

### 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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Audrey Legendre, Sébastien Jacques, Florent Dumont, Jérôme Cotton, Patrick Paullier, et al.. 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. Toxicology in Vitro, 2014, 28 (5), pp.1075-1087. 10.1016/j.tiv.2014.04.008 . hal-03820688

### HAL Id: hal-03820688 https://hal.science/hal-03820688

Submitted on 1 Aug2023

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#### 24 Abstract

25 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-26 27 androgenic drug. Two flutamide concentrations, 10µM and 100µM, were used to expose the hepatocytes for 24h under perfusion. Thanks to the maintenance of hepatocyte 28 29 differentiation phenotype and to the biotransformation performance in the microfluidic cultures, the metabolic ratio analysis of hydroxyflutamide, flutamide-gluthatione and 30 hydroxyflutamide-gluthatione productions demonstrated saturation of the drug's 31 biotransformation process and the maintenance of a high level of flutamide at 100µM 32 when compared to 10µM. A microarray analysis comparing flutamide (10 or 100µM) with 33 controls revealed a common response for both concentrations illustrated by modulating 34 the expression of the mRNA of genes associated with mitochondrial perturbation, of the 35 proliferator-activated receptors (*Ppar*) signaling, lipid and fatty acid metabolism, 36 antioxidant defense, and cell death pathways, consistently with in vitro and in vivo 37 reports. Additionally to literature reports, our integration of the transcriptomic profiles 38 demonstrated a specific dose dependent response. We found at 10µM a typical pro-39 survival/apoptosis network activation (through IGF/PDGFD upstream route and via a 40 downstream up regulation in CREB5, BCL2, IKBKG routes in the PI3K/signaling). We 41 also found a down regulation of mRNA levels in sugar and amino acid metabolism 42 pathways. At 100µM a typical necrosis switch was observed associated with a down 43 44 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 45 46 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 47 synthetic networks to describe the response of rat hepatocytes to both exposure 48 concentrations of flutamide. 49

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51 **Keywords:** Microfluidic biochips, rat primary hepatocytes, flutamide, hepatotoxicity, 52 transcriptomic, microarray, metabolism

#### 53 **1. Introduction**

54 Pharmaceutical companies are facing an Research and Development (R&D) productivity crisis with ever increasing drug development costs and time to market. A 55 large part of the problem lies in high failure rates of drugs during the expensive clinical 56 trial phase (Bains, 2004). Currently ~90% of all drugs that enter clinical trials end up 57 failing due to unpredicted toxicity (~30%), low efficacy (~40%) and Absorption 58 Distribution Metabolism Elimination (ADME) profile (~10%). The test methods available 59 (traditional *in vitro*, *in vivo*, *ex vivo* and *in silico*) are not accurate enough (e.g. traditional 60 in vitro) and/or not practical enough (e.g. ex vivo) for pharmaceutical companies to 61 62 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 63 facing increasingly strict regulations regarding animal trials – with a ban already in place 64 for cosmetics in Europe. For many test endpoints there are no sufficiently accurate 65 alternatives to animal trials and both the industry and its regulators are keen to find 66 alternatives. 67

The new technology provided by microfluidic liver biochips can, for several 68 important application areas, meet this unmet need in pharmaceutical companies as well 69 as chemicals, cosmetics and personal care industries. By replicating key aspects of in 70 vivo conditions, such as three dimensional cell structures (Powers et al., 2002), 71 circulatory flow (Baudoin et al., 2011) and zonation (Tilles et al., 2001; Cheng et al., 72 2012), multi organs (Viravaydia et al., 2004; Choucha et al., 2013a) or multi cellular co-73 74 cultures (Novik et al., 2010), leading to a closer representation when compared to 75 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 76 77 microstructure cell cultures that become "bioartificial organs" capable of simulating human physiology. Within this context, our group has developed an integrated platform, 78 79 the IDCCM box (for Integrated Dynamic Cell Culture in Microsystems), which makes parallel connection of biochips possible, thus increasing throughput and facilitating the 80 investigations (Baudoin et al., 2012; 2013). 81

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; 84 Legendre et al., 2013). In addition, more in-depth investigations of hepatotoxicity have 85 also been made possible thanks to the compatibility of liver biochip cultures with 86 conventional biochemical analysis processes such as metabolomics, proteomics and 87 transcriptomics (Shintu et al., 2012; Prot et al., 2011a, 2012). Thus, basal analysis on 88 primary rat hepatocytes and human liver cell lines has shown that cell defense 89 90 mechanisms including drug metabolism were over expressed in the microfluidic liver biochips (Prot et al., 2011b, Legendre et al., 2013). When applied to acetaminophen 91 92 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). 93

94 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 95 96 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 97 98 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 99 receptors leading to impairment of testosterone signaling and modulation of the 100 testosterone-dependent pathways. However, flutamide presents secondary toxicity in 101 102 the liver. The mechanisms for liver damage associated with flutamide use are currently 103 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 104 105 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 106 production of hydroxyflutamide via the cytochrome P450-1A (CYP1A) biotransformation 107 (Schulz et al. 1988, Shet et al., 1997). More particularly, flutamide was reported to have 108 human hepatotoxic properties in post-marketing studies (Aizawa et al., 2003, Coe et al., 109 2006). The precise mechanism behind hepatic dysfunction in humans has not yet been 110 111 clarified because it is probably the result of metabolic idiosyncrasy (Aizawa et al., 2003, Coe et al., 2006; 2007). 112

113 The plasmatic concentrations of flutamide and hydroxyflutamide after therapeutic 114 doses ranging from 250mg to 750mg of flutamide have shown maximal values varying

between 0.4 and 3 µM for flutamide and 3-10 µM for hydroxyflutamide (Schulz et al., 115 1988; Radwanski et al., 1989; Anjum et al., 1999). The in vitro IC<sub>50</sub> of flutamide 116 (including some data extracted in microchip cultures, Zhang et al., 2011), is reported to 117 range between 30 to 90 µM for 24h of exposure according to the culture methods and 118 assays used (total protein synthesis in Kostrubsky et al., 2007; live dead assays in 119 Zhang et al., 2011). In addition, primary rat hepatocyte exposure (0.36 to 1µM) led to an 120 increase in CYP1A2 mRNA levels, aspartate aminotransferase (AST) and alanine 121 aminotransferase (ALT) activities, and reduced glutathione (GSH) contents (Wang et al., 122 123 2002).

In order to investigate low and high dose effects of flutamide and to enhance the 124 knowledge on the potential mechanisms of hepatotoxicity, we have exposed rat 125 hepatocytes in microfluidic conditions for 24h to 10 and 100µM concentrations of 126 flutamide. The selection of those doses was based on the in vivo and in vitro data 127 described above. Hepatocellular functions were evaluated in terms of glucose 128 consumption, urea and albumin secretions. The metabolism performance of the 129 hepatocytes was also confirmed by metabolite detection and CYP1A activity. The 130 transcriptomic profile was then analyzed in order to identify the mechanism of action of 131 flutamide in microfluidic biochips. Finally the data was compared with literature reports. 132

133

#### 134 **2. Material and methods**

#### 135 2.1 IDDCM bioreactor

The entire culture set-up was called the IDCCM for "Integrated Dynamic Cell 136 Culture in Microsystems". The concept and details of the IDCCM box and biochips are 137 presented in detail in our previous work (Baudoin et al., 2011; 2012). Briefly, the 138 polydimethylsiloxane (PDMS) biochips were connected to the bottom of the IDCCM box 139 by a simple series of "plugging" ports (Fig 1A). This format made for an easy "plug and 140 display" of the biochips in external set-ups such as microscopes. The IDCCM is a 141 142 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 143

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.

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#### 150 **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 48h which included three different phases: hepatocyte extraction (day-0, post extraction), the adhesion phase (day-1, 24h of adhesion), which was performed at 37°C, and a 24h 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 hours in a seeding 158 medium composed of William's E Glutamax medium (Fisher Scientific, Illkirch, France) 159 supplemented with bovine insulin (5µg/ml, Sigma-Aldrich, Saint-Quentin Fallavier, 160 France) and fetal bovine serum (10%v/v). To enhance cell adhesion, the inner surface of 161 the biochips was coated with rat tail type 1 collagen (0.3mg/ml, BD Biosciences) 162 prepared in phosphate-buffered saline (PBS) for 1 hour at 37°C in a humidified 163 atmosphere supplied with 5% CO<sub>2</sub>. After washing with the seeding medium, the cells 164 were inoculated inside the biochips at  $0.5 \times 10^6$  cells per biochip (*i.e.*  $0.25 \times 10^6$  per cm<sup>2</sup>). 165 After the 24h adhesion period, cells were cultured in DMEM-F12 medium (Fisher 166 Scientific) supplemented with 15mM Hepes, 1.2g/L sodium bicarbonate (Sigma-Aldrich), 167 168 6.25µg/ml insulin-transferrin-selenium solution (Becton Dickinson, Biosciences, Le Pont de Claix, France), 100 units/ml of penicillin and 100mg/ml of streptomycin (Fisher 169 Scientific). 170

The IDCCM was prepared for the perfusion phase. Medium reservoirs at the inlet and outlet were filled with a volume of 2mL of culture medium to ensure the correct

medium supply during dynamic culture resulting in a total volume of 4mL for each 173 biochip. Then, the polytetrafluoroethylene (PTFE) tubes were connected to the fluidic 174 cover of the IDCCM box and to the peristaltic pump. PTFE tubes were used for 175 maximum prevention of the loss of chemical compounds through diffusion or adsorption 176 to the tubing in the fluidic circuit. The entire system (pump and IDCCM box with the 177 biochips) was placed in the incubator (32°C, 5% CO<sub>2</sub>) and the perfusion was started 178 179 (25µl/min flow rate). Culture medium was sampled and stocked at -20°C for posterior assays. Cytochrome assays and cell counting were performed on the biochips (see 180 181 below). The cells were photographed using a Leica DMI 6000B microscope together with the Leica camera and LAS life software (Leica Microsystems). 182

#### 183 **2.3 Cellular assays**

Details of the protocols of the cellular assays are presented in supplementary file 184 1 and in our previous works (Baudoin et al., 2011; 2012, Choucha Snouber et al., 2013). 185 186 Briefly, cell counting was performed on a Malassez cell following cell detachment with trypsin-EDTA (Fisher Scientific). Cell viability was quantitatively analyzed using trypan 187 blue staining. Functional metabolism was analysed using CYP1A2 activity, glucose 188 consumption, urea and albumin productions. CYP1A2 activity was determined using 5-189 190 ethoxyresorufin (10µM) as the substrate. Glucose consumption and urea production 191 were analyzed using a biochemical analyzer, the Konelab 20 (Thermo Fisher Electron 192 Corporation, Courtaboeuf, France). Albumin was measured using an ELISA sandwich test in a 96-well plate according to the rat albumin ELISA Quantitation Set protocol 193 194 (Bethyl Laboraries, Euromedex, Souffelweyersheim, France). Then, cell viability and necrosis was assessed by double staining using calcein AM (2µM) and propidium iodide 195 196 (PI) (10µg/mL). Finally reactive oxygen species (ROS) were detected by DCFDA staining. Those stainings were performed in the biochip at the end of the perfusion in 197 198 control and flutamide treated conditions.

199

#### 200 **2.4 Transcriptomic analysis**

Total RNA was extracted using the Nucleospin® RNA XS isolation kit (Macherey-Nagel EURL, Hoerdt, France). The quantity and quality of the RNA were evaluated using a Nanodrop ND-1000 spectrophotometer (Nyxor Biotech,Paris, France) and the Agilent 2100 Bioanalyzer (using the Agilent RNA 6000 Nano and Pico kits), respectively. All RNA samples had an RNA Integrity Number of more than 8 (between 9.3 and 10). cDNA was synthesized from 500ng of total RNA with oligodT Primer, using SuperScript II (Life Technologies), according to the manufacturer's protocol.

208 After validating the guality of the RNA with Bioanalyzer 2100 (using the Agilent RNA6000 nano chip kit), 5 ng of total RNA was reverse transcribed in accordance with 209 the Ovation PicoSL WTA System (Nugen). Briefly, the resulting double strand cDNA was 210 used for amplification based on SPIA technology. After purification according to the 211 Nugen protocol, 2 µg of single strand DNA were used to generate target DNA using the 212 Ovation Exon Module kit (Nugen). 4 µg of target DNA were fragmented and biotin 213 214 labeled using the Encore Biotin Module kit (Nugen). After controlling fragmentation using the Bioanalyzer 2100, cDNA was then hybridized to GeneChip® Rat Gene 1.0 ST 215 216 (Affymetrix) at 45°C for 17 hours.

After overnight hybridization, the chips were washed on the fluidic station FS450 following specific protocols (Affymetrix) and scanned using the GCS3000 7G. The scanned images were then analyzed with Expression Console software (Affymetrix) to obtain raw data (.cel files) and metrics for Quality Control. The observations of some of these metrics and the study of the distribution of raw data did not reveal any out-ofrange experiments.

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#### 224 2.5 Bioinformatics

#### 225 **2.5.1 Quality assessments, normalization and statistics**

The raw data were normalized using the Robust Multichip Algorithm (RMA) in the Bioconductor R. All quality controls and statistics were then carried out using Partek GS®. First we established hierarchical clustering (Pearson's dissimilarity and average linkage) and Principal Component Analysis to control the data. To find differentially expressed genes, we used a classic variance analysis for each gene and made paired Tukey's *post hoc* tests between groups. We then used p-values and fold changes to filter and select differentially expressed genes.

#### 233 **2.5.2 Functional analysis**

Interactions, pathways and functional enrichment analyses were carried out using
 the IPA (Ingenuity® Systems, USA www.ingenuity.com) and KEGG (Kyoto Encyclopedia
 of Genes and Genomes) databases.

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#### 238 **2.6 Mass Spectrometry analysis**

239 To detect and confirm the metabolism of flutamide into hydroxyflutamide and the glutathione conjugations by the hepatocytes in the microfluidic biochips, we performed 240 241 an additional mass spectrometry analysis. Detailed protocol is given in supplementary file 1 and in our previous works (Baudoin et al. 2014). To achieve our internal quality 242 243 criteria the quantification of positively identified analytes was made using an external calibration curve in blank matrix. Nine standards concentrations corresponding to 244 245 0.1 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL, 500 ng/mL, 1000 ng/mL 246 and 5000 ng/mL with three replicated injections were carried out for build of the 247 calibration curve of flutamide and hydroxyflutamide. The quantification was considered valid only within the measured linear range. Thanks to these standard curves, flutamide 248 and hydroxyflutamide concentrations were estimated. The HF metabolic ratio was 249 defined by the ratio between the hydroxyflutamide and the flutamide concentrations. 250 251 Concerning the flutamide metabolites identified by the fragmentation experiments, a metabolic ratio was calculated using the area under curves (AUC) ratio. 252

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#### 254 **2.7 Statistic analysis**

Three independent experiments (i.e 3 euthanized rats) were assessed. Each experiment was repeated at least three times in triplicate biochips. Data were plotted as mean  $\pm$  the standard error of the mean (SEM). Statistical analyses were performed by unpaired t test using GraphPad statistical analysis (Prism 5, version 5.02). The statistically significant differences are reported on the histograms and tables (\*\*\*
 P<0.001, \*\* P<0.01, \* P<0.05).</li>

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263 **3. Results** 

## 3.1 Morphology and cell viability of rat hepatocytes in IDCCM biochips after flutamide treatment

The hepatocyte morphologies at the end of the experiments (after adhesion and 266 after 24h of perfusion) in control, with 10µM and 100µM of flutamide are presented in 267 Fig. 2A to 2C. The hepatocytes adhered and covered the microfluidic channels. After 268 269 24h of perfusion, the control and the 10µM treated biochips presented similar morphologies. The hepatocytes were refringent between their cell junctions, with a 270 typical cuboid shape. The hepatocyte-like morphology was conserved over time and the 271 hepatocytes were still located in the biochips, forming a dense cell layer. At 100µM, the 272 273 hepatocytes were less dense and were not confluent. Finally, no difference was 274 observed between the counted cell in control and 10 µM-flutamide biochips. The number of hepatocytes was greatly decreased after 100µM flutamide treatment, resulting in a 275 276 significant reduction of 35% of the total collected cells (Fig. 3A).

## 3.2 Basal metabolism of rat hepatocytes in IDCCM biochips after flutamide treatment

Mitochondrial respiration was assessed using the alamar blue kit. It revealed intense mitochondrial activity with flutamide treatments which was statistically different from the controls. Both  $10\mu$ M and  $100\mu$ M flutamide exposures led to an increase of up to 60% of the mitochondrial metabolism (Fig. 3B). The glucose consumption and urea production increased when the flutamide concentration increased and significant differences were observed in comparison with control cultures (Figs. 3C and 3D). However, we did not detect any effect on albumin (Figs 3D and 3E).

# 3.3 Drug metabolism of rat hepatocytes in IDCCM biochips after flutamide treatment

CYP1A is the major cytochrome P450 involved in the flutamide metabolism. The 288 289 hepatocyte metabolism performances were confirmed by EROD biotransformation (EROD being a CYP1A substrate). The levels of resorufin production increased when 290 291 the flutamide concentration increased (Fig. 3F). In addition, the mass spectrometry analysis confirmed, in both the 10µM and 100µM treatments, the production of the 292 293 hydroxyflutamide metabolite. We estimated a production of hydroxyflutamide of 14±10 and 9±1 ng/h/10<sup>6</sup> cells (n=6) after 24h of perfusion during the exposures of 10µM and 294 295 100µM of flutamide respectively. The hydroxyflutamide metabolic ratio was found equal to 18.7% and 2.7% for the 10µM and 100µM flutamide exposures respectively. 296

297 Further research into the data was performed (overall value of full scan analysis) on the exact masses of flutamide and hydroxyflutamide complexed with glutathione in 298 299 negative mode at 580.13306 and 596.12797 respectively. The flutamide-glutathione and the hydroxyflutamide-glutathione conjugates were identified (Supplement Fig. 1). The 300 area under curve (AUC) analysis showed similar metabolism of glutathione conjugates 301 for both flutamide treatments. This led to hypothesis a higher metabolic ratios for 302 303 glutathione adducts when 10µM of flutamide was loaded when compared to 100µM conditions. 304

Finally, additional stainings were performed to evaluate the cell viability and the presence of the reactive oxygen species. In control and  $10\mu$ M-treated biochips, the level of fluorescence of propidium was weak and corresponded to background noise. At  $100\mu$ M, the cell population was stained positively illustrating important proportion of necrotic cells (Fig 4A to 4B). The DCFDA stainings showed that the level of ROS were high in experiments performed at  $100\mu$ M whereas few ROS positive cells were detected at  $10\mu$ M. Any DCFDA positive cells were observed in control (Figs 4E – 4 F).

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313 **3.4 Transcriptomic analysis** 

The transcriptomic analysis performed by microarray revealed a difference at the 314 mRNA level between the treated and control conditions. The selected working gene lists 315 from the transcriptomic analysis resulted from two subsequent mathematical selections. 316 A first selection was applied via a criterion based on a *p*-value of less than 0.05. The 317 comparisons between 10µM-treated biochips and controls (10vsctrl), and between the 318 100µM-treated biochips and the controls (100vsctrl) resulted to perturb respectively 319 320 2,597 and 1,553 genes. Then, to refine the lists, a second selection was performed by using a fold change criterion. Filtering with a fold change value of ±1.5, we reduced the 321 two lists to 899 and 471 genes respectively for the 10vsctrl and 100vsctrl conditions 322 (Supplement Tables 1 and 2). 252 genes were common to both flutamide exposures. In 323 324 order to identify the significantly affected genes pathways and networks, these lists were analyzed with Ingenuity Pathway Analysis (IPA) and Kegg databases. 325

The IPA applied to the 10vsctrl comparison highlighted the "LPS/IL-1 mediated 326 327 inhibition of RXR function", "Acute Phase Response Signaling", "Xenobiotic metabolism signaling", "Positive Acute Phase Response Proteins", and "Fatty acid metabolism" as 328 the main modulated pathways (Table 1). In addition, IPA identified the following 329 upstream regulators: LEP, PPARA, ACOX1, HNF4A and HNF1A (IPA p-values are 330 1.09x10<sup>-8</sup>, 1.75x10<sup>-8</sup>, 2.18x10<sup>-8</sup>, 8.03x10<sup>-7</sup>, 1.03x10<sup>-6</sup> respectively). When applied to the 331 100vsctrl comparison, IPA extracted a modulation in the pathways "LPS/IL-1 mediated 332 inhibition of RXR function", "Acute Phase Response Signaling", "Complement system", 333 "LXR/RXR activation", "Fatty acid beta oxidation I and Fatty acid metabolism", and 334 "Cytochrome P450 panel related to Fatty acid" (Table 1). The related upstream 335 regulators were PPARA, POR, LEP, HNF4A and ACOX1 (IPA p-values are 3.8x10<sup>-28</sup>, 336 1.25x10<sup>-12</sup>, 6.49x10<sup>-11</sup>, 5.72x10<sup>-9</sup>, 9.96x10<sup>-9</sup> respectively). 337

The treatments using Kegg databases complemented the results found with IPA. Running the database for the "Human+Disease/Drug" conditions, we extracted 73 and 30 pathways with at least 5 genes modulated in the pathway for the 10vsctrl and 100vscrtl gene lists respectively (Supplement Tables 3 and 4). In order to elicit a mechanistic interpretation for each flutamide treatment (10µM and 100µM), we have represented these pathways in a network of pathways by using common genes as bridges (Figs 5 and 6). The networks created a topology of pathways in the biochips cultures. The bioinformatics treatment and the Kegg analysis extracted for both flutamide treatments the activation of cell survival pathways (*via* JAK-STAT, PI3K/AKT, MAPK, signalings, Figs 5A and 5B, table 2) and a down regulation of mRNA levels in the Ppar signaling and fatty acid metabolism (Fig 5C and 5B, table 3), drug and glutathione metabolisms (table 3).

Specific modulations were found for both 10 and 100µM conditions. In the 350 10vsctrol comparisons, cell survival was more considerably affected when compared to 351 the situation extracted at 100vsctrl (Figs 4A and 4B, table 3). We found at 10µM a cell 352 353 survival response through an IGF/PDGFD upstream route and via a downstream up regulation in CREB5, BCL2, IKBKG routes in the PI3K/AKT signaling. We also detected 354 a down regulation TP53 mRNA levels and a cell cycle perturbation (via HDAC1, 355 CDKN2C, GADD45A, CDC14A, CDC26 and ANAPC5). In parallel, a down regulation of 356 357 genes of the pathways such as the TCA cycle, amino acid metabolism (down regulation of ALDH7A1, DAO, LAP3, MAOA, MAOB, NAGS for example in the arginine proline 358 pathway), and sugar metabolism (down regulation of ALDOB, MPI, TSTA3, SORD in 359 fructose and mannose metabolism and down regulation of GALK2, HEXB, MPI, NAGK, 360 TSTA3, UGP2 in amino sugar and nucleotide sugar metabolism) were observed (Figs 361 6A, 6B, 6C). This modulation was associated with the disturbances in the "genetic 362 information processing" related pathways such as those involving ribosome and mRNA 363 translation, and protein folding, sorting and degradation (Figs 6D and 6E). More 364 specifically, we found a RNA transport mRNA levels down regulation (via EIF1AX, 365 EIF3B, EIF4G3, POP1, POP7, PRMT5) and a down regulation in protein ubiquitin 366 proteolysis (via ANAPC5, CDC26, CUL4A, HERC3 PPIL2, RHOBTB1). Finally, we found 367 an up regulation of the HIF-1 signaling via FIGF, IGF1, PFKFB1, SERPINE1, TCEB2 368 mRNA levels. 369

In the 100vsctrl comparison, a modulation of the tight junctions' pathway was specifically extracted in contrast to 10vsctrl *via* the down regulation at 100µM of *CGN*, *CLDN2*, *GNAI1*, *IGSF5*, *MAGI3*, *PPP2R1B*, *SPTAN1* mRNA when compared to controls (fold change and p-values are presented in supplement table 2). A down regulation in the tryptophan, leucine, isoleucine, valine metabolism pathways was detected. Weak
 perturbations (on contrary to 10µM exposure) of the pathways related the TCA cycle,
 sugar metabolism, and protein processes was observed.

Based on these IPA and Kegg treatments, we propose two synthetic representations of the mechanism of action of flutamide, at  $10\mu$ M and  $100\mu$ M exposures, as shown in Figs 7A and 7B.

380

#### 381 **4. Discussions**

Absorption Distribution Metabolism and Elimination genes are enhanced in 382 microenvironment and microfluidic cultures (Sivaraman et al., 2005; Prot et al., 2011; 383 Legendre et al., 2013). The maintenance of the drug metabolism genes were used to 384 predict hepatic clearances (Novik et al., 2010; Chao et al., 2009; Baudoin et al., 2014). 385 Integrations of transcriptomics, proteomics and metabolomics profiles from cell lines 386 (such as HepG2/C3a and MDCK) in microfluidic cultures have demonstrated the 387 potential to investigate the mechanism of xenobiotic toxicity (Choucha et al., 2012; Prot 388 et al., 20). In this paper, we have successfully extended our feasibility study of our 389 390 microfluidic methodology for biological investigations by integrating the transcriptomics profiles of rat primary hepatocytes when exposed to flutamide. Our results illustrated the 391 possibility to culture primary cells and to collect pertinent data for mechanistic analysis. 392 Using rat hepatocytes, we found three types of cellular response to the two flutamide 393 394 treatments. The responses corresponded to a common flutamide response and two specific ones for each exposure conditions. 395

Commonly to both treatments, we found a down regulation of the Ppar signaling and of the fatty acid and lipid metabolism signaling. This type of down regulation has been already observed as a cell stress response in defense to the increase of Reactive Oxygen Species (Cabrero et al., 2002). Furthermore this down regulation was concomitant in the biochips with activation of *NFKB* related genes (such as *IKBKB* and *NFKB2* at 10 $\mu$ M, and *NFKB2* at 100 $\mu$ M) as reported in literature (Cabrero et al., 2002). In both exposure cases, we detected mitochondrial disturbance illustrated by the

perturbation of *mGSTA3*, *NDUFA9*, *NDUFA10*, *APAF1* and *mGSTA3*, *NDUFB4*, *LHPP*. 403 HTRA2, SLC25A4, APAF1 at 10µM and 100µM respectively. These results may appear 404 consistent with the literature in which flutamide was presented as blocking electron 405 transfer in the mitochondria (Coe et al., 2006; Kashimshetty et al., 2009). The 406 mitochondrial disturbance has been be related to a subsequent disturbance in cells such 407 408 as in calcium homeostasis (Rasola et al., 2011), illustrated in our study via PSEN2 gene 409 down regulation. Mitochondrial disturbances are also related to the modulation of some heat shock genes (Fulda et al., 2010), to the LXR/RXR modulation (Alaynick, 2008), to 410 the Ppar signaling (Anderson et al., 2004) and in the lipid metabolism and fatty acid 411 oxidation (Degli Esposti et al., 2012) that appeared consistent with our results. However 412 413 alamar blue levels in our tests at 10 and 100µM were increased when compared to controls. This result can illustrated an alternative respiration process (via complex II and 414 415 III in the mitochondria, Metha et al., 2008, or *via* glycolysis bypass, Coe et al., 2007) associated with alamar blue reduction by cytosol or microsomal enzymes (Morten et al., 416 417 2013, Gonzalez et al., 2001; Petrenko et al., 2005). Another route of exploration for a common response was to search for the potential effect of hydroxyflutamide itself. 418 Despite hydroxyflutamide being a well-known anti-androgen, we did not identify specific 419 disturbances related the androgen pathway in our study. Finally, when compared to 420 421 others in vitro flutamide hepatic characterization (Coe et al., 2007), we consistently 422 found modulated expression of genes associated with oxidative phosphorylation, fatty acid  $\beta$ -oxidation, antioxidant defense, and cell death pathways. 423

424 At 10µM of flutamide, we observed a global cellular defense response and a 425 down regulation of mRNA levels related to basal cell functions. The mechanisms related to cell survival (*via* the AKT/PI3K and MAPK signaling up to the p53 signaling, Fig. 5A) 426 427 were activated. Under stress conditions, this network played a part in stopping the cell cycle and enhancing the cellular repair mechanism. Nevertheless at the 24h time point, 428 429 the overall response appeared to illustrate a down regulation of the pro-apoptotic signaling and pro survival activation. In addition, a down regulation of two heat shock 430 431 proteins mRNA levels has been found (HSP40 and HSP70). It has been reported that general protein transcription and translation are halted in the initiation of the heat shock 432 433 response. The chaperone proteins, such as HSP40 and HSP70, are involved through

inhibition of cell death pathways (by preventing protein aggregation for instance, or by 434 forming co-chaperone complex modulating apoptosis, Fulda et al., 2010). This might be 435 consistent with the modulation of the protein network (Figs 6D and 6E) that we 436 highlighted at 10µM. However, additional proteomic analysis and other time endpoint 437 would be required to gain additional insight on this process as far as the perturbation 438 found in the microarray was not correlated at the functional level (albumin and urea 439 440 synthesis were not decreased in our flutamide exposures). After 24h of 10µM treatment, the activation of several cell defense routes (ROS responses, PI3K/AKT responses, 441 442 heat shock response) may explain the limitation of the toxicity insofar as limited cell death was observed when compared to controls and to treatment with 100µM. This is 443 444 coherent with the levels of the metabolic ratio illustrating a high flutamide biotransformation and the glutathione conjugations leading to detoxication of the drug 445 446 and its metabolites. This is also confirmed by the calcein AM - PI double staining and the ROS staining by DCFDA at 10µM. Finally, the overall 10µM response was 447 448 associated with an up regulation of the HIF-1 signaling (via FIGF, IGF1, PFKFB1, SERPINE1, TCEB2 mRNA levels) illustrating high oxygen request. 449

At present, it is difficult to state whether the disturbance in sugar, amino acid and 450 protein metabolism pathways at 10µM is a response due to cell defense activation 451 (namely a reduction of none necessary biological processes and a subsequent reduction 452 453 of the protein transcription and translation) or due to a specific response to flutamide. It 454 has effectively been reported that flutamide modulates extracellular sugar uptake and the sugar metabolism routes (Naftalin et al., 2003; Coe et al., 2007). In our experiments, 455 456 at the transcriptomic levels, we did not detect GLUT1 glucose receptor modulation, and the glycolysis pathway was not itself disturbed for both concentrations. However, the low 457 458 glucose consumption and the down regulation of sugar and amino acid metabolism pathways results at 10µM of flutamide appeared consistent with our previous 459 460 metabolomics profiling in human liver HepG2/C3a in which extracellular glucose and amino acid uptakes were reduced at similar exposure conditions (Choucha et al., 461 462 2013b). In parallel, we found intense glucose consumption at 100µM without any disturbance in sugar homeostasis when compared to controls. As we observed a 463 464 transcriptomic modulation in the sugar pathways only at 10µM, it seems that our

observations are a dose dependent response but not an illustration of specific glucose
 transporter binding by flutamide.

At 100µM we observed a switch in the stress response from "apoptotic survival 467 response" to "necrotic response". At the biochip level it was first detected by a reduction 468 in the number of cells collected. In addition, the lower metabolic ratios illustrated a 469 470 saturation of the flutamide metabolism (flutamide metabolism saturation, via a Km value reported between 2.48 and 4.1 µM and a Vmax value ranging between 200 and 472 471 pmol/min/mg, have been reported by Sjorgren et al., 2009). This led to a weak 472 detoxication process and thus to maintaining a high level of flutamide in the culture 473 medium (metabolism analysis) or inside the cell cytosol. At the transcriptomic level, the 474 necrosis was associated with the desperation of the "protein degradation network" route 475 and of most of the bridges in the "survival/apoptosis" pathways (Fig 5B, Fig 7B) whereas 476 ROS cell stress response was maintained. Survival cell responses can be switched to 477 necrosis during intense oxidative stress by two types of mechanisms: cellular ATP level 478 depletion or caspase inactivation due to ROS accumulation at active sites (Fulda et al., 479 2010). Maintaining the high level of flutamide, due to a lack of its biotransformation, may 480 thus play a part in modulating mitochondrial ATP production. After 24h of 100µM 481 treatment, the systemic stress led to significant cell death, illustrated by the reduction in 482 the final cell number, the PI positive cells and the level of ROS. Specifically, at the 483 484 transcriptomic level, the cellular organization appeared to be specifically altered as 485 shown by the down regulation of the tight junction pathway. This result correlated with the hepatic aggregation morphologies and cell culture deterioration that we observed. 486

Then, we compared our *in vitro* data with the *in vivo* report performed on rats and 487 mice. In our in vitro model, we found a reduction in hydroxyflutamide production at 488 100µM when compared to the 10µM treated cases. This was consistent with the 489 490 observations made by Wang et al. 2005 and Matsuzaki et al., 2006 in which giving rats flutamide induced liver CYP1A2 expression (protein, mRNA, activity) in a dose-491 dependent manner (this was also reported in vitro by Shet et al. 1997). In vivo, the gene 492 response of the flutamide appeared to be a classical Ahr ligand response (via the 493 substantial modulation of 15 genes over the consensus of 40 genes involved in the Ahr 494

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495 pathway, Coe et al., 2006). The flutamide also contributed to a down-regulation of the cfos gene and modulated about 50 ADME-related genes including CYP2B and CYP3A 496 497 (Coe et al., 2006). Although, we observed down regulation by flutamide of Ahr related genes (inconsistently with in vivo rat tests of Coe et al., 2006), we measured an 498 increasing CYP1A activity, as demonstrated by the EROD assays. We also found with 499 the microfluidic model the ADME genes modulation and an increase in CYP3A and 500 501 CYP2C as observed in vivo (as shown table 2 at 10µM). Despite in the biochips we did not detect any similar direct c-fos down-regulation, we found a significant modulation in 502 503 the related pathways, such as the MAPK signaling. However, the disruption of liver tissue integrity and ischaemia-reperfusion injury, both occurring during the isolation 504 505 procedure, are reported to activate inflammatory and proliferative responses, mediated by nuclear factor kappaB and mitogen-activated protein kinase, respectively (Fraczek et 506 507 al., 2013). In addition, pentobarbital used for the extraction is known to cause induction of specific cytochromes including CYP1A2 (Sakuma et al., 1999). Consequently, the 508 509 biochip cultures did not yet eliminate all common afferent drawbacks of *in vitro* cultures. Therefore, potential cross talks between several phenomenons still conduct to the 510 511 necessity of wider investigation with the biochips for more pertinent in vivo extrapolations. 512

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#### 514 **5. Conclusion**

515 In summary, we investigated the metabolism, biochemical functions and transcriptomic profiles of the effect of two flutamide concentrations on rat hepatocytes 516 cultivated in microfluidic biochips. The hepatic response to 10µM flutamide exposure led 517 to a pro-survival stress response. The response to 100µM flutamide exposure resulted 518 in necrotic activation. This result demonstrated the potential for extracting from the liver 519 520 biochip cultures certain typical toxicity profiles. As additional results, we proposed two integrated networks of toxicity in rat hepatocytes after 24h of exposure to 10 µM and 521 100µM of flutamide. Nevertheless, additional functional measurements of specific 522 biomarkers (such as quantification of ROS levels, caspases, and quantitative glutathione 523 524 depletion) are required to confirm our metabolic and transcriptomic profilings. Flutamide has idiosyncratic specific toxicity in humans that we could not detect in the present culture model because of the used of rat cells. However, the liver microfluidic biochip cultures might be adequate new tools for reproducing various types of liver and hepatocyte physiology (such as liver zonation, enhanced drug metabolism activities, etc...) using human cells. Better *in vitro* models may help to resolve and to address the idiosyncratic particularity especially by repeating our experiments with several human primary hepatocytes donors.

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#### 534 Acknowledgments:

535 This work was supported by the "Fondation pour la Recherche et l'Innovation" at 536 the Université de Technologie de Compiègne (project ToxOnChip), and by the UTEAM-537 Carnot grant (project Parachip).

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 Table 1: Most relevant pathways extracted by the Ingenuity analysis based on the microarray data.

Comparison	Pathway name	Pathway type	P-value
Control vs 10µM of Flutamide after 24h of cultures (899 genes)	LPS/IL-1 mediated inhibition of RXR function Acute Phase Response Signaling LPS/IL-1 mediated inhibition of RXR function Xenobiotic metabolism signaling Positive Acute Phase Response Proteins Fatty acid metabolism	Top canonical pathway Top canonical pathway Top Tox List Top Tox List Top Tox List Top Tox List Top Tox List	8.17x10 <sup>-5</sup> 1.17x10 <sup>-4</sup> 7.96x10 <sup>-5</sup> 2.9x10 <sup>-4</sup> 1.58x10 <sup>-3</sup> 1.95x10 <sup>-3</sup>
Control vs 100µM of Flutamide after 24h of cultures (471 genes)	Fatty acid b oxidation I LPS/IL-1 mediated inhibition of RXR function Acute Phase Response Signaling Complement system LXR/RXR activation Fatty acid metabolism LPS/IL-1 mediated inhibition of RXR function Cytochrome P450 panel related to Fatty acid LXR/RXR activation	Top canonical pathway Top canonical pathway Top canonical pathway Top canonical pathway Top canonical pathway Top Top Canonical pathway Top Tox List Top Tox List Top Tox List Top Tox List	$\begin{array}{r} 2.4 \times 10^{-7} \\ 5.93 \times 10^{-7} \\ 6.03 \times 10^{-6} \\ 7.35 \times 10^{-6} \\ 1.91 \times 10^{-5} \\ 2.1 \times 10^{-11} \\ 2.26 \times 10^{-7} \\ 1.44 \times 10^{-6} \\ 2.38 \times 10^{-5} \end{array}$

**Table 2:** Genes extracted by Kegg analysis concerning PI3K/AKT, MAPK and JAK/STAT signaling (fold changes are ±1.5 and *p*-values are below 0.05; details are given in Supplement Tables 5 and 6). Underline genes denote up regulation.

	10µM flutamide	100µM flutamide
Networks and pathways	Gene symbol	Gene symbol
PI3K/AKT signaling	BCL2L11, BCL2L13, CREB5, FIGF, FN1, GNG3, IGF1, IKBKG, JAK2, OSMR, PCK2, PDGFC, STK11, TP53, TP53RK	<u>CREB5,</u> FIGF1 <u>, FIGF2</u> , FN1, GYS2, ITGA6, JAK2, OSMR, PPP2R1B, <u>VEGF</u>
JAK/STAT signaling	CTF1, <u>DDIT3,</u> IL22RA1, IL23A, JKA2, OSMR, SOCS2, SOCS5, STAT1, STAT6	CTF1, IL22RA1, IL23A, JKA2, OSMR, SOCS2
MAPK signaling	<u>DUSP8, DDIT3,</u> FLNA, FLNB, <u>GADD45A,</u> <u>IKBKG,</u> NFKB2, MAP3K4, PPP3CB, RASA1, TP53, TP53RK	NFKB2, MAP3K4, MAP3K6, MKNK1, FIGF1 <u>, FIGF2</u>

**Table 3:** Genes extracted by Kegg analysis concerning drug metabolism consensus,\*mapping using ingenuity pathway analysis (fold changes are  $\pm 1.5$  and *p-values* are below 0.05; details are given in Supplement Tables 7 and 8). Underline genes denote up regulation.

	10µM flutamide	100µM flutamide
Networks and pathways	Gene symbol	Gene symbol
Drug and xenobiotic metabolism	ITPA, <u>NAT1</u> ,ADH4, AOX1 , MAOA, MAOB, AKR7A3, <u>CYP2C9, CYP3A2</u> , HNF4a, <u>G6PD</u> , GSTA3 <u>, GSTK1</u> , GSTA4, <u>GSTT2</u> , MGSTA3, UGT1A6, UGT1A7c, UGT2B17, UGT2B28	AOX1, FMO5, GSTA3, <u>GSTK1, GSTT2</u> , , GSTA4 <i>,</i> HNF4a, MGST3, PXR
Glutathione metabolism	<u>G6PD</u> , GPX2, GSS, GSTA3, <u>GSTK1,</u> GSTA4, <u>GSTT2</u> , IDH1, LAP3, MGST3	GPX2, GSTA3, <u>GSTK1, GSTT2</u> , GSTA4, MGST3
Ppar signaling	ACSL3, ACSL5, ANGPTL4, <u>APOA2</u> , AQP7 , CD36, CPT1A, CPT1B, PCK2, SCP2, <u>UBC</u>	ANGPTL4, <u>APOA2</u> , CD36, CPT1A, CPT1B, CYP4A11, CYP27A1, GK, HMGCS2, SCP2, <u>UBC</u> , SLC27A2
Ahr signaling*	ALDH6A1, ALDH7A1, APAF1, GSTA3, GSTK1, GSTA4, GSTT2, GSST2B, MGSTA3, UGT1A6, UGT1A7c, UGT2B17, UGT2B28	ALDH1A1, APAF1, GSTA3, <u>GSTK1,</u> <u>GSTT2</u> , GSTA4, MGST3, NCOA7, SUL1B1
Lipid and fatty acid	AGPAT2, ADH4, ALDH7A1, DGKZ, LIPC, MGLL, PPAP2C, CPT1A, CPT1B, ACSL3, ACSL5, PNPLA7, PTDSS2	ACAA2, ACADVL, AGPAT2, DGAT2, DGKZ,GK, LIPN1, MGLL,CPT1A,CPT1B,CYP4A11,ECHS1, ECI1, ECI2, HADH, HADHA
Bile acid synthesis	ABCB1, ABCC3, ABCC4,,AKR1D1, AQP8, BAAT, <u>CYP3A2</u> , CYP39A1, HSD17B4, SCP2, HMGCR,SLC4A4	ABCB4, AQP8, ATPB1, BAAT, HMGCR, SCL10A2, SCL01B3
ABC transporters	ABCA8 ,ABCA1, ABCB6, ABCB7, ABCC3, ABCC4, ABCC5	ABCB4
Steroid hormone	AKR1D1, <u>CYP3A2,</u> HSD17B6, UGT1A6, UGT2B17, UGT2B28	



**Figure 1**: (A) Principle of the IDCCM biochip and perfusion cultures; (B) IDCCM box with working pump; (C) Experimental procedures.



**Figure 2**: Hepatocyte morphologies in the IDCCM biochip at the end of the perfusion cultures; (A) controls; (B) after 24h of 10µM-flutamide; (C) after 24h of 100µM-flutamide. Scale bar is 100µm.



**Figure 3**: Comparison of control (vehicle 0.1% DMSO) of  $10\mu$ M and of  $100\mu$ M flutamide biochip cultures: (A) Final collected cell number; (B) Alamar blue metabolism; (C) Glucose consumption; (D) Urea production; (E) Albumin production; (F) Resorufin production *via* CYP1A activity (n=6 biochips, mean±SEM; \* p <0.05).



**Figure 4:** Calcein AM and propidium iodide double staining in biochips after 24h of flutamide exposures at  $10\mu M$  (A, B) and of  $100\mu M$  (C, D) ; ROS staining via DCFA probe in biochips after 24h of cultures in  $100\mu M$  (E), in  $10\mu M$  (F) and control (G)



**Figure 5**: Network reconstructions; Survival/apoptosis networks at (A)  $10\mu$ M and (B)  $100\mu$ M; Lipid, fatty acid and Ppar signaling networks at (C)  $10\mu$ M and (D)  $100\mu$ M; common genes are used as bridges; fold changes are ±1.5 and *p*-values are below 0.05; *GENE* = downregulated; <u>*GENE*</u> = upregulated; (number of gene in the pathways).

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Figure 6: Network reconstructions at 10µM exposure; (A) TCA network, (B) amino acid metabolism network; (C) sugar network; (D) Ribosome network; (E) Protein network; common genes are used as

**Ribosomes network (Fig 5D)** 

bridges; fold changes are  $\pm 1.5$  and *p*-values are below 0.05; *GENE* = downregulated; <u>*GENE*</u> = upregulated; (number of gene in the pathways).



**Figure 7**: Global network reconstruction illustrating flutamide toxicity at  $10\mu$ M (A) and  $100\mu$ M (B). Superscript 1 denotes the network we elicited from the Kegg database; Superscript 2 denotes the network we elicited from the Ingenuity database. fold changes are ±1.5 and *p*-values are below 0.05

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**Supplement Figure 1**: Structural confirmation of the presence of flutamide covalently complexed with glutathione. The extract chromatogram shows the presence of the molecule in the sample, while the mass spectra from fragmentation experiments show two specific ion fragments D and G.