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Investigation of the hepatotoxicity of flutamide: Pro-survival/apoptotic and necrotic switch in primary rat hepatocytes characterized by metabolic and transcriptomic profiles in microfluidic liver biochips

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ABSTRACT

We investigated the effects of the liver damage induced by flutamide in primary rat hepatocytes using liver microfluidic biochips. Flutamide is a non-steroidal anti-androgenic drug. Two flutamide concentrations, 10 μM and 100 μM, were used to expose the hepatocytes for 24 h under perfusion. Thanks to the maintenance of hepatocyte differentiation phenotype and to the biotransformation performance in the microfluidic cultures, the metabolic ratio analysis of hydroxyflutamide, flutamide-gluthatione and hydroxyflutamide-gluthatione productions demonstrated saturation of the drug's biotransformation process and the maintenance of a high level of flutamide at 100 μM when compared to 10 μM. A microarray analysis comparing flutamide (10 or 100 μM) with controls revealed a common response for both concentrations illustrated by modulating the expression of the mRNA of genes associated with mitochondrial perturbation, of the proliferator-activated receptors (*Ppar*) signaling, lipid and fatty acid metabolism, antioxidant defense, and cell death pathways, consistently with *in vitro* and *in vivo* reports. Additionally to literature reports, our integration of the transcriptomic profiles demonstrated a specific dose dependent response. We found at 10 μM a typical pro-survival/apoptosis network activation (through *IGF/PDGFD* upstream route and *via* a downstream up regulation in *CREB5, BCL2, IKBKG* routes in the PI3K/signaling). We also found a down regulation of mRNA levels in sugar and amino acid metabolism pathways. At 100 μM a typical necrosis switch was observed associated with a down regulation of the tight junctions' pathway, a cellular aggregation and a reduction of the cell viability. Altogether our data demonstrated the potential and the sensitivity of our liver microfluidic cultures to evaluate xenobiotic toxicity by improving *in vitro* analysis and reproducing both *in vitro* and *in vivo* results. Finally, we proposed two integrated synthetic networks to describe the response of rat hepatocytes to both exposure concentrations of flutamide.

1. Introduction

Pharmaceutical companies are facing an Research and Development (R&D) productivity crisis with ever increasing drug development costs and time to market. A large part of the problem lies in high failure rates of drugs during the expensive clinical trial phase (Bains, 2004). Currently ~90% of all drugs that enter clinical trials end up failing due to unpredicted toxicity (~30%), low efficacy (~40%) and Absorption Distribution Metabolism Elimination (ADME) profile (~10%). The test methods available (traditional *in vitro*, *in vivo*, *ex vivo* and *in silico*) are not accurate enough (*e.g.* traditional *in vitro*) and/or not practical enough (*e.g. ex vivo*) for pharmaceutical companies to improve prediction for the human condition, and thus make better decisions earlier on in the process. At the same time, chemical, cosmetics and personal care companies are facing increasingly strict regulations regarding animal trials – with a ban already in place for cosmetics in Europe. For many test endpoints there are no sufficiently accurate alternatives to animal trials and both the industry and its regulators are keen to find alternatives.

The new technology provided by microfluidic liver biochips can, for several important application areas, meet this unmet need in pharmaceutical companies as well as chemicals, cosmetics and

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personal care industries. By replicating key aspects of *in vivo* conditions, such as three dimensional cell structures (Powers et al., 2002), circulatory flow (Baudoin et al., 2011) and zonation (Tilles et al., 2001; Cheng et al., 2012), multi organs (Viravaidya and Shuler, 2004; Choucha-Snouber et al., 2013a) or multi cellular co-cultures (Novik et al., 2010), leading to a closer representation when compared to traditional *in vitro* methods, the human-like environment can be mimicked. The concept of the technology consists of a micro environment for dynamic three dimensional microstructure cell cultures that become "bioartificial organs" capable of simulating human physiology. Within this context, our group has developed an integrated platform, the IDCCM box (for Integrated Dynamic Cell Culture in Microsystems), which makes parallel connection of biochips possible, thus increasing throughput and facilitating the investigations (Baudoin et al., 2012, 2013).

Functionality and the potential for hepatic metabolism in such biochips have been illustrated *via* analysis of phases I and II xenobiotic metabolism-related genes and by drug clearances and biotransformation (Chao et al., 2009; Baudoin et al., 2013; Legendre et al., 2013). In addition, more in-depth investigations of hepatotoxicity have also been made possible thanks to the compatibility of liver biochip cultures with conventional biochemical analysis processes such as metabolomics, proteomics and transcriptomics (Shintu et al., 2012; Prot et al., 2011a, 2012). Thus, basal analysis on primary rat hepatocytes and human liver cell lines has shown that cell defense mechanisms including drug metabolism were over expressed in the microfluidic liver biochips (Prot et al., 2011b; Legendre et al., 2013). When applied to acetaminophen injury with cell lines, this led to reproducing a substantial sequence of the mechanism of actions when compared to Petri analysis (Prot et al., 2012).

In order to investigate the effect of drug treatment, we focused our study on the non-steroidal anti-androgenic drug, flutamide. Flutamide is one of several beneficial drugs used in the treatment of prostate cancer and in combination with oral contraceptives for the treatment of hirsutism and benign prostatic hyperplasia (Wang et al., 2002). In prostate cancer, tumor cells need testosterone to proliferate. Flutamide and its active metabolite, 2-hydroxyflutamide, compete with testosterone to bind to androgen receptors leading to impairment of testosterone signaling and modulation of the testosterone-dependent pathways. However, flutamide presents secondary toxicity in the liver. The mechanisms for liver damage associated with flutamide use are currently unknown, with many hypotheses for the mechanisms liable to play a part in the liver damage (*e.g.* P450-mediated bioactivation, mitochondrial dysfunction, generation of reactive oxygen species (ROS), inhibition of the bile salt transporter; Coe et al., 2006, 2007; Iwanaga et al., 2007). In addition, the toxicity of flutamide is also due to the production of hydroxyflutamide *via* the cytochrome P450-1A (CYP1A) biotransformation (Schulz et al., 1988; Shet et al., 1997). More particularly, flutamide was reported to have human hepatotoxic properties in post-marketing studies (Aizawa et al., 2003; Coe et al., 2006). The precise mechanism behind hepatic dysfunction in humans has not yet been clarified because it is probably the result of metabolic idiosyncrasy (Aizawa et al., 2003; Coe et al., 2006, 2007).

The plasmatic concentrations of flutamide and hydroxyflutamide after therapeutic doses ranging from 250 mg to 750 mg of flutamide have shown maximal values varying between 0.4 and 3 μM for flutamide and 3–10 μM for hydroxyflutamide (Schulz et al., 1988; Radwanski et al., 1989; Anjum et al., 1999). The *in vitro* IC_{50} of flutamide (including some data extracted in microchip cultures, Zhang et al., 2011), is reported to range between 30 and 90 μM for 24 h of exposure according to the culture methods and assays used (total protein synthesis in Kostrubsky et al., 2007; live dead assays in Zhang et al., 2011). In addition, primary rat hepatocyte exposure (0.36–1 μM) led to an increase in CYP1A2 mRNA levels, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, and reduced glutathione (GSH) contents (Wang et al., 2002).

In order to investigate low and high dose effects of flutamide and to enhance the knowledge on the potential mechanisms of hepatotoxicity, we have exposed rat hepatocytes in microfluidic conditions for 24 h to 10 and 100 μM concentrations of flutamide. The selection of those doses was based on the *in vivo* and *in vitro* data described above. Hepatocellular functions were evaluated in terms of glucose consumption, urea and albumin secretions. The metabolism performance of the hepatocytes was also confirmed by metabolite detection and CYP1A activity. The transcriptomic profile was then analyzed in order to identify the mechanism of action of flutamide in microfluidic biochips. Finally the data was compared with literature reports.

2. Materials and methods

2.1. IDDCM bioreactor

The entire culture set-up was called the IDCCM for "Integrated Dynamic Cell Culture in Microsystems". The concept and details of the IDCCM box and biochips are presented in detail in our previous work (Baudoin et al., 2011, 2012). Briefly, the polydimethylsiloxane (PDMS) biochips were connected to the bottom of the IDCCM box by a simple series of "plugging" ports (Fig. 1A). This format made for an easy "plug and display" of the biochips in external set-ups such as microscopes. The IDCCM is a manufactured polycarbonate box using the conventional format of the 24-well plate. Each microfluidic biochip is connected between two wells. The 24 wells were used as an inlet and outlet reservoir leading to the parallelized culture of 12 biochips (Fig. 1B). A specific cover was designed so that the polycarbonate box could be closed hermetically for continuous flow perfusion. The cover included ports for fluid perfusion and sampling. The hermetic closure of the IDCCM box and the pressure in the IDCCM box prevented any leakage or reservoir drain.

2.2. Primary rat hepatocyte cultures in the IDCCM

The hepatocytes extraction is given in detail in the Supplementary file 1 and in our previous work (Legendre et al., 2013). The experiments were performed over a period of 48 h which included three different phases: hepatocyte extraction (day-0, post extraction), the adhesion phase (day-1, 24 h of adhesion), which was performed at 37 °C, and a 24 h perfusion phase at 32 °C (Fig. 1C). The sterilization of the biochips, the IDCCM box and the perfusion circuit was achieved by autoclaving the whole set-up. The biochips were then connected to the box under sterile conditions.

Freshly isolated hepatocytes were cultured for the first 24 h in a seeding medium composed of William's E Glutamax medium (Fisher Scientific, Illkirch, France) Supplemented with bovine insulin (5 μg/mL, Sigma–Aldrich, Saint-Quentin Fallavier, France) and fetal bovine serum (10%v/v). To enhance cell adhesion, the inner surface of the biochips was coated with rat tail type 1 collagen (0.3 mg/mL, BD Biosciences) prepared in phosphate-buffered saline (PBS) for 1 h at 37 °C in a humidified atmosphere supplied with 5% CO_2. After washing with the seeding medium, the cells were inoculated inside the biochips at 0.5×10^6 cells per biochip (*i.e.* 0.25×10^6 per cm^2). After the 24 h adhesion period, cells were cultured in DMEM-F12 medium (Fisher Scientific) supplemented

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