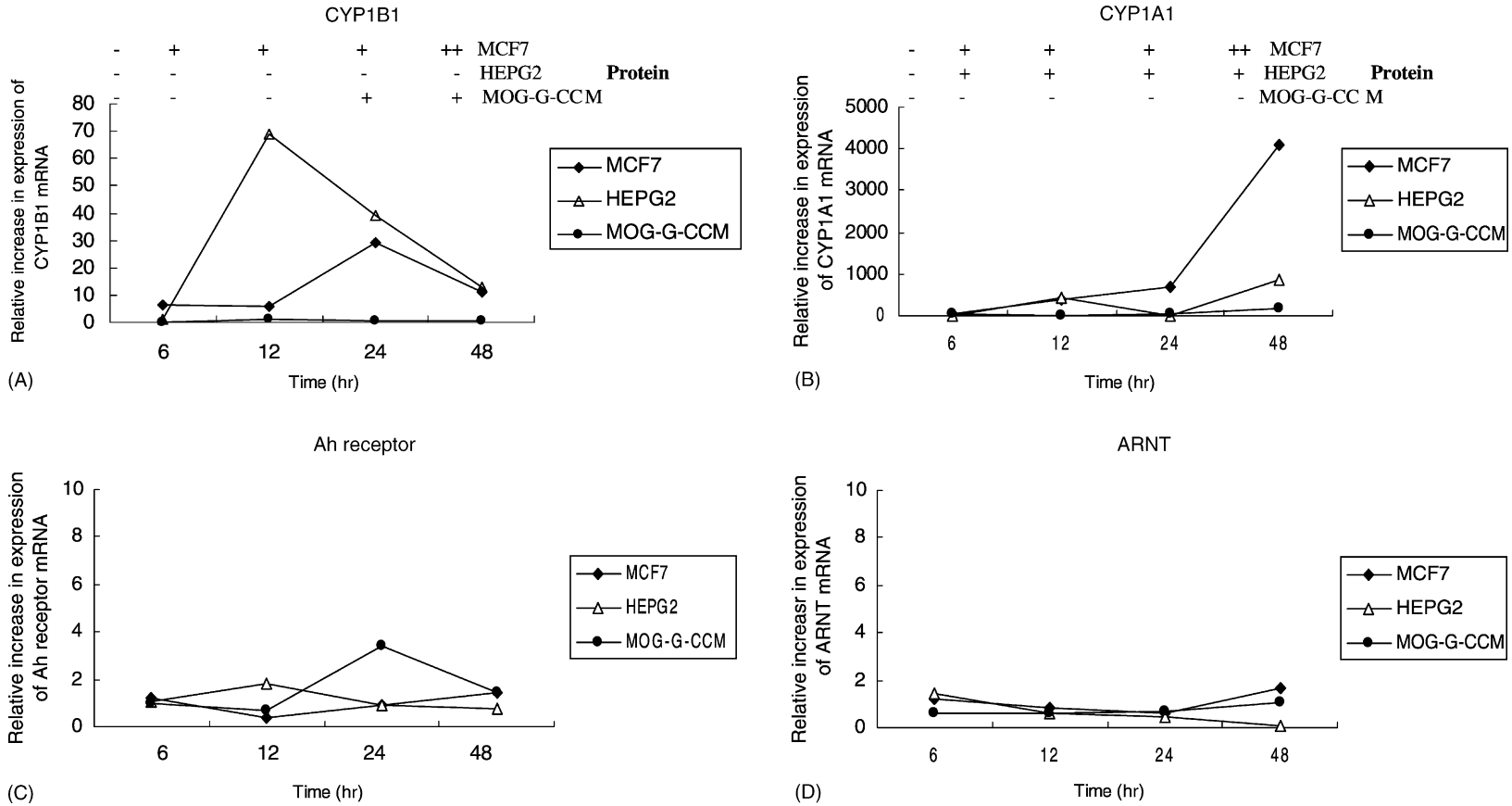

Fig. 3. Relative increase in mRNA expression of components of the Ah receptor signalling pathway in three cell lines (MCF7, HEPG2 and MOG-G-CCM) exposed to 3-MC over 48-hr time period expressed as a fraction of the DMSO control. Abbreviations: U = DMSO control, I = 3-MC-treated cells. Expression of CYP1B1 (A), CYP1A1 (B), Ah receptor (C) and ARNT (D) mRNA. Expression normalised against rRNA for all samples. Protein findings are displayed for CYP1A1 and CYP1B1: '−' indicates the absence of protein, '+' indicates the presence of the appropriate protein.

with the higher level of CYP1B1 mRNA observed in this cell line (Fig. 2). This result contrasted with that observed for both the MCF7 and HEPG2 cell lines which demonstrated more than 10-fold lower level of Ah receptor mRNA at the zero time point than the MOG-G-CCM cell line (Table 3, Fig. 2), but both the MCF7 and HEPG2 cell lines expressed greater basal CYP1A1 mRNA expression (2.6- and 110-fold, respectively) than the MOG-G-CCM cell line (Table 5, Fig. 2).

3.2. Protein expression

A sensitive enhanced chemiluminescent method was used in this study; immunoreactive protein was considered to be present if a band at the correct size was demonstrated. No constitutive expression of CYP1A1 protein was observed in any of the cell lines investigated. However, on exposure to 3-MC the presence of CYP1A1 protein was observed in both the HEPG2 and MCF7 cell lines (Fig. 3). The presence of CYP1A1 protein in the HEPG2 cell line remained constant over the 48-hr time period following exposure to 3-MC (Fig. 3). In contrast, in the MCF7 cell line the level of CYP1A1 protein expression increased over the time course with the strongest product observed at the 48-hr time point (Fig. 3).

The presence of CYP1B1 protein was only demonstrated in the MCF7 and MOG-G-CCM cell lines following exposure to 3-MC (Fig. 3). CYP1B1 protein was detectable in the MCF7 cell line as a faint product at 6 hr after exposure to 3-MC (Fig. 3) with an increase in intensity of the protein band observed over the 48-hr time course. The MOG-G-CCM cells in contrast, demonstrated the presence of CYP1B1 protein only following 24 hr exposure to 3-MC [3] as with the MCF7 cells an increase in intensity of the protein band was observed at the 48-hr time point.

4. Discussion

Three cell lines, which differentially express basal and induced CYP1A1 and CYP1B1, were used in this study to quantitatively analyse the importance of components of the Ah receptor/CYP pathway, in the expression of CYP1A1 and CYP1B1 in human cells and tissues. A number of previous studies have investigated the presence of CYP1A1 and CYP1B1 mRNA in normal tissue and extrapolated this finding to suggest that the protein is also present. In this study, the use of quantitative real-time RT–PCR, in conjunction with a sensitive immunoblotting technique, has enabled the quantifiable measurement of basal and induced CYP1A1 and CYP1B1 mRNA to be compared with the level of expressed protein.

Our previous investigations by qualitative PCR and Western blotting have demonstrated differential expression of CYP1A1 and CYP1B1 in normal and tumour cells; for example, in human breast cancers CYP1B1 mRNA and protein displayed consistent constitutive expression whereas CYP1A1 was only detected in 25% of samples [27]. A similar pattern of expression has also been shown in kidney tumour samples [10]. CYP1B1 appears to be a tumour-specific protein constitutively expressed in a variety of human tumours [13,15,18] but absent in normal tissue although CYP1B1 mRNA is present. As CYP1B1 shows enhanced expression in tumour cells [13], this P450 is rapidly being established as an important target in cancer research [30]. Therefore, it is important to have a greater understanding of the factors regulating the distinct cell-type-specific expression observed for both CYP1A1 and CYP1B1.

Previous studies have shown low constitutive expression of CYP1B1 mRNA and immunoreactive protein, but not CYP1A1 mRNA or protein in untreated MCF7 cells [14]. Immunoreactive CYP1A1 and CYP1B1 proteins have been identified following exposure to TCDD in MCF7 cells [31]. Generally, in untreated HEPG2 cells neither CYP1A1 nor CYP1B1 mRNA is believed to show constitutive expression [16], but following exposure to TCDD, CYP1A1 (mRNA and protein) but not CYP1B1, are highly induced [16]. Low levels of constitutive CYP1B1 mRNA have been shown in the untreated MOG-G-CCM cell line [32]; the presence of immunoreactive protein has only been reported following exposure to the Ah receptor agonist dimethyl benzanthracene [28]. The presence of CYP1A1 mRNA or protein at a basal or induced level in the MOG-G-CCM cell line has not previously been studied.

Studies using qualitative and semi-quantitative RT–PCR have not been helpful with regards to the presence and amount of CYP1B1 mRNA in HEPG2 cells [33,34]. In this current study, the low level of CYP1B1 mRNA and the lack of CYP1B1 protein in the HEPG2 cell line following exposure to the Ah agonist 3-MC suggests that in certain cells a threshold of mRNA expression must be obtained before the protein is expressed. Indeed, although the basal levels of CYP1B1 were higher in the MCF7 and MOG-G-CCM cell lines, it was only following exposure to 3-MC that detectable CYP1B1 protein was observed in these cells. There was no marked difference in CYP1B1 mRNA expression between the untreated and 3-MC-treated MOG-G-CCM cells. The induction of CYP1B1 protein following exposure to 3-MC suggests the presence of cell-type-specific post-transcriptional factors in the MOG-G-CCM cell line. Studies with cultured mouse embryo fibroblasts suggest substrate stabilisation of the CYP1B1 protein may contribute to its regulation [2]. It is not yet known whether a similar mechanism of CYP1B1 regulation exists in humans. The differences observed between the levels of CYP1B1 in the HEPG2 compared to the other two cell lines suggest the involvement of both transcriptional and post-transcriptional factors. Wo *et al.* have previously demonstrated positive and negative regulatory elements in the upstream region of the 5′-end of the *CYP1B1* gene

[35]. This region contains both basal regulatory and dioxin-responsive elements. Thus, a combination of transcriptional, post-transcriptional and cell-type-specific factors may be required which regulate the differential expression of CYP1B1 protein in distinct cell types. Stabilisation of the CYP1B1 protein could provide an answer to the apparently contradictory results of CYP1B1 mRNA and protein in human tissues and tumours [7,13,15,36]. Our findings suggest that the MOG-G-CCM cell line may be an appropriate model to study the mechanisms regulating CYP1B1 expression in human tissues.

CYP1A1 is not constitutively expressed in human tissues but CYP1A1 mRNA and protein have been shown to be induced in liver and lung [37,38]. In this study, the use of quantitative RT–PCR provided an accurate mechanism for quantifying the presence of native and induced levels of CYP1A1. This approach demonstrated low levels of CYP1A1 in the MOG-G-CCM cell line even on exposure to 3-MC. The low level of CYP1A1 mRNA detectable in the MOG-G-CCM cell line highlights similar cell-type-specific factors, which may be controlling the expression of CYP1B1 mRNA in the HEPG2 cell line [16]. Although the negative regulatory elements within the CYP1B1 promoter are distinct from those found in the CYP1A1 promoter [18,35], both may require cell-type-specific factors to bind in order to control transcriptional processing of these P450s in human tissues and cells [16].

In addition to quantitative analysis of the effector molecules of the activation pathway, the receptor molecules, namely, the Ah receptor and the ligand-activated transcription factor ARNT, were also assessed by quantitative real-time RT–PCR. However, as this study was centred upon the effector molecules rather than the ligand dimerisation partners, the levels of protein expression of the Ah receptor and ARNT were not investigated. High-affinity TCDD binding studies have suggested that the level of the Ah receptor can be a rate-limiting factor in the induction of CYP1A1.

Our findings suggest that the Ah receptor may be differentially expressed in the cell lines examined in this study with the MOG-G-CCM cell line exhibiting a higher constitutive and induced level of the Ah receptor than either the MCF7 or HEPG2 cell lines. Although the amount of Ah receptor was not a limiting factor for CYP1B1 expression in this cell line, it may be less sensitive to the inducing ligand than the Ah receptor in the other two cell lines. Moreover, the tissue-specific factors regulating transcriptional processing in the MOG-G-CCM cells may be different from those found in the MCF7 cell line.

Our current findings agrees with the findings from Okey's laboratory where they found that Ah receptor mRNA levels were not altered by TCDD exposure in either the Hepa-1 hepatoma or human colon carcinoma cell line LS180 [39].

Although there is established species-specific mechanisms of P450 regulation, other non-Ah receptor-mediated mechanisms may also be involved in CYP1B1 regulation. For instance, in both the mouse and the rat CYP1B1 is also under the regulatory control of cAMP [15]. Indeed, in Ah knockout mice, Ryu *et al*. have shown weak induction of CYP1B1 mRNA but not CYP1A1 by piperonyl butoxide [40]; again it is not known whether such a mechanism exists in human tissues.

Quantitative real-time RT–PCR in this study provided a tool to distinguish between traces of mRNA, which results in protein expression. Our findings have shown that CYP1A1 and CYP1B1 mRNA and protein expression are not necessarily linked and that there are likely to be other regulatory mechanisms controlling expression of CYP1A1 and CYP1B1 mRNA and protein in specific cell types. In addition, the sensitivity of the Ah receptor may play a role in the transcriptional up-regulation of the P450 enzymes in specific cell types.

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