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RESEARCH ARTICLE

Assessment of cytochrome P450 (1A2, 2B6, 2C9 and 3A4) induction in cryopreserved human hepatocytes cultured in 48-well plates using the cocktail strategy

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Abstract

1. A fast, straightforward and cost-effective assay was validated for the assessment of CYP induction in cryopreserved human hepatocytes cultured in 48-well plates. The cocktail strategy (*in situ* incubation) was used to assess the induction of CYP1A2, CYP2B6, CYP2C9 and CYP3A4 by using the recommended probe substrate, i.e. phenacetin, bupropion, diclofenac and midazolam, respectively.
2. Cryopreserved human hepatocytes were treated for 72 h with prototypical reference inducers, β-naphthoflavone (25 μM), phenobarbital (500 μM) and rifampicin (10 μM) as positive controls for CYP induction. The use of a cocktail strategy has been validated and compared to the classical approach (single incubation). The need of using phase II inhibitor (salicylamide) in CYP induction assay was also investigated.
3. By using three different batches of cryopreserved human hepatocytes and our conditions of incubations, we showed that there was no relevant drug–drug interaction using the cocktail strategy. The same conclusions were observed when a broad range of enzyme activity has to be assessed (wide range of reference inducers, i.e. EC_{50}–E_{max} experiment). In addition, the interassay reproducibility assessment showed that the day-to-day variability was minimal.
4. In summary, the study showed that the conditions used (probe substrates, concentration of probe substrate and time of incubation) for the cocktail approach were appropriate for investigations of CYP induction potential of new chemical entities. In addition, it was also clear that the use of salicylamide in the incubation media was not mandatory and could generate drug–drug interactions. For this reason, we recommend to not use salicylamide in CYP induction assay.

Keywords: CYP induction, cocktail strategy, human hepatocytes

Introduction

Drug–drug interactions (DDIs) represent a serious problem in clinical practice. To reduce its occurrence, great importance has been placed on *in vitro* studies as tools for predicting *in vivo* DDIs, particularly those resulting from cytochrome P450 (P450) induction. Indeed, the induction of P450 can reduce the exposure to parent compound and increase formation of metabolite(s) and as a consequence can alter the safety and efficacy profile of a co-administered drug. *In vitro* screening for potential induction of human P450s is therefore a crucial part of the drug discovery and should be the more reliable possible to deselect drug candidate before they reach development or clinical trials. Primary human hepatocytes are widely recognized as the gold standard *in vitro* assay to study a drug's induction potential (LeCluyse et al. 2000; Huang & Stifano 2006; Hewitt et al. 2007). Indeed, other test systems that could be used as alternatives to hepatocytes suffer from several drawbacks. For example, the receptors PXR and CAR controlling the expression of CYP2 and CYP3A families are strongly down-regulated or absent in cell lines, such as HepG2 or Hepa-1c1c7 (Pascussi

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et al. 2001). Moreover, Gerbal-Chaloin et al (2006) have also demonstrated the presence of cross-talk between PXR/CYP3A4 and AhR that could result in misleading interpretation when using hepatoma cell lines for induction assay. In addition, due to significant species differences in CYP isoforms, substrate specificities and induction response, human CYPs induction evaluation can only be accurately performed with human tissues (Lu & Li 2001). The use of cryopreserved hepatocytes for long-term studies has always been challenged compared to the fresh hepatocytes. However, in the last couple of years, cryopreservation techniques have been improved, allowing a high percentage of viable and plateable hepatocytes after thawing (Gómez-Lechón et al. 2006; Li 2007). Cryopreserved hepatocytes are now accepted by FDA as a valid alternative to fresh human hepatocytes and they have been shown to be comparable to fresh hepatocytes in terms of their response to prototypical CYP1A2, CYP2B6 and CYP3A4 inducers (Huang & Stifano 2006; Kafert-Kasting et al. 2006; Roymans et al. 2005). The recent EMA guidance on drug interactions does not make any distinction between the use of fresh versus cryopreserved hepatocytes for CYP induction study. However, it is clearly stated that cultured hepatocytes is the preferred *in vitro* system for these studies. In addition to the general consensus on the use of cryopreserved hepatocytes for enzyme induction, the following advantages over fresh hepatocytes are not negligible: (i) ease of experimentation (planning of experiments), (ii) repeat experimentation and historical data available (iii) choice of donor.

The work described in this publication is part of all the steps undertaken in the context of the CYP induction international validation project, managed by the European Union Reference Laboratory for Alternative Methods to Animal Testing (ECVAM) (2010). The cocktail approach, used in this validation project for endpoint measurement, was first described by Kanebratt and Andersson in (2008). The basis for the general applicability of the cocktail approach has been developed within the EU FP6 project "Vitrocellomics" (Project No. 018940) to which JRC-ECVAM participated together with the Karolinska Institute, Pharmacelsus GmbH and Astra Zeneca Sweden (Mandenius et al. 2011).

More specifically, the main objective of the present study was to validate the use of the cocktail strategy to assess CYP P450 inducibility. CYP activities were measured at the end of the induction period by an *in situ* approach (addition of substrates to the washed hepatocyte monolayer). Even if the *in situ* method has the main disadvantage of a low sensitivity of the activity measurement, this method required much fewer hepatocytes than the measurement of CYP activities in microsomes prepared from the monolayer and gives also a global view of hepatocytes functionality following treatment with an unknown test compound. In addition, several studies have already compared CYP activities determined in microsomes and monolayers and have shown that responses to CYP inducers were similar using both approaches (Lecluyse 2001; Richert et al. 2010). The use of the cocktail strategy consisted of a simultaneous evaluation of selected CYP activities by concomitant incubation of the selected probes. One of the main challenges of this method remains the biological validation to ensure that no DDIs occurred during the incubation step and also the development of a fast, sensitive and robust analytical method allowing the simultaneous measurement of each metabolite without any cross-interference from parent compound and other metabolites present in the samples. Selective probes for CYP1A2, CYP2B6, CYP2C9 and CYP3A4 were selected to be part of the cocktail. The advantage of this cocktail is twofold. First, induction of P450s under the regulation of the three major transcription factors, AhR (CYP1A), CAR and PXR (CYP2 and CYP3A) will be detected (Hewitt et al. 2007) and secondly, these four P450s enzymes belong to the major P450s involved in drug metabolism. Indeed, 60% of marketed drugs are metabolized by these enzymes (Zuber et al. 2002; Guengerich 2006), the other 30% being metabolized by CYP2D6, not known as an inducible CYP (Li & Kedderis 1997; Rae et al. 2001; Westerink & Schoonen 2007).

This study summarizes the validation of a straightforward CYP induction assay using cryopreserved human hepatocytes cultured in 48-well plates with *in situ* enzymatic activities determination using the cocktail strategy.

Methods

Chemicals and reagents

All compounds and reagents were of analytical grade. The reference inducers, probe substrates and metabolites used in the present study were purchased from Sigma-Aldrich (Saint Louis, MO, USA) except phenobarbital which was obtained from Certa (Braine-L'Alleud, Belgium), midazolam obtained from AAPIN Chemicals (Oxfordshire, UK) and hydroxybupropion obtained from BD Gentest (Franklin Lakes, NJ, USA). All the other chemicals were purchased from different commercial sources.

Hepatocyte culture and treatment with CYP450 inducers

Cryopreserved human hepatocytes from 6 different donors (see characteristics in Table 1) were purchased from CellzDirect (Pittsboro, NC, USA) and were stored in vapours of liquid nitrogen until use. The hepatocytes were thawed using cryopreserved hepatocytes recovery medium (CHRM™) and seeded (250 µL of a cell suspension at 10^6 cell/mL) in a 48-well plate pre-coated with a single film of collagen (in house coating, *ca* 50 µg/mL). The hepatocytes were allowed to attach for *ca.* 6 h in cryopreserved hepatocytes plating medium (CHPM™) in a 5% CO_2:95% air humidified atmosphere at 37°C before starting an overnight pre-incubation period in WBL medium (Williams E medium containing

Table 1. Human hepatocytes donor demographics.

Batch	Age (years)	Sex	Race	Cause of Death	Smoker	Alcohol Use	Drug Use	Medications	Serological Data
Hu4037	64	♀	Caucasian	Stroke	Yes	No	No	Avonex Thyrosine HCTZ	Negative except CMV positive
Hu4122	19	♂	Caucasian	GSW to head	Yes	Yes	Yes	Hydroxycut	All negative
Hu4198	9	♀	Caucasian	Anoxia/head trauma	No	No	No	None	CMV positive
Hu4237	57	♀	Caucasian	Anoxia	No	No	No	Vitamins	CMV positive
Hu8063	4	♂	Caucasian	Anoxia	No	No	No	None	All negative
Hu8127	31	♀	Caucasian	CV event after heart transplant	Yes	Yes	Yes	Anti-rejection meds for heart transplant	CMV positive

Glutamax I, 100 IU/mL penicillin, 100 µg/mL streptomycin, 4 µg/mL bovine insulin and 50 µM hydrocortisone hemissuccinate).

At the end of the overnight pre-incubation period, the monolayers of cryopreserved human hepatocytes were treated with the reference inducers β-naphtoflavone (β-NF, 25 µM), phenobarbital (PB, 500 µM) or rifampicin (RIF, 10 µM). The reference inducers were dissolved in DMSO, which reached a final concentration of 0.1% in the culture medium. In control hepatocytes, culture medium was spiked with 0.1% DMSO. The cells were then incubated in a 5% CO_2:95% air humidified atmosphere at 37°C for 72 h with medium renewal every *ca.* 24 h. All conditions were performed in triplicate.

Human liver microsomes

Mixed gender pooled (n = 50) human liver microsomes (HLM) from Xenotech (Lenexa, KS, USA) were used. The incubations of HLM (0.1 mg/mL) with phenacetin (60 µM) in presence or absence of salicylamide were carried out in polypropylene containers at *ca* 37°C in a shaking water bath, with 50 mM potassium phosphate buffer (pH 7.4) containing a NADPH regenerating system [NADP (1.3 mM), glucose 6-phosphate (3.3 mM), $MgCl_2$ (3.3 mM) and glucose 6-phosphate dehydrogenase (0.4 U/mL)]. Incubations were performed in triplicate. Final solvent concentration in incubates was ≤ 1%.

After 20-min incubation, reactions were stopped by 1 volume (buffer incubations) of ice-cold acetonitrile. The tubes were then thoroughly mixed using a vortex mixer and centrifuged (*ca* 10000×*g*, 4°C) for 10 min. Clear supernatants were stored at ≤–80°C prior to analysis for acetaminophen by LC/MS-MS.

Cytochrome P450 activity assays

After a 72-h exposure to vehicle control (0.1% DMSO) and reference inducers, the hepatocyte monolayers (vehicle control and induced hepatocytes) were washed twice with prewarmed PBS and incubated *in situ* in the presence of phenacetin, bupropion, diclofenac and/or midazolam in HBSS (0.4% MeOH final concentration), either individually (determination of kinetic parameters) or as a cocktail (final concentrations in Table 2). In some conditions, the incubation was performed in the presence of 1 mM salicylamide as phase II inhibitor, that was solubilized in HBSS. When different conditions were tested (cocktail vs single incubation or with vs without salicylamide), different wells were used. However, they were treated in the same way up to the final incubation step. At the end of the incubation period, the supernatants were collected and centrifuged for 10 min at 10000×*g*. The clear supernatants were stored at –80°C until analyzed.

Protein determination

The hepatocytes were dissolved in 0.1 N sodium hydroxide for 2 h at room temperature and the protein content in each well after the treatment period was determined using a Pierce BCA (bicinchoninic acid) Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) using bovine serum albumin as a standard.

Analytical methods

The formation of acetaminophen, hydroxybupropion, 4'-hydroxydiclofenac and 1'-hydroxymidazolam was measured by LC-MS/MS analytical method validated according to Shah et al. (2000). Basically, three validation batches were processed on three separate days. In each batch, three sets of 8-point standard curves were constructed by plotting peak area ratios of analytes/IS vs nominal analytes concentrations. Back-calculation of the concentrations using the calibration curves and linear regression analysis were conducted by using a weighted $1/(\text{concentration})^2$. Regression parameters of the slope, y-intercept, and correlation coefficient (r^2) were calculated to evaluate linearity and reproducibility. In addition, three levels of QC samples (low, medium and high) were used to evaluate intra-day and inter-day assay accuracy and precision. Concentrations of QC sample replicates (n = 5) were determined using a calibration curve obtained on the same day. Relative errors (REs [%]) and coefficients of variations (CVs [%]) of the measured concentrations were calculated to evaluate the validity of the method. The intraday and interday precisions were required to be less than 15%, and the accuracy to be within ±15%. Validation parameters are shown in Table 3. Preparation of sample was straightforward.

Table 2. Marker reaction for CYP induction assay.

P450	Probe susbtrate	Enzymatic reaction	Metabolite monitored	Concentration in cocktail (μM)
CYP1A2	Phenacetin	Phenacetin *O*-deethylation	Acetaminophen	10
CYP2B6	Bupropion	Bupropion hydroxylation	Hydroxybupropion	250
CYP2C9	Diclofenac	Diclofenac hydroxylation	4′-Hydroxydiclofenac	10
CYP3A4	Midazolam	Midazolam hydroxylation	1′-Hydroxymidazolam	3

Table 3. Analytical method parameters.

	Acetaminophen	Hydroxybupropion	1′-Hydroxymidazolam	4′-Hydroxydiclofenac
Linearity (r^2)	0.9974	0.9982	0.9960	0.9981
LOD (nM)	0.696	0.260	0.0708	1.38
LLOQ (nM)	6.63	3.73	2.90	9.61
ULOQ (nM)	1330	747	579	1920
Accuracy (%)*				
Intraday	–3.7/6.6	–7.0/4.6	–15.0/3.7	–9.8/0.6
Interday	–0.9/0.7	–1.0/2.3	–13.0/0.0	–6.9/5.7
Precision (%)*				
Intraday	4.0/4.2	2.2/4.5	1.8/4.2	3.2/5.5
Interday	6.2/7.1	4.0/6.0	2.8/4.3	3.2/5.5

LOD: Limit of detection; LLOQ: Lower limit of quantification; ULOQ: Upper limit of quantification.
*Minimum and maximum values observed are reported in table.

Briefly, 50 μL of clear supernatant was added to 10 μL of a mixture of acetaminophen–D4, hydroxybupropion–D6, 4′-hydroxydiclofenac–13C6 and 1′-hydroxymidazolam–D4, used as internal standards. The final mixture was directly injected into the HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a Zorbax Eclipse XDB-C18 (5 μm, 50 × 2.1 mm). Detection and quantification of acetaminophen, hydroxybupropion, 4′-hydroxydiclofenac and 1′-hydroxymidazolam were performed with a Quattro Micro mass spectrometer (Waters-Micromass, Manchester, UK). Elution was performed using a mixture of solvent A consisting of water containing 0.1% trifluoroacetic acid, adjusted to pH 2.4 with ammonium hydroxide and solvent B consisting of acetonitrile. The proportion of solvent B in the mobile phase was increased from 5 to 25% for 1 min, increased from 25 to 50% from 1 to 4.5 min, increased from 50 to 90% from 4.5 to 5.5 min and decreased to 5% from 7.5 to 11 min. Mass spectrometric conditions for each analyte and its internal standard are described in Table 4. The chromatogram of probe substrates, their metabolites and salicylamide is presented in Figure 1.

For assessment of acetaminophen formation in clear supernatant coming from liver microsomes incubations, internal standard were first added to samples and then they were evaporated to dryness under nitrogen at 45°C. The residue was reconstituted in 60 μL using a mixture of water containing 0.1% TFA pH 2.4/acetonitrile (95:5; v/v). The mixture was then injected onto HPLC as described above.

Data analysis

Metabolite formation rates (hepatocytes and microsomal incubation) were normalized to protein content and incubation time and expressed as pmol/min/mg protein. Enzyme activities expressed as pmol/min/mg prot were plotted against the substrate concentration for the determination of K_m and V_{max} according to the biphasic saturation (CYP1A2 – vehicle control and treated cells and CYP2B6, vehicle control cells) or Michaelis–Menten models (all others) using Erithacus GraFit Software (East Grinstead, UK). Results are presented as individual value (kinetic parameters) or as the mean ± SD from triplicate sample for each donor (effect of incubation conditions).

Statistical analyses were performed to compare both approaches (cocktail and single incubation). T test was used on log-transformed data to compare absolute enzymatic activities determined either by classical approach (single incubation) or by cocktail approach. Two-factor ANOVA with replication was used on log-transformed data to compare both approach on the fold induction parameter. Statistical analysis was performed using Microsoft Excel 2003. ANOVA with Dunnett post test were performed using GraphPad Prism (Version 5.02).

Results

The first work of the present study was the validation of an analytical method allowing the quantification of the four metabolites of interest in a same run using adequate chromatographic conditions to obtain maximum separation of parent compound from their metabolites within an acceptable run time. Probe substrate selected for the assessment of CYP1A2, CYP2B6, CYP2C9 and CYP3A4 activities are described in Table 2 and are those recommended by FDA guidance (Huang & Stifano 2006). The chromatographic conditions, as described in Material and Methods section, allowed the separation of all peaks of interest as shown in the chromatogram presented in Figure 1. Following the development of this analytical method, it was validated according to FDA criteria and used for the present study.

Table 4. Mass spectrometric conditions.

Compound	Monitored transitions (Da)	Cone voltage (V)	Collision energy (eV)	Dwell time (s)
Hydroxybupropion	256 → 139	15	20	0.060
Hydroxybupropion – D6	262 → 139	15	25	0.060
Acetaminophen	152 → 110.1	25	15	0.060
Acetaminophen – D4	156 → 113.7	22	17	0.060
1'-Hydroxymidazolam	342→ 203	30	25	0.060
1'-Hydroxymidazolam – D4	346 → 203	30	25	0.060
4'-Hydroxydiclofenac	312 → 231	20	17	0.060
4'-Hydroxydiclofenac – 13C6	318 → 237	20	17	0.060

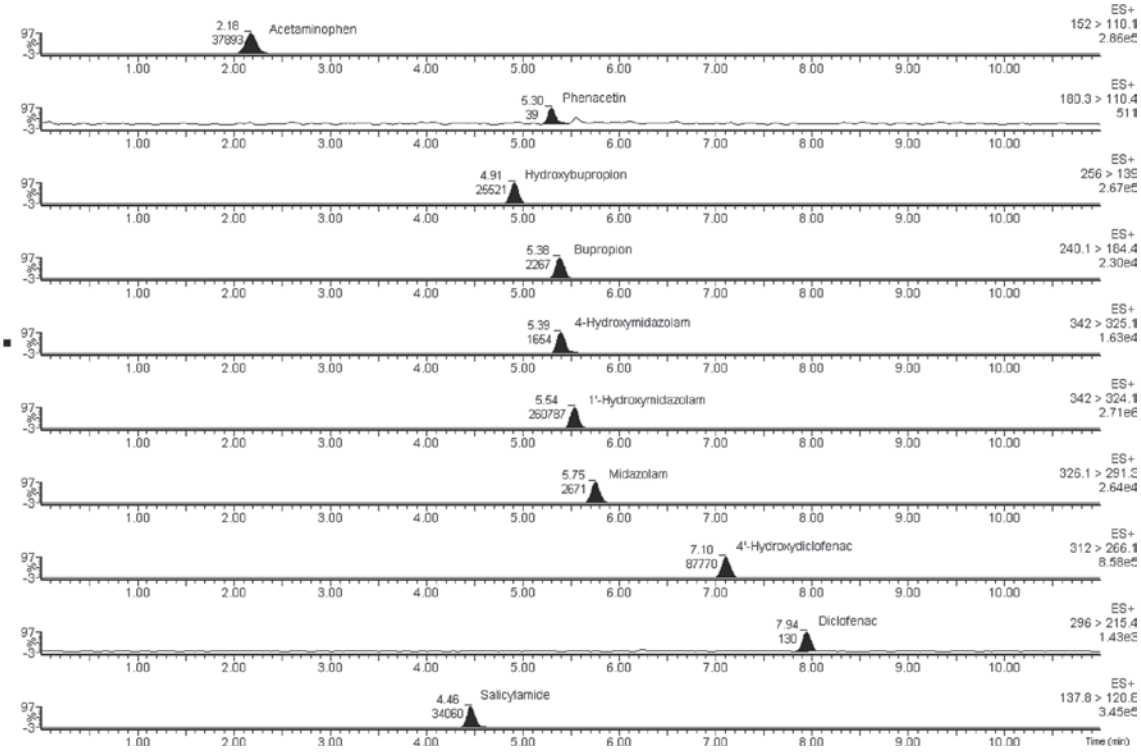

Figure 1. MRM chromatograms of a supernatant sample following the *in situ* incubation of probe substrates with cryopreserved human hepatocytes seeded in a 48-well plate format (250,000 cells, 30 min incubation with 10 μM phenacetin, 250 μM bupropion, 10 μM diclofenac and 3 μM midazolam, salicylamide = 10 μM diclofenac, 3 μM midazolam, and 1 mM salicylamide).

Preliminary development

A first set of experiments were conducted in order to assess the linearity of metabolite formation in control hepatocytes as well as in induced hepatocytes (72 h treatment with reference inducers, i.e. β-naphthoflavone for CYP1A2, phenobarbital for CYP2B6 and CYP2C9, and rifampicin for CYP3A4 and CYP2C9). Probe substrates were incubated from 15 to 60 min at 37°C and metabolites were quantified. Formation of the corresponding metabolite was linear up to 60 min for phenacetin, bupropion and diclofenac in control and induced hepatocytes whereas for midazolam, it was linear up to 60 min only in control hepatocytes (linear up to 30 min in induced hepatocytes) (data not shown).

Kinetic parameters of probe substrates

As second step, the estimation of the kinetic parameters for each probe substrate in control and induced hepatocytes have been investigated under linear conditions as determined in the preliminary developments. The two main objectives of this assay were (i) to check that the fold induction is independent on the probe substrate concentration and (ii) to select the concentration of the probe substrate to be used in the cocktail according to the limit of quantification of the analytical method. To this aim, cryopreserved human hepatocytes from one representative donor (Hu8063, see characteristics in Table 1) were treated for 72 h (medium renewal every 24 h) with DMSO (0.1% v/v final concentration, acting as dose vehicle control), β-NF (25 μM), PB (500 μM) or RIF (10 μM), as reference inducers of CYP1A2, CYP2B6 (and CYP2C9) and CYP3A4 (and CYP2C9), respectively. Following 72 h treatment, relevant CYP activities were evaluated *in situ* using the specific probe substrate for each P450 enzymes, i.e. phenacetin for CYP1A2 (vehicle

control and β-NF), bupropion for CYP2B6 (vehicle control and PB), diclofenac for CYP2C9 (vehicle control, PB and RIF) and midazolam for CYP3A4 (vehicle control and RIF). Figure 2 shows for each P450, the direct plot of the enzymatic activity (expressed in pmol/min/mg protein) in relation with the concentration of the probe substrate. Table 5 describes the model used as well as the kinetic parameters derived from the fitting of the data presented in Figure 2. For the formation of acetaminophen (CYP1A2), the biphasic saturation model had to be used to fit accurately the data. In addition, the fact that this model had to be used to describe the formation of acetaminophen was expected and has already been described previously (von Moltke et al. 1996). For the formation of hydroxybupropion (CYP2B6), the biphasic saturation model was also used but only in control hepatocytes whereas in induced hepatocytes, the classical Michaelis–Menten model better fitted the data. For the formation of hydroxydiclofenac (CYP2C9) and hydroxymidazolam (CYP3A4), both enzymatic kinetics were perfectly fitted with the Michaelis–Menten equation in both conditions (control and induced hepatocytes). As expected, the K_m value for each reaction was quite similar in control and induced hepatocyte whereas the V_{max} and CL_{int} were always increased in induced hepatocytes compared to control hepatocytes. In *in vitro* CYP induction studies, the "fold induction" parameters is the final endpoint classically used for the assessment of the potential of a NCE to induce P450 enzymes. In Table 6, the fold induction parameter has been calculated by using 4

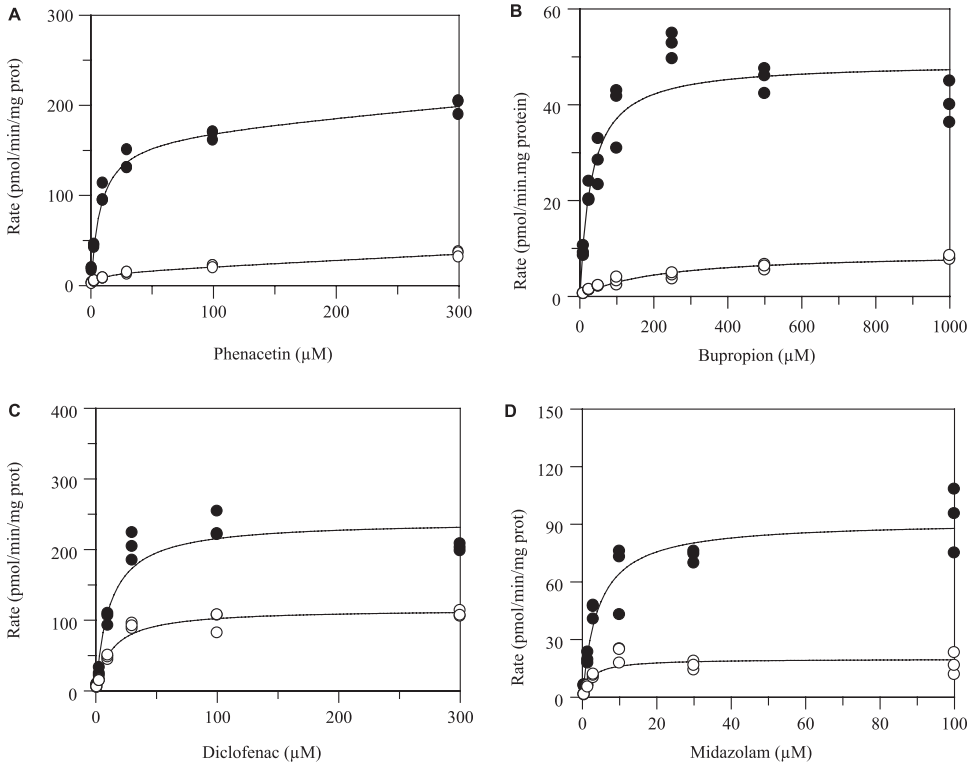

Figure 2. Cryopreserved human hepatocytes from 1 representative donor (Hu8063) were treated with vehicle (0.1% DMSO; ○) or reference inducer (●) for 3 days at 37°C with medium renewal every *ca.* 24 h. Reference inducer was β-NF 25 μM (panel A), PB 1 mM (panel B), RIF 10 μM (panel C and D). At the end of the treatment, human hepatocytes were incubated for 60 min *in situ* with phenacetin (panel A), bupropion (panel B) and diclofenac (panel C) or 30 min with midazolam (panel D) as probe substrates of CYP1A2, CYP2B6, CYP2C9 and CYP3A4, respectively.

Table 5. Determination of kinetic parameters.

Target CYP	Treatment	Model used	K_m (μM)	V_{max} (pmol/min/mg prot)	CL_{int} (μL/min/mg prot)	CL_{int2} (μL/min/mg prot)
CYP1A2	Vehicle control	Biphasic saturation	8.8	15.5	1.76	0.0675
	β-NF treated	Biphasic saturation	7.7	169	21.9	0.115
CYP2B6	Vehicle control	Biphasic saturation	67	4.8	0.071	0.0038
	PB treated	Michaelis–Menten	31	48.8	1.55	–
CYP2C9	Vehicle control	Michaelis–Menten	13	116	8.85	–
	RIF Treated	Michaelis–Menten	12	240	20.9	–
CYP3A4/5	Vehicle control	Michaelis–Menten	2.4	19.9	8.29	–
	RIF Treated	Michaelis–Menten	4.2	91.6	21.8	–

different approaches: (i) using Vmax ratio, (ii) using CL_{int} ratio, (iii) using mean of all enzymatic activities ratio (calculated for each probe substrate concentration), and (iv) using the enzymatic activity ratio at the anticipated concentration of probe substrate in the cocktail. The data showed that fold-induction parameters were similar whatever the approach used for all P450 tested excepted for CYP2B6 and CYP3A4 when the CL_{int} ratio was used. Indeed, as CL_{int} is V_{max}/K_m ratio, this discrepancy is easily explained by the different K_m values observed in control and induced hepatocytes (within twofold) for these two P450 enzymes. These results strongly confirmed that the determination of the fold-induction parameter is independent of the probe substrate concentration. Therefore, the only limitation for the selection of the probe substrate concentration in the cocktail remained the sensitivity of the analytical method that should be high enough to detect the formation of the metabolite in control hepatocytes. Indeed, the activity in control hepatocytes is important to calculate the fold-induction parameter.

According to these values, the selection of probe substrate concentration in the cocktail was mainly driven by the sensitivity of the analytical method. Indeed, the lowest concentration possible of probe substrate was selected in order to reduce to a maximum the risk of DDI between probe substrates. The final composition of the cocktail is described in Table 2.

Validation of the cocktail strategy

The most important objective of the present study was to validate the use of the cocktail strategy for CYP induction assessment with regards to the classical approach (incubation with single substrate). To this aim, at the end of the induction period (as described in Material and Methods section), the P450 activities (CYP1A2, CYP2B6, CYP2C9 and CYP3A4) were determined either individually (one CYP assessed per sample) or concomitantly by using the cocktail strategy (four CYPs assessed in the same sample). The concentration of probe substrate was the same in both conditions (and described in Table 2). In addition, to firm up the validation, the experiment was performed on three different human donors (see characteristics in Table 1). The results for CYP1A2, CYP2B6, CYP2C9 and CYP3A4 are shown in Figures 3–6, respectively. In each figure and for each human donor, the results were expressed as enzymatic activity in control and induced hepatocytes (panels A, B, C) and as fold induction over control (panels D, E, F).

As shown in Figure 3, CYP1A2 activities were induced 4- to 35-fold, depending on the donor, after 72 h treatment with 25 μM β-NF. Interestingly enough, the absolute CYP1A2 activity determined in control hepatocytes and in treated hepatocytes using the cocktail approach (grey bar) were not statistically different from those determined by the classical approach (open bars) except for one donor (panel C) in treated hepatocytes where the CYP 1A2 activity was 20% lower using the cocktail approach. However, when looking at the final endpoint for CYP induction (fold-induction parameter), the results were similar among the three donors in the sense that no statistical difference was observed between the two approaches for the determination of CYP1A2 induction.

Table 6. Fold-induction calculation using several approaches.

	Fold-induction calculated based on ratio (induced/control) of			
P450	V_{max}	CL_{int}	Enzymatic activities (mean)*	Enzymatic activities (concentration in cocktail)†
CYP1A2	11	12	9.1 ± 2.3	13 ± 1
CYP2B6	10	22	12 ± 2	12 ± 4
CYP2C9	2.1	2.4	2.2 ± 0.2	2.0 ± 0.3
CYP3A4	4.6	2.6	4.1 ± 1.0	4.1 ± 0.4

*For each substrate concentration, the ratio of enzymatic activities in induced hepatocytes over control was calculated and the mean of all these ratios is represented in this column.

†The ratio of enzymatic activities in induced hepatocytes over control at the intended probe substrate concentration in the cocktail is represented.

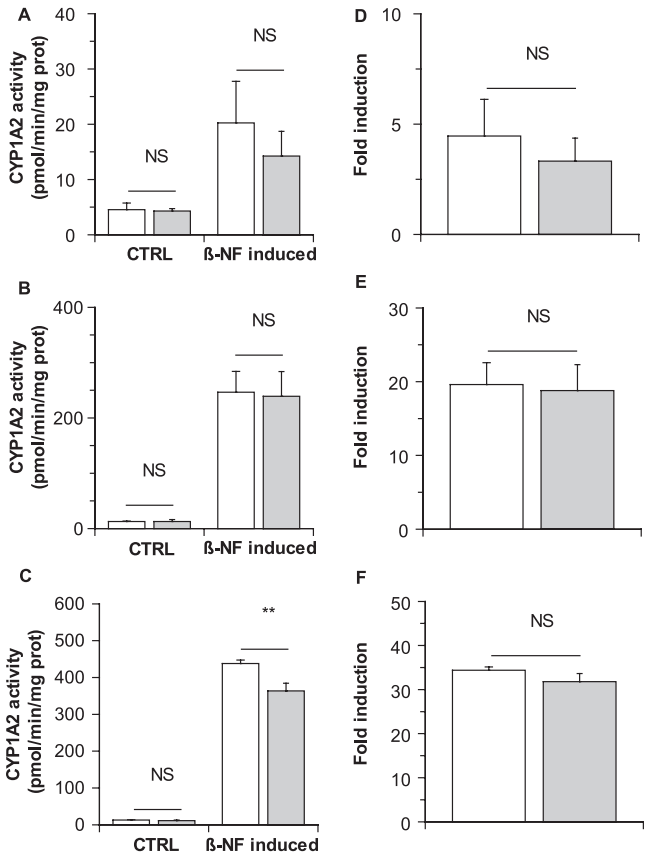

Figure 3. Cryopreserved human hepatocytes from 3 donors (Hu4037: A and D; Hu4122: B and E; Hu8063: C and F) were treated with vehicle (0.1% DMSO) or β-NF (25 μM) for 3 days at 37°C with medium renewal every *ca.* 24 h before the determination of acetaminophen as marker of CYP1A2 after 30 min incubation *in situ* with phenacetin (10 μM) (open bar: single incubation; grey bar: incubation using the cocktail strategy [Table 2]). Results are the mean of a triplicate determination and are expressed as enzymatic activity (A, B, C) or fold-induction over control cells (D, E, F). NS: Not significant ($p > 0.05$), **$p < 0.01$.

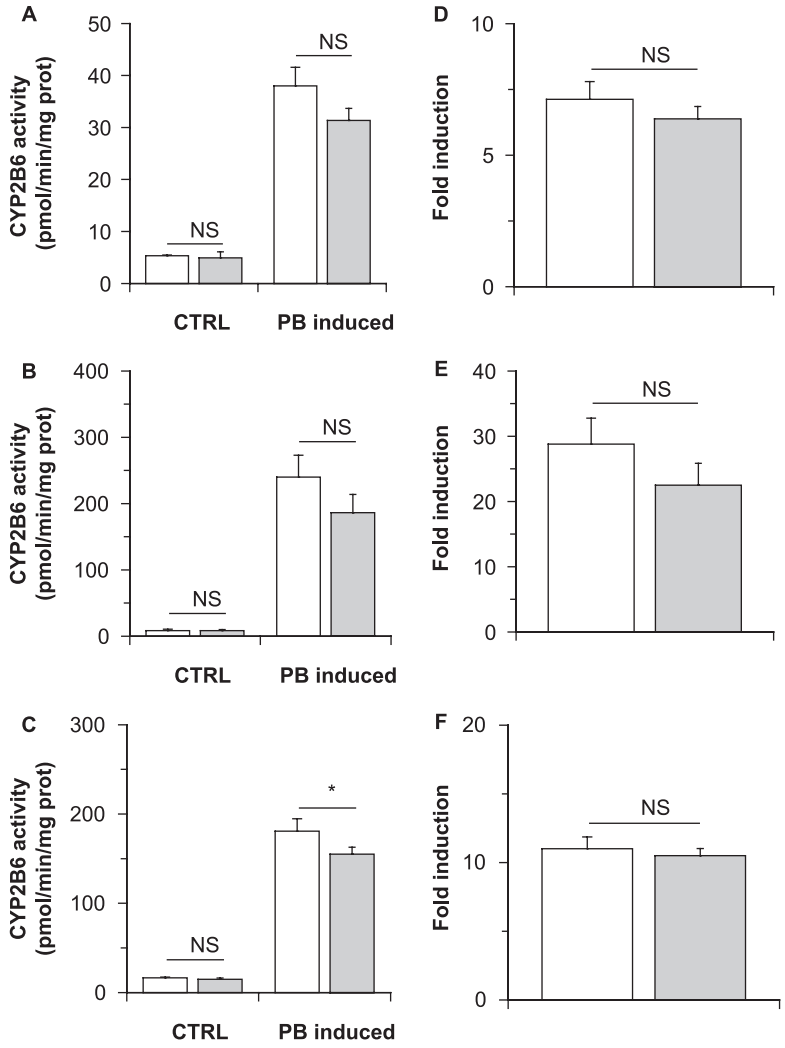

Figure 4. Cryopreserved human hepatocytes from 3 donors (Hu4037: A and D; Hu4122: B and E; Hu8063: C and F) were treated with vehicle (0.1% DMSO) or PB (500 μM) 3 days at 37°C with medium renewal every *ca*. 24 h before the determination of hydroxybupropion as marker of CYP2B6 after 30 min incubation *in situ* with bupropion (250 μM) (open bar: single incubation; grey bar: incubation using the cocktail strategy [Table 2]). Results are the mean of a triplicate determination and are expressed as enzymatic activity (A, B, C) or fold-induction over control cells (D, E, F). NS: Not significant ($p > 0.05$), *$p < 0.05$.

In Figure 4, the results showed that CYP2B6 activities were induced 6- to 30-fold, depending on the donor following 72 h treatment with PB (500 μM). As for CYP1A2, no statistical difference was observed between both approaches, except in one donor in treated hepatocytes only (20% of apparent decreased activity using the cocktail approach). However, as for CYP1A2, this statistical difference becomes not significant when the fold-induction parameter was used as endpoint. Results for CYP2C9 are shown in Figure 5. Since both PB and RIF are known as prototypical inducers of CYP2C, CYP2C9 activities were reported following 72 h treatment with PB (500 μM) and RIF (10 μM). Panel D, E and F showed that the level of CYP2C9 induction was roughly similar regardless of the inducer used (PB or RIF) and reached twofold to threefold induction over control. In some cases, a significant statistical difference was observed between the classical approach and the cocktail strategy. However, no clear trend was observed and the statistical differences were not always in the same direction (e.g. CYP activity higher with classical approach compared to cocktail strategy). These statistical differences probably result from a lower experimental variability in these conditions suggesting that these differences were likely not related to a specific DDI occurring with cocktail approach. The results for CYP3A4 are shown in Figure 6. For this P450 enzyme, for all conditions, no statistical difference was observed between incubation with midazolam alone or using the cocktail strategy whatever the endpoint used (absolute enzyme activity or fold-induction). Regarding the level of CYP3A4 induction, it reached 2- to 4-fold over control depending on the human donor which was in the usual range.

Having shown that the cocktail approach could be used for CYP induction and that no significant DDI occurred during the incubation step, additional validation studies were performed to further firm up the use of the cocktail. First, we wanted to confirm that the cocktail

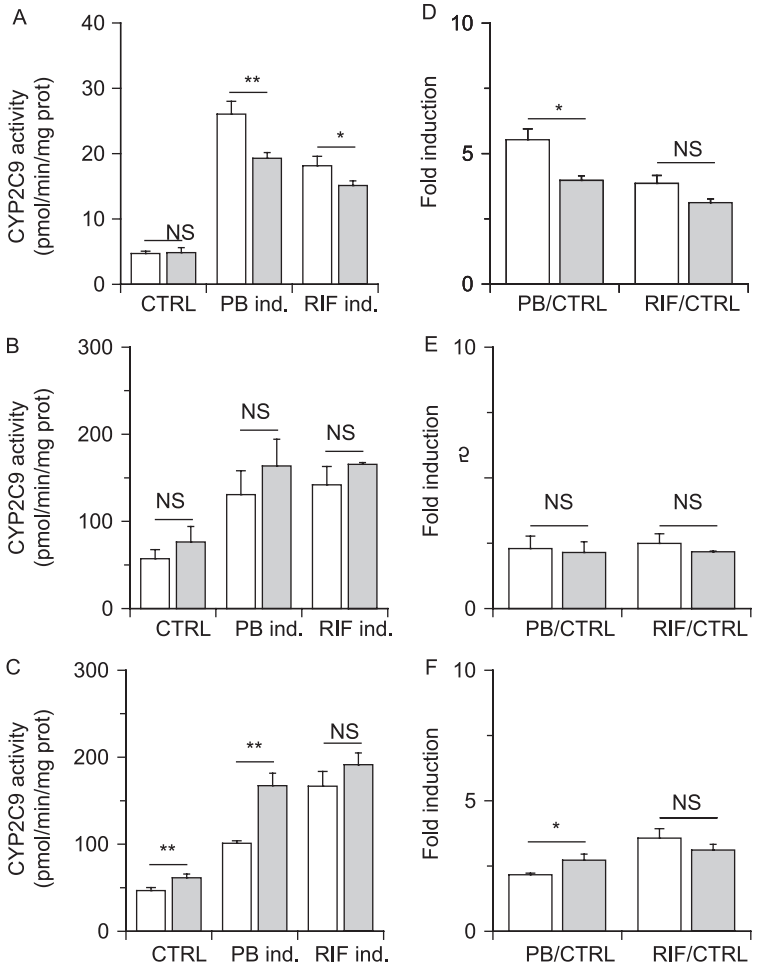

Figure 5. Cryopreserved human hepatocytes from 3 donors (Hu4037: A and D; Hu4122: B and E; Hu8063: C and F) were treated with vehicle (0.1% DMSO), PB (500 μM) or RIF (10 μM) for 3 days at 37°C with medium renewal every *ca.* 24 h before the determination of 4'-hydroxydiclofenac as marker of CYP2C9 after 30 min incubation *in situ* with diclofenac (10 μM) (open bar: single incubation; grey bar: incubation using the cocktail strategy [Table 2]). Results are the mean of a triplicate determination and are expressed as enzymatic activity (A, B, C) or fold-induction over control cells (D, E, F). NS: Not significant ($p > 0.05$) *$p < 0.05$, **$p < 0.01$.

approach could also be used to derive EC_{50} and E_{max} values when different concentrations of reference inducers are used. To this aim, hepatocytes were incubated with increasing concentrations of reference inducers and then, enzymatic activities were determined, either using cocktail approach or single incubation. As shown in Figure 7, no difference between fold-induction determined using cocktail approach (open symbol) and using single incubation (closed symbol) was observed for CYP1A2 (panel A), CYP2B6 (panel B), CYP2C9 (panel C) and CYP3A4 (panel D). Even though final EC_{50} and E_{max} values could not be determined accurately for CYP2B6 and CYP2C9 mainly because of high concentration of phenobarbital to be used, the results showed no difference between both approaches on a wide range of concentration of reference inducers. For CYP1A2, EC_{50} and E_{max} values were, for single incubation, 3.5 ± 0.6 μM and 169 ± 16 fold increase, respectively and, for cocktail incubation, 3.6 ± 0.4 μM and 134 ± 9 fold increase, respectively. For CYP3A4, EC_{50} and E_{max} values were, for single incubation, 1.6 ± 1.3 μM and 26 ± 4 fold increase, respectively and, for cocktail incubation, 0.8 ± 0.3 μM and 18 ± 4 fold increase, respectively.

As last validation experiment, we evaluated the inter-day reproducibility of the present CYP induction assay. Hepatocytes from three different donors were treated with reference inducers for 72 h according the classical protocol. At the end of induction period, fold induction was assessed using the cocktail approach only. The full assay (from hepatocytes thawing up to enzymatic activities measurement) was repeated on three different occasions to check for inter-day reproducibility. Results are shown on Figure 8 and clearly demonstrated minimal difference between assays for all CYPs as well as for the three donors.

Use of salicylamide in CYP induction assay

Salicylamide has sometimes been used in CYP induction assay in order to inhibit phase II metabolism, especially in the case of midazolam. Indeed, 1'OH-midazolam is further metabolized by phase II enzymes to midazolam-*O*-glucuronide. To prevent this phase II metabolism and to obtain the true CYP3A4 activity, salicylamide has been used by some authors (Ring et al. 2005). In this paper, we investigated the impact of salicylamide on the

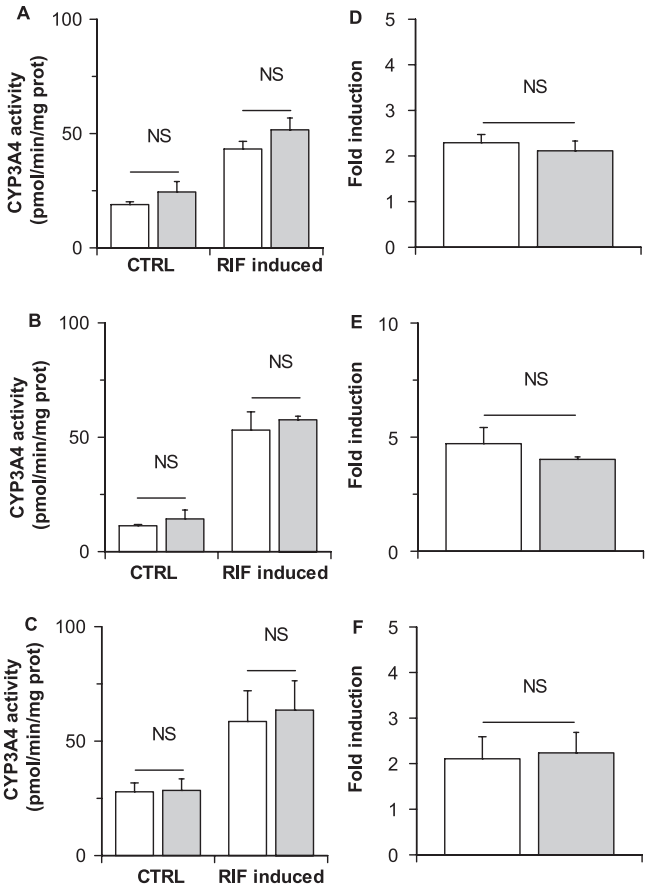

Figure 6. Cryopreserved human hepatocytes from three donors (Hu4037: A and D; Hu4122: B and E; Hu8063: C and F) were treated with vehicle (0.1% DMSO) or RIF (10 μM) for 3 days at 37°C with medium renewal every *ca.* 24 h before the determination of 1'- hydroxymidazolam as marker of CYP3A4 after 30 min incubation *in situ* with midazolam (3 μM) (open bar: single incubation; grey bar: incubation using the cocktail strategy [Table 2]). Results are the mean of a triplicate determination and are expressed as enzymatic activity (A, B, C) or fold-induction over control cells (D, E, F). NS: Not significant ($p > 0.05$).

absolute CYP activity and on the fold-induction parameters to clearly identify if it was worth to use it, since, to our knowledge, no available data showed the advantage of salicylamide in CYP induction studies.

To this aim, three donors (Hu4037, Hu4122, Hu8063) were used and salicylamide (1 mM) was added during the incubation with probe substrates (classical and cocktail approach were compared). For sake of clarity, only the results from one representative donor using the classical approach for the measurement of CYP activities are represented in Figure 9. Results for CYP1A2, CYP2B6, CYP2C9 and CYP3A4 are represented in panels A-E, B-F, C-G, and D-H, respectively. In panels A, B, C and D, results are expressed as absolute CYP activities (pmol/min/mg prot) whereas in panels E, F, G and H, results are reported in fold-induction. Results showed that salicylamide significantly inhibited CYP1A2 activities in control and induced hepatocytes (approximately 60% inhibition, panel A). However, no significant difference was observed between incubation with or without salicylamide when the fold induction parameter was used as endpoint (panel E). In order to confirm the inhibition of CYP1A2 by salicylamide, human liver microsomes were used to rule out potential effect of salicylamide on phase II enzymes. Interestingly enough, the results presented in Figure 10 showed that salicylamide dose dependently inhibits CYP1A2 activities with approximately 50% inhibition at 2 mM which is in accordance with our results obtained in hepatocytes. Regarding CYP2B6 (panels C and G from Figure 9), no significant effect of salicylamide was observed on CYP activities and on the fold induction parameter. For CYP2C9 (panels C and G from Figure 9), there was a trend of increased CYP activities in presence of salicylamide. Even though this increase was moderate (maximum 30%), it reached statistically significance only in induced hepatocytes. However, when looking at the fold induction parameter, this statistical difference disappeared. As expected for CYP3A4, the presence of salicylamide increased the absolute CYP3A4 activities in both control and induced hepatocytes. However, when results were processed to obtain the fold induction parameters, there was no effect of salicylamide.

Discussion

As of today, the scientific community (industry, regulatory, academia) unanimously accepted and considered that primary human hepatocytes are the gold standard

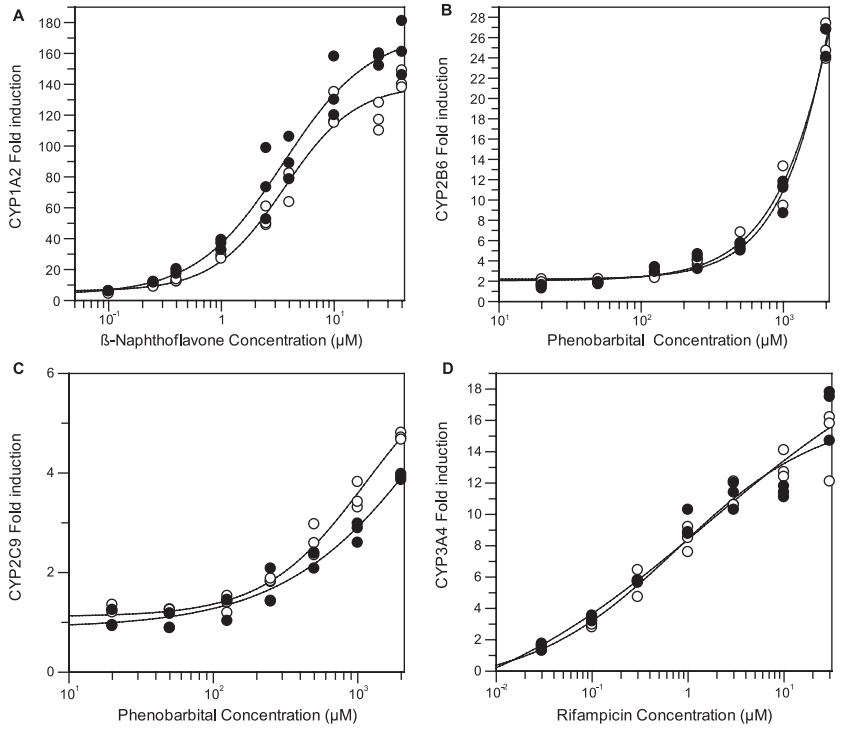

Figure 7. Cryopreserved human hepatocytes from one representative donor Hu4237 were treated with vehicle or with increasing concentrations of reference inducers, i.e. β-NF (1-40 µM, panel A), phenobarbital (20–2000 µM, panel B and C), rifampicin (0.03–30 µM, panel D). CYP1A2 (panel A), CYP2B6 (panel B), CYP2C9 (panel C) and CYP3A4 (panel D) activities were determined following *in situ* incubation using cocktail strategy (open symbol ○) or using single incubation (closed symbol ●). Data are expressed as fold induction over vehicle treated hepatocytes.

in vitro assay to assess the potential for drug candidates to induce human CYP expression. Human hepatocytes are a complete cellular system with properties analogous the liver function, which is essential to effectively model the inducibility of drug candidates. Indeed, primary human hepatocytes have been shown to efficiently model human *in vivo* induction responses and are well recognized by FDA and EMA as the preferred *in vitro* tool for assessing induction potential (Huang & Stifano 2006; European Medecines Agency and Committee for Human Medicinal Products, 2010). Regarding the use of fresh or cryopreserved hepatocytes for these CYP induction studies, the consensus has never been so clear-cut mainly because of two major drawbacks, i.e. the platebility and viability after thawing. However, these last years, considerable improvements in the cryopreservation process have been made making that cryopreserved hepatocytes do no longer suffer from their historical inconveniences. In addition to that, the convenience of cryopreserved human hepatocytes resulted in considerable regain of interest and demonstrated their ability to be an adequate model for the *in vitro* assessment of CYP induction. Among the different endpoints that could be used for the evaluation of CYP induction, enzyme activity is by far and in most cases, the most recommended approach for a reliable prediction of the *in vivo* situation. In the present study, *in situ* determination of the enzyme activity has been preferred for two major reasons, (i) use of less hepatocytes and (ii) increased throughput compared to microsomes that required additional preparation steps. In order to minimize the use of hepatocytes, a so-called cocktail approach was investigated. In addition to the lower total amount of hepatocytes required which is not negligible knowing the price of hepatocytes, the cocktail approach has many other interesting advantages. Indeed, since multiple CYPs are measured simultaneously in one samples, there is much less samples to prepare and handle as well as to analyze, therefore saving human ressources and reducing utilization time of mass spectrometer which is critical in discovery.

Even though the use of cryopreserved human hepatocytes is becoming more common these days, we did not find robust validation in the literature for the use of cocktail approach in CYP induction assay. Indeed, most of the study described in the literature using the cocktail approach, were for CYP inhibition experiment in liver microsomes (Dierks et al. 2001; Testino & Patonay 2003; Floby et al. 2004; Kim et al. 2005). Having a deeper look in the CYP induction studies, it appeared that they all have some gaps that the present study tries to fill. For example, Dixit et al. (2007) determined the enzyme activity using microsomes and had to use two different cocktails to avoid DDI. In the study of Mohutsky et al. (2005), CYP2B6 was not included in the P450 enzymes investigated. In the study from Lahoz et al. (2008), benzoxyresorufin was used as probe substrate for CYP2B6 which is not considered by regulatory agencies as preferred substrate for CYP2B6. In addition, the incubation time used for the CYP activity determination was quite long (2 h) even though they claimed that it was under linear condition. This is in

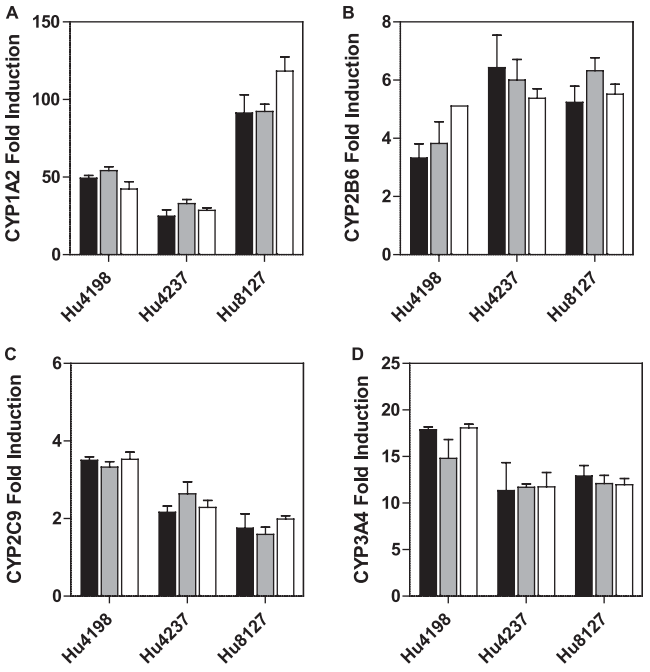

Figure 8. Cryopreserved human hepatocytes from three different donors (Hu4198, Hu4237 and Hu8127) were used for interassay variability assessment. Human hepatocytes were incubated with vehicle (0.1% DMSO), β-NF (25 μM – panel A), PB (500 μM – panel B and C) or RIF (10 μM – panel D) for 3 days at 37°C with medium renewal every *ca.* 24 h. CYP450 activities were determined following 30 min incubation *in situ* with cocktail solution (composition described in Table 2). Results are expressed as fold induction over vehicle treated hepatocytes for CYP1A2 (panel A), 2B6 (panel B), 2C9 (panel C) and 3A4 (panel D). The experiment was repeated on three different occasions represented by three different colour codes (■ assay 1, ■ assay 2, □ assay 3). Results are the mean of a triplicate determination with error bars being the standard deviation.

contradiction with our data where we showed that in induced hepatocytes, the formation of 1'OH-midazolam was linear up to 30 min. However, it was not clear if they checked the linearity also in induced hepatocytes where we observed that this could be different from the control hepatocytes. Recently, Feidt et al. (2010) issued another study with the use of cocktail for CYP induction. However, in order to rule out potential risk of DDI during the development of its cocktail, other probe substrate than those classically used and recommended by FDA and EMA were used (e.g. use of atorvastatin as marker for CYP3A4 instead of midazolam or testosterone, propafenone for CYP2D6 instead of dextromethorphan or bufuralol). Indeed, as already mentioned, the major issue with this cocktail approach remained the potential cross-interaction between probes during the incubation process.

Since the objective of the present study was the development of a straightforward CYP induction assay that could be used for submission, classical probes were used and limited to those strictly required, i.e. phenacetin for CYP1A2, bupropion for CYP2B6, and midazolam for CYP3A4. Even if not strictly needed, diclofenac (CYP2C9) was also added as a marker for CYP2C.

Another critical point with the use of cocktails is the selection of the incubation conditions like probe concentration and time of incubation. Incubation time is important because linearity of the enzymatic reaction is not the same for all probes and a compromise between linearity and detection should be made in the case of the cocktail approach. In some studies, the linearity of the enzymatic reaction was not taken into account in the validation of the cocktail (Testino & Patonay 2003; Tolonen et al. 2007). Based on our data, we selected an incubation time as shorter as possible for two reasons (i) first to remains within the linear conditions for midazolam and (ii) to limit phase II metabolism of 1'HO-midazolam. Regarding the selection of the concentration of each probe substrate, this was a compromise between limit of detection of the analytical method and the risk of DDI between probes. In order to select the concentration that will be used in the cocktail, we first performed a K_m/V_{max} experiment under control and treated conditions (with reference inducers). Our results (K_m values) were in accordance with published values (von Moltke et al. 1996; Faucette et al. 2000; Brown et al. 2007; Hallifax et al. 2008). Enzymatic kinetic for acetaminophen formation was biphasic as expected due to the involvement of low affinity P450 enzymes for this reaction (CYP2C9, CYP2C19, CYP2E1, CYP2A6, CYP2D6) (Venkatakrishnan et al. 1998; Kobayashi et al. 1999). For CYP3A4 and CYP2C9, enzymatic kinetics was classical Michaelis–Menten reaction whereas for CYP2B6, it was a biphasic kinetic in control hepatocytes and Michaelis–Menten in induced hepatocytes. On a first instance, this could be surprising to observe two different kind of kinetics within the same batch of hepatocytes. However, an easy explanation is that under induced conditions, the expression CYP2B6 has been so up-regulated by the inducer that it masks the activity of the other low affinity enzymes making that the biphasic model is no longer the adequate model to fit the data. In addition, this K_m/V_{max} experiment also clearly showed, as expected, that the fold-induction parameters is independent of the substrate concentration. On that basis, concentration of each probe substrate in the cocktail was selected to find a compromise between the lower concentration of probe substrate achievable to minimize the risk of DDI and a concentration allowing detection of formation of metabolite in control conditions to calculate the fold-induction parameters. Now that the conditions for the use of the cocktail approach were selected (time of incubation and concentration of each probe substrate), the final step of the study was the validation, strictly speaking, of this approach compared to classical one (single incubation of probe substrate).

First, even though this was not the primary objective of the present study, our results are in accordance with other studies and confirmed that in the conditions we used, cryopreserved human hepatocytes are a suitable model to evaluate the potential of new chemical entities to induce the expression of P450 enzymes. Indeed, β-NF elicited a 4- to 35-fold induction of CYP1A2, PB a 6- to 30-fold induction of CYP2B6 and RIF a 2- to 5-fold induction of CYP3A4. These values were obviously

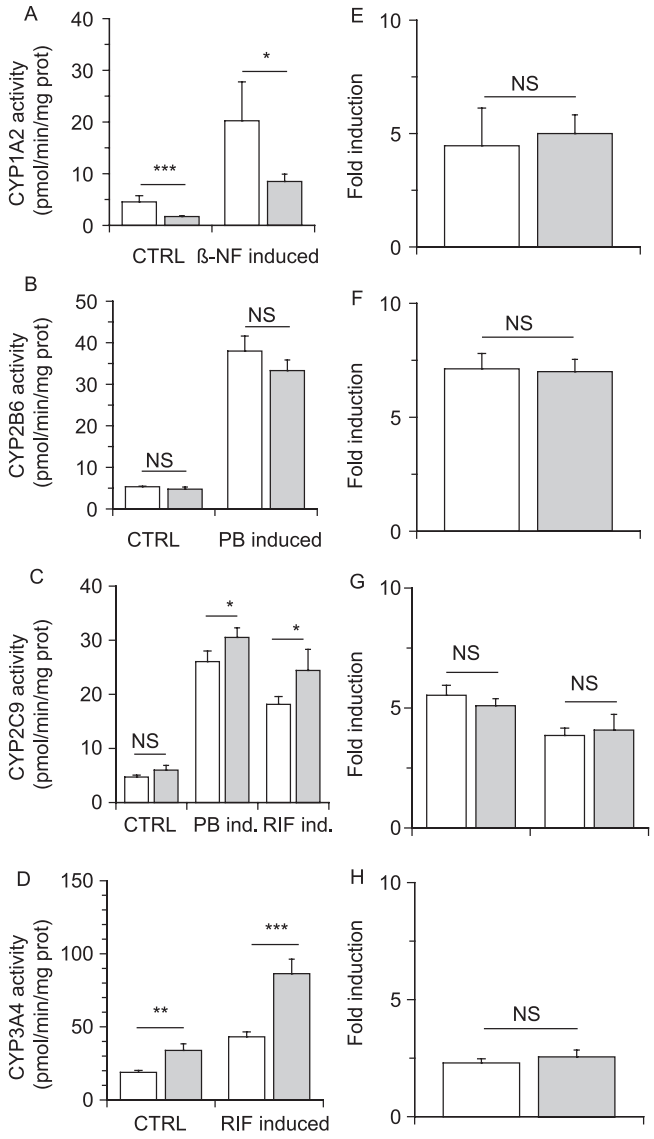

Figure 9. Cryopreserved human hepatocytes from one representative donor Hu4037 were treated with vehicle (0.1% DMSO), β-NF (25 μM), PB (500 μM) or RIF (10 μM) for 3 days at 37°C with medium renewal every *ca.* 24 h. CYP450 activities were determined following 30 min incubation *in situ* with phenacetin (10 μM), bupropion (250 μM), diclofenac (10 μM) or midazolam (3 μM) (single incubation) as substrate marker for CYP1A2, 2B6, 2C9 and 3A4, respectively, in the absence (open bar) and in the presence of 1 mM salicylamide (grey bar). Results are the mean of a triplicate determination and are expressed as enzymatic activity (A, B, C, D) or fold-induction over control cells (E, F, G, H). NS: Not significant ($p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

dependent on the donor but remained in the classical range of fold-induction usually described (Faucette et al. 2000; Lu & Li, 2001; Madan et al. 2003; Zhang et al. 2010).

Most importantly are the results of the cocktail which clearly and unambiguously showed that the use of cocktail strategy as defined in the present study is adequate and optimal for CYP induction assay. Indeed, in these conditions, no significant and biologically relevant DDIs were observed between probe substrates since the absolute CYP activities were similar using both methods. In addition, this was further confirmed by using three different batches of cryopreserved human hepatocytes. Even though if in some conditions, statistical differences were observed, they were mainly ascribed to a lower variability in those conditions. Indeed, for a specific P450 enzyme, the statistical difference observed was not reproduced in all donors tested and was not in the same direction between donors. These observations strengthen the hypothesis that these statistical differences were without or of low biological relevance. In addition, the only P450 enzymes for which a statistical difference was observed on the fold induction endpoint, was CYP2C9 which is not *per se*, mandatory in the CYP induction assay. Moreover, when observed, the difference never exceeded 30% which remained within the variability of the assay.

The adequacy of cocktail approach was also confirmed in an EC_{50}-E_{max} experimental setting where a huge range of enzyme activity has to be assessed. Indeed, the results showed no difference between cocktail and

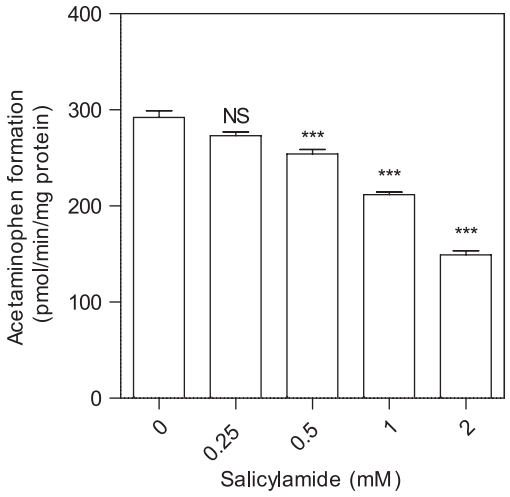

Figure 10. Effect of salicylamide on the formation of acetaminophen in human liver microsomes. Human liver microsomes were incubated 20 min at 37°C with 60 μM phenacetin and salicylamide. Results are represented as mean ± SD (n = 3). ANOVA with Dunnett post test (***$p < 0.001$).

single incubation whatever the concentration of reference inducers used.

Finally, the final assay (using cocktail incubation) was shown to be reproducible as no significant difference were observed among results when performed on three different occasions. To our knowledge, the present study is the first validation of the cocktail strategy for its application in CYP induction assay with the advantage of using the FDA and EMA recommended probe substrate for assessment of CYP1A2, 2B6, 2C9 and 3A4 activities. However, the recent new FDA draft guidance (Huang 2012) on DDI changes things since in the current version, it is recommended to use mRNA as endpoint for CYP induction. Compared to mRNA, the main drawback of measuring CYP activities as endpoint in CYP induction is in the case where the test compound is a CYP3A4 mechanism-based inhibitor and therefore could generated erroneous conclusions that such compound will not cause clinical induction, yet the other induced but non-inactivated enzymes will still have increased expression and activity. However, this scenario will only occurred if the two following conditions are met: (i) the compound is a potent mechanism-based inhibitor of CYP3A4 and (ii) mechanism-based inhibition potential of the compound is not known at the time of CYP induction. Usually, the CYP inhibition profile of NCE is identified before CYP induction meaning that, in the latter case, the right interpretation of CYP induction data could be drawn using enzymatic activities. In addition, mechanism-based inhibition do not always translate into no observed effect in CYP induction assay, it really depends on the potency of inactivation and affinity for CYP3A4 as well as on the affinity for PXR. Moreover, potent mechanism-based inhibitor of CYP3A4 are dismissed during development meaning that they should not be tested in CYP induction assay. However, this scenario really depends on the screening strategy put in place in the company. Even though the current recommendation in FDA DDI guidance is adopted in the final document, we strongly believe that measurement of CYP activities in CYP induction assay still deserve a place in the overall assessment of DDI potential for all the reasons explained previously. Even if mRNA data are used for CYP induction assay, measuring enzymatic activities in parallel will provide more mechanistic insight in the overall picture of DDI and brings a better knowledge of the kind of interaction that can be expected.

In order to close the debate on the real need to use phase II inhibitor in CYP induction study, we evaluated the effect of salicylamide on (i) absolute CYP activity and (ii) on the fold induction parameters, the final endpoint used in this CYP induction study. Our data clearly demonstrated that (i) at the concentration used (1 mM), salicylamide inhibits CYP1A1/2, (ii), as expected, the absolute CYP3A4 activities are enhanced due to decrease of phase II metabolism of 1'HO-midazolam and (iii) the use of salicylamide did not increase significantly the fold induction parameter. To our knowledge, the inhibition of phenacetin O-deethylation reaction by high concentration of salicylamide (≥1 mM) has never been reported. MacDonald et al. (2004) reported that salicylamide (250 μM) had no effect on EROD activities (marker of CYP1A) in HepG2 cells, which is in accordance with our results but they did not investigate higher concentration of salicylamide which are needed to inhibit phase II reaction.

In conclusion, the cocktail assay validated in the present study using cryopreserved human hepatocytes cultured in 48-well plate is a rapid, straightforward and cost-effective tool to assess induction of CYP1A2, 2B6, 2C9 and 3A4. In addition, the use of salicylamide is not recommended in CYP induction because of it can generate some DDI without providing any added value.

Declaration of interest

The authors report no declarations of interest

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