Metabolic characterization of cell systems used in *in vitro* toxicology testing: Lung cell system BEAS-2B as a working example

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ABSTRACT

The bioactivation of pro-toxicants is the biological process through which some chemicals are metabolized into reactive metabolites. Therefore, *in vitro* toxicological evaluation should ideally be conducted in cell systems retaining adequate metabolic competency and relevant to the route of exposure. The respiratory tract is the primary route of exposure to inhaled pro-toxicants and lung-derived BEAS-2B cell line has been considered as a potentially suitable model for *in vitro* toxicology testing. However, its metabolic activity has not been characterized.

We performed a gene expression analysis for 41 metabolism-related genes and compared the profile with liver- and lung-derived cell lines (HepaRG, HepG2 and A549). To confirm that mRNA expression was associated with the corresponding enzyme activity, we used a series of metabolic substrates of CYPs (CYP1A1/1B1, CYP1A2, CYP2A6/2A13 and CYP2E1) known to bioactivate inhaled pro-toxicants. CYP activities were compared between BEAS-2B, HepaRG, HepG2, and A549 cells and published literature on primary bronchial epithelium cells (HBEC).

We found that in contrast to HBEC, BEAS-2B and A549 have limited CYP activity which was in agreement with their CYP gene expression profile. Control cell lines such as HepG2 and HepaRG were metabolically active for the tested CYPs. We recommend that similar strategies can be used to select suitable cell systems in the context of pro-toxicant assessment.

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1. Introduction

The need for *in vitro* cell systems as alternatives to animal models for toxicological testing is increasing in response to new regulations, such as the EU 7th Amendment Directive (European Commission, 2003), and to ethical considerations like the 3Rs principle (Schechtman, 2002). Due to their relative homogeneity and ability to be maintained in culture indefinitely, established cell lines have been one of the preferred cell systems employed in the development and validation of *in vitro* toxicology assays. Most continuous cell lines, however, have been derived from malignant or transformed tissue and fail to replicate the physiology and morphology of normal cells. Historically, hepatic cell lines have been thoroughly characterized, as they are the prime systems used for drug metabolism and toxicity testing in pre-clinical development

(Guguen-Guillouzo and Guillouzo, 2010; Brandon et al., 2003). For instance, the hepatoma cell line HepG2 lacks normal metabolic activity and has been engineered to express hepatic cytochrome P450 (CYP) enzymes (CYP3A4, CYP2E1) to study *in vitro* drug hepatotoxicity caused by compounds such as paracetamol (Yoshitomi et al., 2001). CYP3A4 and CYP2E1 catalyze the transformation of paracetamol into a highly reactive metabolite responsible for the tissue specific toxicity of the drug. This process of metabolic transformation of a chemical (pro-toxicant) into a toxic species is defined as bioactivation and is a key element in many toxicity studies. In contrast, many other cell lines used in toxicology, and in particular non-hepatic cells, have not been extensively characterized for their metabolic competency. The deficiencies in the metabolic capabilities of cell lines could lead to inaccurate evaluation of test compounds (Kirkland et al., 2007). This is the case for benzo[a]pyrene (B[a]P), a well-known tobacco smoke chemical that is ultimately metabolized to a diol-epoxide carcinogen by the inducible lung CYPs, CYP1A1/1B1. The formation of B[a]P DNA adducts has been reported *in vitro* using lung carcinoma-derived A549 cells (Feldman et al., 1978) but the role of CYP1A1/1B1 in the formation of such adducts in A549 was not demonstrated at the time. In 2000, Hukkanen and colleagues reported the expression and inducibility of CYP1A1/1B1 in the A549 cell line

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but activity was not verified. In 2008, Quinn established that CYP1A1 was not required in A549 for the oxidation of B[a]P to its reactive form and that this reaction could be catalyzed by AKR1B10 (Quinn et al., 2008). However CYP1A1 activity was reported the same year by EROD assay after A549 induction (Billet et al., 2008). In contrast, in a comparison between CYP1A1/1B1 activity in A549 and HBECS Newland et al. showed that the CYP1A1 activity in A549 was limited when compared to a culture of human primary lung epithelial cells when incubated with a luminogenic probe substrate. Thus the mechanism of adduct formation in A549 can potentially follow multiple metabolic routes different than what would be expected in a normal lung epithelium. CYP2B6 activity has also been reported in A549 together with mRNA expression of CYP2D6, 2E1, 3A5, and CYP3A7, the latest is not expected to be present in normal adult tissue. Other key lung epithelium CYPs such as CYP2A6, CYP2A13, and CYP2F1 involved in the bioactivation of toxicants such as nitrosamines were not detected in this cell line. This example highlights the importance of characterizing the metabolic enzyme profile in cell lines used for toxicological evaluation, with the possibility to restrict such study to enzymes relevant to the metabolic pathway of specific toxicants. However, to date, there is no standard approach to metabolic characterization. Where some researchers focus on gene expression only (Jennen et al., 2010) others may combine gene expression with enzyme activity (Westerink and Schoonen, 2007).

The aim of our investigation was to describe an experimental strategy combining quantitative real time PCR (qPCR) and functional enzymatic assays applied to the lung-derived BEAS-2B cell line. Initially, we profiled the gene expression of a panel of oxidative and conjugative metabolism-related genes involved in xenobiotics metabolism, more specifically related to the toxicity of cigarette smoke to human lung (Hecht, 2006). Next, we used a series of metabolic substrates and inhibitors to test the enzyme activity of key CYP enzymes. The selection was made taking into account the relevance of the target organ and the nature of the test article. The human lung-derived BEAS-2B cell line was first described in 1988, when normal bronchial epithelial cells obtained from autopsy of non-cancerous individuals were isolated, then infected with a replication-defective 12-SV40/adenovirus hybrid and cloned to create an immortalized phenotype (Reddel et al., 1988). The non-cancerous phenotype of BEAS-2B cells is an advantage in the investigation of carcinogenic processes such as DNA damage and cell transformation (Sun et al., 2011). Therefore, BEAS-2B cells have been considered as a relevant cell line for in vitro toxicology testing in the field of pollutants, tobacco products and nanomaterials (Persoz et al., 2012; Veljkovic et al., 2011; Haniu et al., 2011). Although, several studies have employed BEAS-2B cells to evaluate the effect of some pro-toxicants such as B[a]P and 4-(N-methylnitrosamino)-1-(3-pyridinyl)-1-butanone (NNK), the metabolic capacity of this particular cell line has not been fully characterized (Ovrevik et al., 2010; Proulx et al., 2005).

Here, BEAS-2B cells were tested and compared to the lung-derived A549 cells broadly used as pulmonary in vitro system but with no cytochrome P450 expression reported for CYP1A2 and the CYP2A family, and inducibility documented for CYP1A1/1B1 genes (Hukkanen et al., 2002; Castell et al., 2005). Cells derived from hepatocarcinomas considered to have a more extensive cytochrome P450 enzyme activity (HepG2 and HepaRG) were used as a more comprehensive control for our CYP assays (Jennen et al., 2010). Moreover, the results were contrasted to those reported in primary human bronchial epithelium culture (Newland et al., 2011; Courcot et al., 2012). The results of this study are considered to be useful for in vitro toxicological testing using the cell line BEAS-2B as cell system. Furthermore, we propose that the outlined strategy can be incorporated in the characterization of cell systems used in in vitro testing.

2. Materials and methods

2.1. Cell culture

The human bronchial epithelial cell line (BEAS-2B), purchased from ATCC (United States), was seeded into culture vessels that had been pre-coated with 0.03 mg/mL PureCol® bovine collagen solution (Nutacon, The Netherlands). Cells were maintained in Bronchial Epithelial Growth Medium (BEGM®) at 37 °C and 5% CO_2 in a humidified incubator. BEGM® was prepared by supplementing Bronchial Epithelial Basal Medium with growth supplements provided in the manufacturer's BEGM® SingleQuot® kit (Lonza Group Ltd., Belgium) containing: bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin-B and retinoic acid.

The human type II alveolar adenocarcinoma-derived (A549) and hepatocarcinoma-derived (HepG2) cell lines purchased from the Global Bioresource Centre (ATCC, USA), were maintained in Dulbecco's modified Eagle medium supplemented with fetal bovine serum (10% v/v), L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 µg/mL) (complete DMEM) at 37 °C and 5% CO_2 in a humidified incubator.

The human hepatocarcinoma-derived cell line (HepaRG) was purchased as a differentiated confluent monolayer from Biopredic International (France). After shipment, the cells were maintained in basal medium supplemented with recovery mix for 24 h followed by basal medium supplemented with maintenance/metabolism mix. Media and supplements were provided by the manufacturer (Biopredic, France).

BEAS-2B, A549 and HepG2 cells were cultured and expanded in-house. Experiments were performed between passages 3 and 12 only. All cultures were negative for mycoplasma. Additionally, the cells were authenticated using the short tandem repeat profiling to confirm the nature of the cell cultures (LGC Standards, United Kingdom) (Nims et al., 2010).

2.2. Gene expression assay

BEAS-2B, A549 and HepG2 cells were plated in 12-well tissue culture plates, at 60% confluency. A total of 6 wells per plate were treated for 48 h with 10 nM of the CYP1A1/1B1 inducer 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). HepaRG cells were not used as positive control cell line for CYP1A1/1B1, therefore, they were not pre-induced with TCDD.

After 96 h from seeding, total RNA was isolated from the cells using the RNeasy mini kit (Quiagen, United Kingdom). The RNA quantity was measured by using the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, USA) and the quality assessed with the Agilent 2100 Bioanalyzer (Agilent, United Kingdom).

The RNA was converted to cDNA using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, United Kingdom). qPCR was carried out using custom TaqMan® array 96-well plates and TaqMan® Fast Universal Master mix (Applied Biosystem, United Kingdom). Each plate contained two assays with the probes of 1 manufacturing control, 5 endogenous controls and 41 metabolism-related genes from both phase I and phase II (Table 1). qPCR amplification mixtures (20 µL) contained 2 µL of cDNA and 18 µL of fast master mix and were amplified using the fast PCR 7500 (Applied Biosystems, United Kingdom). The cycle conditions comprised 2 min at 50 °C, 20 s at 95 °C, then 40 cycles of 3 s at 95 °C and 30 s at 60 °C.

Table 1
Panel of genes classified by phase of metabolism and function. Standard gene nomenclature taken from (HGNC, 2012).

Controls		Phase I			
Manufacturing Control	Endogenous control	Cytochrome P450		Aldo–keto reductases	Others
RN18S1	GAPDH	CYP1A1	CYP3A4	AKR1B10	EPHX1
	HPRT1	CYP1B1	CYP3A5	AKR1C1	CBR1
	GUSB	CYP1A2	CYP2C9	AKR1C2	DCXR
	B2 M	CYP2A6	CYP2D6	AKR1C3	AOX1
	RPLP0	CYP2A13	CYP2E1	AKR7A3	FMO3
		CYP2B6	CYP2F1		HSD11B1
		CYP2B7P1			NQO1

Phase II		
Glutathione S-transferases	UDP-glucuronosyltransferase	Others
GSTA1	UGT1A4	SULT1A1
GSTM1	UGT1A6	NAT1
GSTP1	UGT1A7	COMT
GSTT1	UGT1A1	
	UGT1A8	
	UGT1A9	
	UGT1A10	
	UGT2B7	
	UGT2B10	

Threshold cycle (Ct) values for the genes were normalized to RPLP0, and relative expression levels were calculated using the $\Delta\Delta$Ct method (Livak and Schmittgen, 2001).

2.3. Cytochrome P450 enzyme activity assays

Range finder experiments were initially carried out to select optimal concentrations for substrates, inducer and inhibitors where maximal activity, induction or inhibition were obtained without cytotoxicity.

For the enzyme activity profiling, phenol free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.5 mM L-Glutamine was used as a basal experimental medium.

CYP1A1/1B1 activity was measured using the specific P450-Glo™ kit (Promega, United Kingdom). The probe used was Luciferin 6′ chloroethyl ether (luciferin-CEE) (50 µM) added directly to the basal medium (0 and 4 h incubation) of 3 wells per condition. For induced conditions, cultures were pre-incubated for 48 h with 10 nM of TCDD. For inhibited conditions, α-naphthoflavone (10 µM) was added to the basal medium 30 min prior to the probe. After the incubation, the luminescence was measured using a LMaxII® luminometer (Molecular Devices, United States). HepG2 cells were used as 'positive control'.

The measurement of CYP2A6/2A13 activity was based on the methodology described previously (Newland et al., 2011). The same methodology was adapted, including probes and inhibitors, for the measurement of CYP1A2 and CYP2E1 activities. A549 cells were used as 'negative control' for CYP2A6/2A13 and CYP1A2, the status of CYP2E1 activity is unknown in A549. HepaRG cells were used as a positive control for CYP1A2 and CYP2A6/2A13. HepG2 cells were used as 'positive control' for CYP2E1.

For the CYP1A2 activity assay, 7-ethoxyresorufin (20 µM) was used as a probe and fluvoxamine (100 µM) was used as inhibitor. The metabolite quantified was resorufin.

In the case of the CYP2A6/2A13 activity assay, coumarin (200 µM) was used as a probe and 8-methoxypsoralen (8-MOP) (100 µM) as inhibitor. The metabolite measured was 7-hydroxycoumarin.

Finally, the CYP2E1 activity assay used chlorzoxazone (100 µM) as probe and disulfiram (20 µM) as inhibitor. 6-hydroxychlorzoxazone was the metabolite quantified.

After the probe incubations, 250 µL of basal medium was adjusted to pH 5.0 with hydrochloric acid and treated with 2.5 µL of β-glucuronidase from *Helix pomatia* for 18 h at 37 °C while shaking. Once the glucuronidase treatment finished, 250 µL of methanol and the internal standard 4-methylumbelliferon (5 µM) was added to the solution. The metabolites where then quantified using an UPLC-AB SCIEX/API 4000 Q-Trap® mass spectrometer using the column Phenomenex Kinetex 2.6 µm PFP, 100 Å (Applied Biosystems, United States).

Once all basal medium was removed, cells were lysed using Mammalian Protein Extraction Reagent (M-PER) lysate buffer (Thermo Fisher Scientific Inc., United Kingdom) and protein content was measured employing the bicinchoninic acid protein assay (BSA) together with a Multiskan Ascent® spectrophotometer (Thermo Fisher Scientific Inc., United Kingdom).

Lactate dehydrogenase (LDH) release was used as a measure of cytotoxicity during the enzyme activity assays. The CytoTox-ONE® homogeneous membrane integrity assay (Promega, United Kingdom) was used following manufacture recommendations and analyzed with a Fluoroskan Ascent® fluorometer (Thermo Fisher Scientific Inc., United Kingdom). The percentage LDH release is inversely proportional to the cell viability which was >85% for all treatments and timepoints.

2.4. Gene expression data analysis

After completion of the qPCR, the threshold cycle (Ct) values were visually inspected using the fast PCR 7500 software v.2.0.5. When required, the threshold setting default (0.2) was manually adjusted to ensure optimal sensitivity was maintained. All Ct > 36, indicative of the plateau phase of qPCR, were considered non-expressed genes.

The Ct values were then normalized against the selected endogenous control gene to generate ΔCt values ($Ct_{gene\ of\ interest} - Ct_{endogenous\ control\ gene}$).

2.5. Statistical analysis

All the experiments were repeated three times containing three replicates per condition and timepoint.

GeneSpring™ GX11.5.1 (Agilent, United Kingdom) was used to perform the gene expression graphical and statistical analysis. Principal Component Analysis (PCA) and hierarchical clustering were selected for graphical representations. For the hierarchical

clustering algorithm, Euclidean distance measured with average linkage was selected for interpretation of the normalized gene expression data (ΔCt). One-way ANOVA was used to analyze the effect of the TCDD induction on the expression of each gene.

The enzyme activity data are represented by the arithmetic mean of three experiments + standard deviation (SD). Minitab v.16 was used to perform Student's t-test. Difference was significant when $p < 0.05$.

3. Results

3.1. Gene expression

The first stage in the metabolic characterization was to quantify the mRNAs of a panel of enzyme-encoding genes involved in oxidative (phase I) and conjugative (phase II) metabolism.

The endogenous control gene RPLP0 showed the most stable expression across the different samples and treatments (data not shown). Furthermore, RPLP0 has been reported as being highly conserved across tissues and species (Akamine et al., 2007). Therefore, RPLP0 was chosen for normalization of data, generating ΔCt values ($Ct_{gene\ of\ interest} - Ct_{RPLP0}$).

PCA was used to visualize the dataset in a 3D scatter plot graph shown in Fig. 1. This analysis demonstrated the segregation of cell lines based on their gene expression profile. The graph shows a clear separation of the different cell lines (represented by colors) indicating that the expression profile differs from cell line to cell line. In addition, only HepG2 cells show a variation between the induced (triangle shaped icon) and non-induced samples (rectangle shaped icon), while there is no apparent separation of the induced from the non-induced BEAS-2B and A549 samples. HepaRG cells were not induced so Fig. 1 only represents the basal gene expression levels.

The hierarchical cluster shown in Fig. 2 was generated to visualize the gene expression and induction profiles of each individual cell line. This graphical representation contained the expression value for each individual gene normalized (ΔCt values); red, blue and yellow indicate increased (positive ΔCt), reduced (negative ΔCt) and undetectable (ΔCt close to or 0), respectively. The details contained in the hierarchical cluster allowed a gene by gene comparison between induced and non-induced treatments but also, between different cell lines. This cluster analysis confirms the observations made above by PCA. Gene expression profiles in

BEAS-2B and A549 cells do not show a significant difference between induced and non-induced samples. In contrast, the HepG2 profile shows some changes between induced and non-induced samples. However, there are many genes that are not differentially expressed. HepaRG cells show a high expression in the majority of the tested genes.

To allow fine observations between TCDD-induced and non-induced samples, $\Delta\Delta$Ct data representing fold-changes in gene expression for BEAS-2B, A549 and HepG2 are detailed in Table 2.

As expected, CYP1A1/1B1 were inducible across the three cell lines. In BEAS-2B cells, CYP1A2 also showed a degree of inducibility. However, no other gene studied in BEAS-2B cells shows a relevant up- or down-regulation.

3.2. Enzyme activity

The enzymatic activities of four cytochrome P450s enzymes involved in the oxidative metabolism of smoke toxicants were further evaluated in BEAS-2B, HepG2, HepaRG, and A549 cells to complement the gene expression data.

Data represent the rate of metabolite formation in pmol/mg protein/min, normalized to soluble protein, except for CYP1A1/1B1 where the metabolite is represented as a measure of luminescence (RLU). Each experiment included data for the cell line intended for characterization (BEAS-2B), A549 and the 'positive control' cell line (Hep-G2 or HepaRG).

Results in Fig. 3A represent CYP1A1/1B1 enzyme activity. In the absence of TCDD, only background activity was detected for BEAS-2B (0.0470 RLU/mg/min ±0.0082). In TCDD-induced BEAS-2B, the activity levels increased 3.7-fold compared to non-induced cells (0.1740 RLU/mg/min ±0.0317) and were inhibited in the presence of the CYP1A1/1B1 inhibitor α-naphthoflavone. The activity increase in TCDD-treated cells was statistically significant with a p value < 0.0001 and was consistent with the CYP1A1/1B1 mRNA induction observed in our gene expression data.

HepG2 cells gave a high level of enzyme activity as expected from the positive control cell line following induction with TCDD. In contrast, A549 cells produced only background activity both in the presence and absence of the inducer TCDD (0.0284 and 0.0121 RLU/mg/min respectively).

The results observed for CYP2E1 enzyme activity (Fig. 3B) showed no statistically significant difference in the levels of enzyme activity between BEAS-2B or A549 cultures treated in the absence or presence of inhibitor disulfiram ($p = 0.793$ and $p = 0.222$
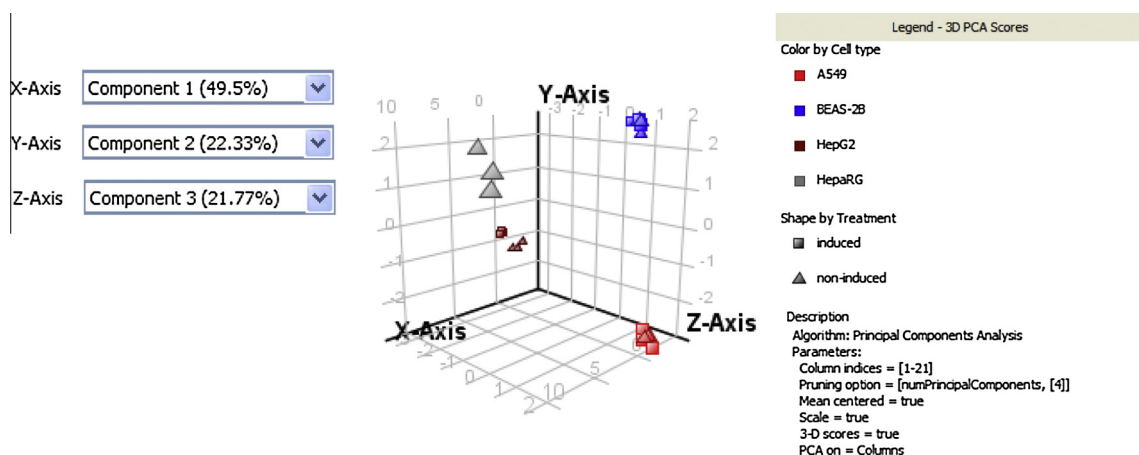

Fig. 1. 3D scatter plot representing principal component analysis (PCA) of qPCR data. Cell type is represented by color – A549 (red), BEAS-2B (blue), HepG2 (maroon) and HepaRG (grey). Shaped icons represent induction status, triangle (▲) represents induced cultures and square (■) represents non-induced cultures. (Note: HepaRG gene expression was only measured in non-induced cultures).
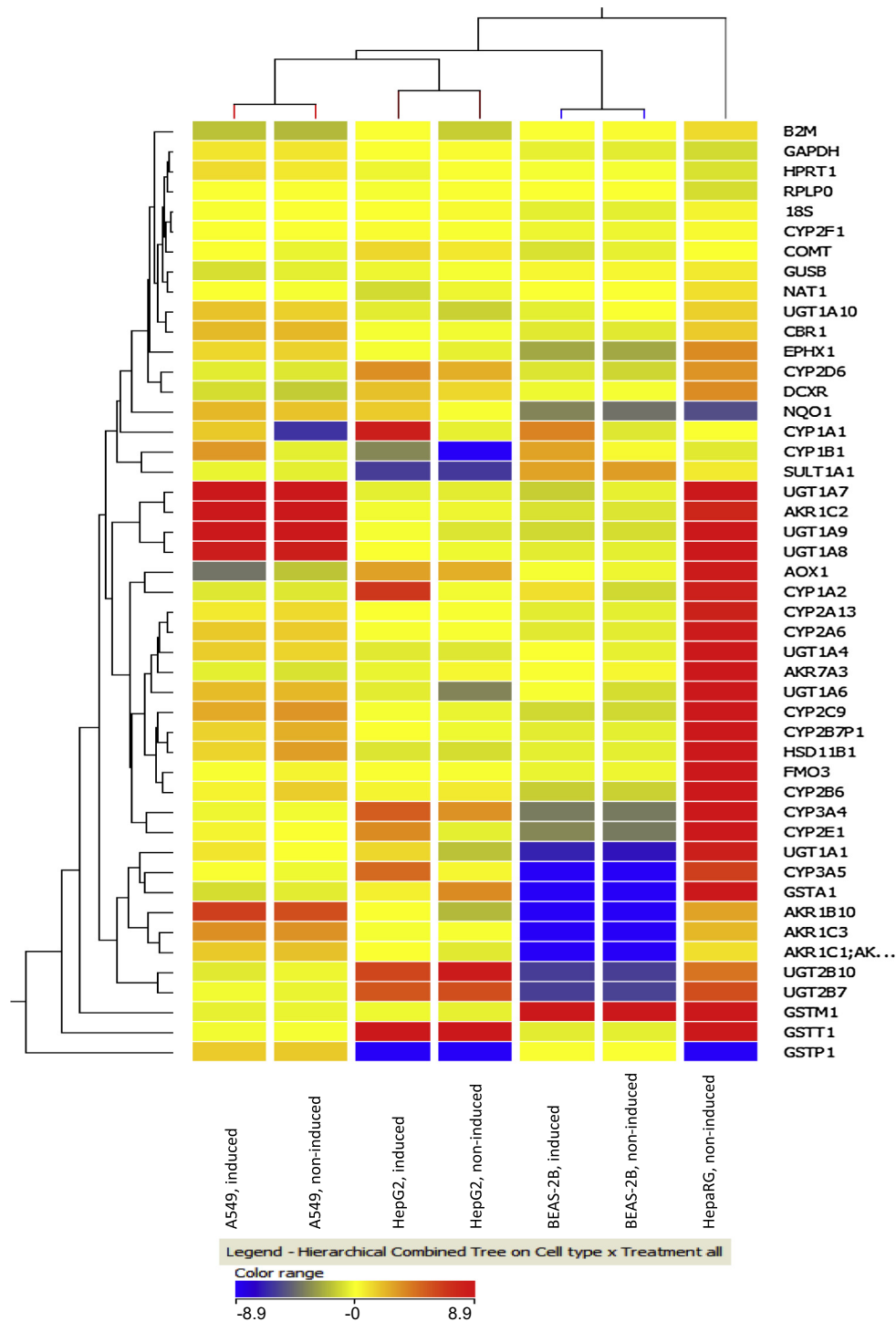
Fig. 2. Hierarchical cluster representing the gene expression profiles of all four cell lines dependent on treatment (induced or non-induced). (*Note*: HepaRG gene expression was only measured in non-induced cultures). Columns represent individual samples and rows represent genes. Red, blue and yellow indicate high signal intensity, low signal intensity or no signal in normalized gene expression data (ΔCt), respectively.

respectively). The positive control cell line (HepG2), on the other hand, showed a significant reduction of enzyme activity in the presence of inhibitor ($p = 0.022$).

CYP2A6/2A13 oxidizes coumarin to 7-hydroxycoumarin. The results presented in Fig. 3C showed no statistically significant difference ($p = 0.741$) in BEAS-2B CYP2A6/2A13 activity in the presence and absence of inhibitor 8-MOP. A similar profile was observed for A549 cells. These results are in agreement with the

lack of CYP2A6/2A13 mRNA expression (Ct > 36). HepaRG cells (positive control cell line) showed a reduction in enzyme activity in the presence of inhibitor ($p < 0.0001$).

Fig. 3D represents the enzyme activity levels measured for CYP1A2. The results showed that the levels of activity detected in BEAS-2B cells were equivalent to those observed when the CYP1A2 inhibitor fluvoxamine was present in the cultures. These results concurred with the results obtained for CYP1A2 gene expression

Table 2
Gene expression data represented as fold change comparing TCDD-induced with non-induced cells. In red: genes with at least 2-fold increase in gene expression after induction (up-regulated). In blue: genes with at least 2-fold decrease in gene expression after induction (down-regulated).

Gene ID	BEAS-2B	A549	HepG2
Phase I			
CYP1A1	25.35 ↑	256.99 ↑	389.97 ↑
CYP1B1	5.79 ↑	13.69 ↑	154.52 ↑
CYP1A2	4.47 ↑	1.02 ↑	124.64 ↑
CYP2A6	−1.06 ↓	1.05 ↑	−1.02 ↓
CYP2A13	−1.05 ↓	−1.42 ↓	1.02 ↑
CYP2B6	−1.05 ↓	−2.11 ↓	−1.22 ↓
CYP2B7P1	−1.05 ↓	−2.11 ↓	1.18 ↑
CYP3A4	1.02 ↑	−1.11 ↓	3.60 ↑
CYP3A5	−1.62 ↓	1.36 ↑	20.43 ↑
CYP2C9	1.02 ↑	−1.52 ↓	1.23 ↑
CYP2D6	1.28 ↑	1.14 ↑	1.96 ↑
CYP2E1	1.37 ↑	1.32 ↑	18.18 ↑
CYP2F1	−1.05 ↓	−1.06 ↓	1.18 ↑
AKR1B10	1.25 ↑	1.55 ↑	4.33 ↑
AKR1C1;AKR1C2	1.21 ↑	−1.14 ↓	1.67 ↑
AKR1C2	−1.05 ↓	1.44 ↑	1.10 ↑
AKR1C3	1.63 ↑	1.04 ↑	−1.04 ↓
AKR7A3	−1.15 ↓	1.28 ↑	−1.72 ↓
EPHX1	−1.01 ↓	−1.10 ↓	1.29 ↑
CBR1	−1.02 ↓	−1.05 ↓	1.017 ↑
DCXR	−1.11 ↓	1.43 ↑	1.54 ↑
AOX1	1.18 ↑	−5.51 ↓	1.34 ↑
FMO3	−1.05 ↓	−1.35 ↓	1.18 ↑
HSD11B1	1.02 ↑	−3.08 ↓	1.18 ↑
NQO1	1.54 ↑	1.30 ↑	3.30 ↑
Phase II			
GSTA1	−1.05 ↓	−1.35 ↓	−8.38 ↓
GSTM1	−1.09 ↓	−1.06 ↓	1.18 ↑
GSTP1	1.03 ↑	−1.02 ↓	87.66 ↑
GSTT1	−1.05 ↓	−1.06 ↓	1.71 ↑
UGT1A4	1.41 ↑	1.13 ↑	1.05 ↑
UGT1A6	1.94 ↑	−1.15 ↓	8.17 ↑
UGT1A7	−1.93 ↓	−1.34 ↓	1.05 ↑
UGT1A1	1.29 ↑	1.82 ↑	8.51 ↑
UGT1A8	−1.05 ↓	1.04 ↑	1.18 ↑
UGT1A9	−1.12 ↓	−1.03 ↓	1.62 ↑
UGT1A10	−1.47 ↓	1.31 ↑	1.72 ↑
UGT2B7	−1.01 ↓	1.16 ↑	−1.26 ↓
UGT2B10	−1.05 ↓	−1.28 ↓	−5.73 ↓
SULT1A1	−1.17 ↓	1.09 ↑	1.13 ↑
NAT1	−1.02 ↓	1.10 ↑	−1.72 ↓
COMT	−1.28 ↓	1.39 ↑	1.40 ↑

that indicate that without induction there is no expression of the CYP1A2 gene in BEAS-2B cells based on a Ct > 36. HepaRG cells (positive control cell line) showed a reduction in enzyme activity (1.6-fold) in the presence of inhibitor, however, this reduction was not statistically significant ($p = 0.127$).

4. Discussion

The lung-derived cell line BEAS-2B has been identified as a cell line of interest in the *in vitro* toxicological testing of inhaled toxicants (Veljkovic et al., 2011; Ansteinsson et al., 2011). However, to date the metabolic capabilities of this cell line have not been thoroughly investigated. In this study, we employed high throughput technology to provide a rapid screening for gene expression and inducibility for a panel of 41 metabolism-related genes, producing a profile of gene expression and gene inducibility. Then, four key CYP enzymes involved in the bioactivation of some smoke pro-toxicants were selected for functional enzyme activity assay. The data obtained from both analysis would confirm if enzyme activity was consistent with gene expression. The scientific approach used in this study is a working example of our proposed

strategy for the metabolic characterization of cell systems used in the context of *in vitro* toxicology testing.

Our gene expression results show that non-induced BEAS-2B cells have high and moderate mRNA expression for, GSTM1 and SULT1A1 respectively, both related to conjugative reactions which mainly act as detoxification mechanisms (Castell et al., 2005). As expected, when cultures were pre-induced with TCDD, CYP1A1, CYP1B1 and CYP1A2 genes showed an up-regulation of 25-fold, 6-fold and 4-fold respectively compared with non-induced cultures. The up-regulation of CYP1A1 and CYP1B1 genes after pre-incubation with TCDD has also been reported in normal human primary bronchial epithelium (NHBE) cells (Newland et al., 2011). Surprisingly, in a recent publication Courcot and colleagues report high levels of CYP1B1 gene expression in non-induced cultures of BEAS-2B cells and high levels of CYP1A1/1B1 gene expression in non-induced cultures of human primary bronchial epithelium cells (HBEC), among other lung cell systems (Courcot et al., 2012). This contrasts with our gene expression results and other published results (Newland et al., 2011; Castell et al., 2005). It is possible that the high levels of CYP1A1 and CYP1B1 reported in HBEC by Courcot and colleagues could be as a result of the smoking habit of the donor; cigarette smoke is known to activate these enzymes in the lung (Nishikawa et al., 2004; Anttila et al., 2011). However, this information is not disclosed in their methodology. In order to confirm the relevance of the gene expression, activity assessment is generally recommended as a follow up experiment.

The gene expression results we obtained were compared with the enzyme activity data obtained for the tested CYPs (CYP1A1/1B1, CYP1A2, CYP2A6/2A13 and CYP2E1). When BEAS-2B cells were pre-incubated with TCDD, CYP1A1/1B1 activity showed a statistically significant increase compared to non-treated cultures (Fig. 3A). This concurs with the gene up-regulation described earlier. TCDD-induced BEAS-2B cells showed an activity of 0.2 RLU/mg/min while HBEC cultures have been reported to show an enzyme activity level between 4.3 and 7.3 RLU/mg protein/min (Newland et al., 2011). No activity was observed in BEAS-2B cells for the other three CYPs analyzed (CYP2E1, CYP2A6/2A13 and CYP1A2) which confirms the findings from our gene expression analysis. Previous studies have also reported no detectable CYP1A2 activity in BEAS-2B cells and lung microsomes (Van Vleet et al., 2002; Shimada et al., 1992), however, CYP1A2 activity could be induced by environmental factors and specific CYP1A2 gene polymorphisms increasing lung cancer risk as recently reviewed (Pavanello et al., 2012). The activity related to CYP2A and CYP2E1 has not been previously reported in BEAS-2B cells, but has been detected in human lung (Hukkanen et al., 2002). Newland et al. also reported that HBEC cultures from three different donors showed a CYP2A6/2A13 activity between 0.15 and 1.33 pmol/mg/min (Newland et al., 2011) a similar study by Runge and colleagues showed that CYP2E1 activity in HBEC (0.6 pmol/mg/min), however substantial inter-individual variability was reported as only two out of the four donors showed CYP2E1 activity (Runge et al., 2001).

Overall, the relative enzyme activity level in BEAS-2B cells appears limited compared with normal tissue. For instance, immunobloting of human lung microsomes have been used to detect CYP1A1, 1B1, 2A6, 2B6, 2C9, 2D6, 2E1, 2F1 and 3A4/5 in normal airway tissue (Hukkanen et al., 2002; Bernauer et al., 2006). In HBEC, these CYPs have been reported to show both gene expression and enzyme activity, however, high interindividual variability between different donors was also noted (Runge et al., 2001; Newland et al., 2011; Anttila et al., 2011; Castell et al., 2005). The lack of gene expression for the majority of metabolizing enzyme-encoding genes tested, with or without induction by TCDD, and the lack of activity for three out of the four selected P450 enzymes indicates that BEAS-2B cells might not be suitable to study the toxicity of
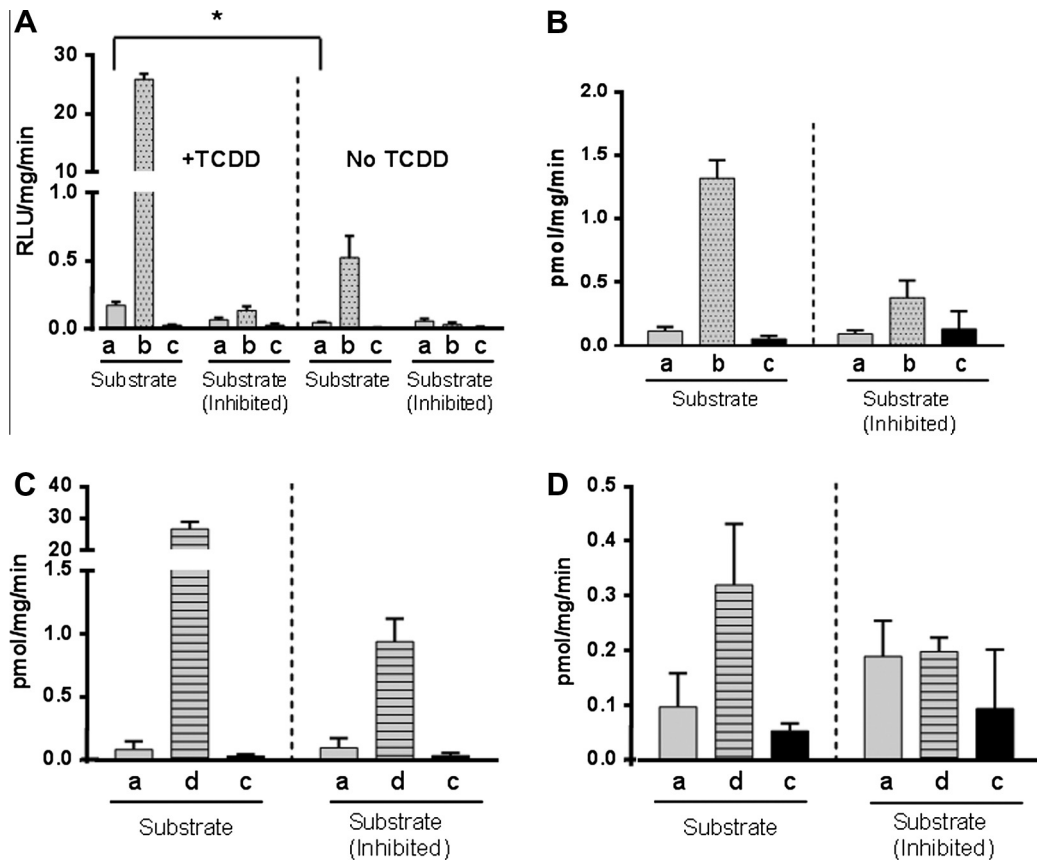
Fig. 3. Enzyme activity represented by metabolite formation. (A) Luciferin formation measured in BEAS-2B (a), A549 (c) and Hep-G2 (b) with and without induction with TCDD in the presence and absence of a specific inhibitor (α-naphthoflavone). Luciferin rate of formation is indicative of CYP1A1/1B1 activity when luciferin-CEE was used as a substrate. (B) 6-Hydroxychlorzoxazone formation measured in BEAS-2B (a), A549 (c) and Hep-G2 (b) in the presence and absence of a specific inhibitor (disulfiram). 6-Hydroxychlorzoxazone rate of formation is indicative of CYP2E1 activity when chlorzoxazone was used as a substrate. (C) 7-Hydroxycoumarin formation measured in BEAS-2B (a), A549 (c) and HepaRG (d) in the presence and absence of a specific inhibitor (8-MOP). 7-hydroxycoumarin rate of formation is indicative of CYP2A6/2A13 activity when coumarin was used as a substrate. (D) Resorufin formation measured in BEAS-2B (a), A549 (c) and HepaRG (d) in the presence and absence of a specific inhibitor (fluvoxamine). Resorufin ratio of formation is indicative of CYP1A2 activity when 7-ethoxyresorufin was used as a substrate. CYP activities are expressed as the mean + SD of three independent experiments with triplicate samples. The asterisk (*) indicates a $p < 0.001$.

some inhaled pro-toxicants without an external source of metabolic activation (S9 fractions, microsomes, co-cultures or *in vitro* liver-like cell lines amongst others) (Brandon et al., 2003). Alternatively to an external source of metabolic activation, Macé and colleagues developed a series of cell lines, derived from BEAS-2B cells, expressing one single human cytochrome P450 cDNA, CYP1A2, 2A6, 2B6, 2C9, 2D6, 2E1, 2F1, 3A4 and 3A5 (Mace et al., 1997a,b). These cell lines have been mainly used for the toxicological assessment of single compounds (Mace et al., 1994; Van Vleet et al., 2002; Nichols et al., 2003). Although useful for the toxicity evaluation of single compounds, genetically engineered cell lines have toxicity testing limitations with complex mixtures and compounds with unknown metabolic pathway. The complex mixture could contain various pro-toxicants bioactivated by multiple CYPs. Nevertheless, pro-toxicants which are metabolised by CYP1A1/1B1 enzymes such as PAHs could be bioactivated in pre-induced BEAS-2B cultures. In this study CYP1A1/1B1 gene expression and enzyme activity were induced using TCDD, however, other xenobiotics such as B[a]P have been used previously to induce these isoforms (Nebert et al., 1993; Tsuji and Walle, 2006).

It is important to consider that the BEAS-2B cell line has a wider application for biological endpoint assessment such as DNA damage and repair mechanisms *in vitro*. The non-cancerous phenotype and wild-type p53 status of the BEAS-2B cell line makes them an ideal cell system in cell transformation research (Reddel et al., 1988; Petitjean et al., 2007; IARC-TP53, 2013). Moreover, the

"oncogenic stress" exhibited by pre-malignant and cancer tissues could affect the measure of certain biomarkers of DNA damage such as the γH2AX (Svetlova et al., 2010). The BEAS-2B cell line has also been selected as a cell system in the study of nanomaterials cellular transport and intracellular response (Gilbert et al., 2012; Ekstrand-Hammarstroem et al., 2012).

During this study a number of well-characterized cell lines were used in parallel with the same treatment conditions. The A549 cell line was selected as a lung carcinoma-derived cell system for comparison purpose while the HepG2 and HepaRG cell lines were used as 'positive control' with a more extensive cytochrome P450 enzyme activity. A549 cells showed a small number of up-regulated genes in basal cultures such as AKR1B10 and AKR1C2 known to be associated with the cell line's tumorigenic origin (Quinn et al., 2008). As expected, in pre-induced cultures CYP1A1 and CYP1B1 genes were up-regulated (260-fold and 14-fold increase respectively). Interestingly, in our study the up-regulation of these genes was not translated into enzyme activity. The lack of CYP1A1/1B1 enzyme activity has been observed previously (Newland et al., 2011). With respect to the results obtained for HepG2 and HepaRG cells, we observed that HepaRG express more genes involved in phase I and phase II metabolism than HepG2. Our results concur with data published previously (Gerets et al., 2012; Jennen et al., 2010).

Our data on BEAS-2B have shown a different profile to the data published recently by Courcot et al. They recommended BEAS-2B

cells as a surrogate for metabolism and bioactivation of toxicants in the lung, based on gene expression similarities between these cells and primary cultured cells (Courcot et al., 2012). Although, our functional experiments include a subset of metabolic enzymes, our results suggest that BEAS-2B cells do not have significant phase I metabolism capabilities. The different results could be explained by variations in the culture conditions and cell origin. These protocol variations have been reported as causes of differences in phase I and phase II activities (Hewitt and Hewitt, 2004). Nevertheless, while qPCR is a sensitive method to measure gene expression, not all mRNAs are translated into active proteins. There are multiple processes that could interfere with the translation and activation of proteins from mRNA one example is the emerging field of microRNA research which has shown the ability to modify the regulation of both gene expression and translation (Lee and Vasudevan, 2013). Thus, mRNA level is not always correlated with protein or activity. For instance, Halladay and colleagues studied the induction of various hepatic cytochrome P450 at the mRNA, protein and activity level from different donors. For CYP1A2, the inducer rifampicin did not increased mRNA and protein levels (1.00 and 1.03-fold induction respectively), however, the activity was induced by an average of 2.55-fold (one donor's activity reaching above 4-fold induction). On the contrary, CYP3A4/5 inducer ritonavir (5 μM) increased mRNA expression by 2.5-fold but protein and activity levels were not induced (<0.3-fold induction) (Halladay et al., 2012). In our study, we observed that the HepG2 cell line showed enzyme activity for both CYP1A1/1B1 and CYP2E1 (Fig. 3A and B) but a low mRNA expression was detected in un-induced HepG2 cultures (Fig. 2). The lack of correlation between activity and mRNA could be caused by post-transcriptional factors and is also a function of the protein stability. Also, it is worth noting that the mRNA expression of both CYP1A1/1B1 and CYP2E1 was upregulated in induced HepG2 cultures, the substrates used during the enzyme activity assays could have had an inducibility effect. For these reasons, key enzymatic activities should be included in any metabolic characterization to confirm the gene expression results prior the use of the cell line for further *in vitro* toxicological testing.

In summary, we would like to outline an experimental strategy that benefits from the high throughput of qPCR but includes key functional assays (i.e. enzymatic activity).

i. Define an experimental design considering the nature of the test article, route of exposure and metabolic pathways. In our study, the experimental design was orientated towards toxicological studies on cigarette smoke toxicants.

ii. Obtain the cell systems from an established supplier such as the European Collection of Cell Cultures (ECACC) to avoid the propagation of misidentified cultures (Lacroix, 2008). Furthermore, whenever the cell line is expanded in-house, simple authentication methods will confirm the authenticity of the cells (Nims et al., 2010).

iii. Select a panel of pathway-relevant metabolism genes to carry out expression analysis.

iv. Assess corresponding enzyme activity.

The metabolic characterization of the cell line BEAS-2B carried out in this study will support future experimental designs, taking into account the cell system limitations. Moreover, we propose that this strategy can be applied to unravel the metabolic capabilities of other cell systems considered for *in vitro* toxicology testing.

Conflict of interest

None.

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