PERFORMANCE OF BIOTRANSFORMATION OF HUMAN PRIMARY HEPATOCYTES EXPOSED TO A PHARMACOLOGICAL COCKTAIL INSIDE A LIVER MICROCHIP

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ABSTRACT
In this paper, we have compared the biotransformation of a cocktail of 7 pharmaceutical molecules by cryopreserved primary human hepatocytes cultivated in a microchip and in Petri dishes. Each molecule corresponds to specific substrate of the key enzymes implied in the xenobiotic metabolism. A dynamic culture condition is used during the incubation period in the microchip and the hepatocyte metabolic activity is followed by LC-MS/MS analysis. The results showed greater activity for CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A and UGT in the microchip when compared to the Petri cultures and confirm the relevance of our microchip in pharmaceutical study.

KEYWORDS: Liver, microchip, CIME cocktail, primary human hepatocyte, drug metabolism.

INTRODUCTION
The liver plays a major role in the biotransformation of drugs and xenobiotics through high enrichment of metabolizing enzymes such as CYPs450 and UGTs. Primary Human hepatocytes are considered to be one of the best models in in vitro pharmacological studies. Unfortunately, hepatocytes rapidly lose a wide range of their specific detoxification functions when they are cultivated in 2D static conditions or in suspension.

In this study, we proposed to use a microchip to cultivate primary human hepatocyte cells in dynamic condition. To assess the specific liver functions, we incubated the cells with the CIME cocktail (Carte d’Identité MEtabolique) which was designed to address the phase I enzymes (CYPs), and phase II enzyme simultaneously. To avoid intensive labour and parallel analysis using each substrate, a simultaneous LC-MS/MS assay of all substrates and the corresponding metabolites in culture media has been developed.

EXPERIMENTAL
The microchip setup represented in Fig.1A consists on a reservoir extended by a perfusion circuit connected to the microchip. It permits the recirculation of the culture medium likewise “blood circulation”. The microchip is made of 2 PDMS layers. The biotransformation experiments consist on the incubation of a cocktail of 7 molecules. The molecules and their substrates are given in table 1. The experiments were realized in parallel using 12 microchips and 24 Petri dishes. A cell density of 1.1±0.1x10^5 cells/cm² was used in the biochip (condition M) whereas two cells densities are used in Petri dishes (P1 1.2±0.1x10^5 cells/cm² ; P2 2.4±0.1x10^5 cells/cm²). Incubation of the molecules is performed for 4h. Analyses of the metabolites are realized by LC/MSMS methods to determine the metabolic ratio of the specific reaction catalyzed by the different enzymes.

The metabolic ratio (MRt) was defined by the ratio of the Area Under the Curve (AUC) of the reaction products divided by the sum of all the AUC of the parent substrate and its metabolites. The equation (1) is as follows:

\[
MRt = \frac{\text{AUC (P)}}{\text{AUC (P)} + \text{AUC (S)}} - MR_0 \times 100
\]

In which MRt is the Metabolic Ratio at time t, AUC is the Area Under the Curve of the P Product of the enzymatic reaction and the S substrate.

At the end of the incubation period, a live/dead assay was performed using CalceinAM/Propidium Iodide probes to assess the viability of cells cultivated in the microchip.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Enzymes</th>
<th>Analyzed metabolites</th>
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<tbody>
<tr>
<td>Midazolam</td>
<td>CYP3A</td>
<td>1-OH-midazolam/ 4-OH-Midazolam</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>CYP2D6 et CYP3A</td>
<td>Dextrophan/ 3-Methoxymorphinan</td>
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<tr>
<td>Caffeine</td>
<td>CYP1A2</td>
<td>Paraxanthine</td>
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<tr>
<td>Tolbutamide</td>
<td>CYP2C9</td>
<td>4-OH-Tolbutamide</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>UGT</td>
<td>Acetaminophen-glucuronide</td>
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<tr>
<td>Amodiaquine</td>
<td>CYP2C8</td>
<td>Desethylamodiaquine</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>CYP2C19 et CYP3A</td>
<td>5-OH-omeprazole/sulfone</td>
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RESULTS AND DISCUSSION

Cell morphology after 12h of adhesion inside the liver microchip is shown in Figs 1B. 85% of the inoculated cells adhered to the microchip. The cells covered 90% of the microchip. The cells displayed a classical polygonal shape demonstrating successful adhesion in the PDMS-coated collagen surface of the liver microchip.

Figure 1: (A) Setup of the liver microchip and perfusion circuit connected to the peristaltic pump; (B) Hepatocytes morphology after adhesion; (C) Live/dead assay after 4h of perfusion in the liver microchip.

At the end of the 4h perfusion period, a Live-Dead assay was performed in order to assess the viability of the cells (Fig 1C). Live cells appeared green and dead cells red. There is a majority of green cells indicating that the primary human hepatocytes were successfully cultivated in a healthy condition inside the microenvironment and in a dynamic situation.

Figure 2: Metabolic ratio comparison between liver microfluidic biochip (M: 1.1±0.1x10^5 cells/cm²) and Petri culture (P1: 1.2±0.1x10^5 cells/cm²; P2 2.4±0.1x10^5 cells/cm²). (A) 1-OH midazolam; (B) 4-OH-midazolam; (C) Dextrorphan; (D) 3-Methoxymorphinan; (E) Paraxanthine; (F) 4-OH-tolbutamide; (G) 5-OH-omeprazole; (H) Omeprazole-sulfone; (I) Acetaminophen glucuronide; (J) Desethylamodiaquine.
Fig. 2 shows the metabolic ratio of all compounds after 4 hours of incubation compared for the 3 conditions tested (microchip “M”, Petri “P1” and “P2”). The results were expressed in terms of metabolic ratio normalized by the initial cell number. When we compared the metabolic ratio in the Petri conditions, we did not observe any significant difference between the 2 groups after 4 hours of culture. We could not separate the two conditions, as illustrated by midazolam or omeprazole metabolism (Fig 2AB-2GH). Interestingly, the metabolic ratio appeared to be higher in the liver microchip when compared to the Petri conditions for 5 out of 7 substrates: dextromethorphan (Fig 2CD), caffeine (Fig 2E), tolbutamide (Fig 2F), acetaminophen (Fig 2I) and omeprazole (Fig 2GH). These results suggest that the enzymatic reactions related to CYP3A4, CYP2D6, CYP1A2, CYP2C9, UGTs and CYP2C19 activities were more effective in the liver microchip when compared to the Petri conditions. On the contrary, 2 substrates appeared less or equally metabolized in the liver microchip than in petri dishes: midazolam and amodiaquine related to CYP3A and CYP2C8 respectively. These polar molecules are probably adsorbed by the perfusion circuit.

Finally, we performed a simple culture protocol, in which a monoculture of hepatocytes was cultivated in the PDMS microchip. We reached a higher activity in our PDMS microchip concerning CYP1A2, CYP2C9, CYP3A, CYP2D6, CYP2C19 and UGT activities when compared to Petri cultures. These enzyme activities appeared consistent with our previous investigations with HepG2/C3a in a PDMS microchip in which the phase I (CYP1A1, 2B6, 3A4, 3A5, 3A7), phase II (SULT1A1, SULT1A2, UGT1A1, UGT2B7) and phase III (MDR1, MRP2) enzymes involved in xenobiotic metabolism were found to be up regulated when compared to Petri static cultures [1]. This highlights the fact that our microchip configuration made possible to enhance the metabolic activity of the hepatocytes by maintaining hepatic differentiation without using complex co-culture systems or spheroid formation.

Furthermore, the CIME cocktail allows the monitoring of phase I and phase II enzymes simultaneously inside the microchip in one set of experiments. Thus, this operation could be used as a routine assay for evaluating microfluidic culture performance before running drug screening analyses. In addition, in vitro exposure to the CIME cocktail can reproduce the in vivo situation resulting in treatments in which several drug substrates from several of these enzymes are often given to a patient simultaneously.

CONCLUSION
In this study, we successfully performed primary human hepatocyte culture inside a liver microchip. The microchip made healthy dynamic culture conditions possible. We investigated the production rate of 10 metabolites, resulting in the conversion of a cocktail of 7 parent substrates. The cocktail made rapid hepatocyte profiling possible, as well as metabolic characterization, using a single detection technique. Seven of the 10 metabolites tested in this study showed a higher production rate in the liver microchip than in conventional culture methods. We showed that dynamic culture conditions and the microenvironment of the liver microchip resulted in higher activity of the enzymes involved in xenobiotic metabolism. This led to higher xenobiotic conversion for 5 of the 7 substrates. Thus, the microchip appeared to be a sensitive new culture method using primary human hepatocytes for future models for pharmaceutical drug screening and toxicity analysis of xenobiotics.

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REFERENCES

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