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Potential involvement of chemicals in liver cancer progression: An alternative toxicological approach combining biomarkers and innovative technologies

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ABSTRACT

Pesticides as well as many other environmental pollutants are considered as risk factors for the initiation and the progression of cancer. In order to evaluate the *in vitro* effects of chemicals present in the diet, we began by combining viability, real-time cellular impedance and high throughput screening data to identify a concentration "zone of interest" for the six xenobiotics selected: endosulfan, dioxin, carbaryl, carbendazim, p′p′DDE and hydroquinone. We identified a single concentration of each pollutant allowing a modulation of the impedance in the absence of vital changes (nuclear integrity, mitochondrial membrane potential, cell death). Based on the number of observed modulations known to be involved in hepatic homeostasis dysfunction that may lead to cancer progression such as cell cycle and apoptosis regulators, EMT biomarkers and signal transduction pathways, we then ranked the pollutants in terms of their toxicity. Endosulfan, was able to strongly modulate all the studied cellular processes in HepG2 cells, followed by dioxin, then carbendazim. While p,p′DDE, carbaryl and hydroquinone seemed to affect fewer functions, their effects nevertheless warrant close scrutiny. Our *in vitro* data indicate that these xenobiotics may contribute to the evolution and worsening of hepatocarcinoma, whether via the induction of the EMT process and/or via the deregulation of liver key processes such as cell cycle and resistance to apoptosis.

1. Introduction

To ensure the safety of new molecules on human health and the environment, both public and private institutions have in recent years greatly promoted the development of alternative methods in toxicology to replace animal experiments deemed both unethical and not always representative of xenobiotic effects in humans (Olson et al., 2000). Current regulations for compound approval require manufacturers to perform numerous tests, many of which use liver cells as toxicological model. Indeed, the liver is the main organ of detoxification within the human body and is where xenobiotics are metabolized prior to their excretion. In addition to being long and costly, such regulatory measures could also be considered as insufficient in light of several molecules being withdrawn from market following suspicions raised by healthcare professionals and pressure from the governments concerned. Indeed, many environmental contaminants including drugs and pesticides have now been recognized as risk factors for hepatic diseases (Mostafalou and Abdollahi, 2013) that affect not only farmers and workers within the agrochemical industry but the entire population (Ferris et al., 2008). Yet, although epidemiologic studies tend to highlight the statistically relevant link between chemicals and hepatocarcinoma (HCC), the molecular mechanisms remain unclear. Our vision of a single molecular pathway is insufficient and should instead incorporate many pathways combined that relate to other perturbations of cellular homeostasis such as apoptosis, cell cycle dysregulation, or epithelial to mesenchymal transition (EMT), three processes involved in carcinoma progression and leading to metastasis (Fabregat, 2009).

The EMT process is complex and comprises a succession of events leading to the loss of epithelial characteristics. This phenomenon can be studied by monitoring the disappearance of

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specific markers, particularly in the dissociation of adherent junctions in which E-cadherin is downregulated by the modulation of expression of many transcription factors such as Snail or Slug known to be controlled by different pathways including TGF-β/Smad, (Dang et al., 2011). This step induces the stabilization of β-catenin protein and the activation of the Wnt/β-catenin pathway (Nambotin et al., 2012). In parallel, the increase of mesenchymal markers (fibronectin, S100a4...) is associated with the gain of migratory and invasive proprieties. It requires two levels of interaction: firstly between the cell and the ECM, and secondly between the same cell and its neighbors (Yang et al., 2011). The contact between cells and the ECM allows the activation of the ERK pathway and the master survival AKT pathway under the control of Fak and ILK respectively (Fuchs et al., 2008). The breakdown of epithelial cell adhesion during EMT is followed by the activation of a survival program (Fabregat et al., 2007). Two potential markers are the anti-apoptotic protein XIAP which has recently been shown to be overexpressed in HCC, and Cyclin D1 which, when coexpressed with XIAP, relates to poor prognosis (Che et al., 2012). On the other hand mesenchymal resistant cancer cells show increased levels of AKT and STAT3 activation (Ji and Wang, 2012), thus highlighting two more potential markers. The consequential inhibition of apoptosis also requires the intervention of survival pathways such as PI3 K/AKT and RAS/ERK pathways described above. Finally, $P21^{waf1}$ was used here as key marker of the cell cycle control.in HCC. It acts as a tumor suppressor by inhibiting cyclin complexes but can also exhibit oncogenic activities in certain contexts (Buitrago-Molina et al., 2013).

In our laboratory, we have recently developed the technology of High Content Screening (HCS) which combines in one device the simultaneous measurements performed by fluorescence microscopy, flow cytometry and microplate reader. Interestingly, all data collected can be processed via a simultaneous high-throughput analysis. This innovative and multi-functional "Cytomic" technology analyses live cells responding to chemical stress by combining assessments of such as proliferation, apoptosis, metabolism, morphology or signaling pathways (O'Brien et al., 2006; Tolosa et al., 2012). In parallel we use the xCELLigence system to measure real-time changes in electrical impedance of culture cells across microelectrodes integrated into the bottom of a 96-well plate. The cell status is then quantified and annotated as a Cell Index (CI), according to a specific equation provided by the manufacturer (Ke et al., 2011). In practice and reflecting uniform physiological conditions, more cells attaching onto the electrodes lead to a larger value for the cell index. Furthermore, for the same number of cells per well, a change in the cell status such as morphology, receptor activation or adhesion will lead to a change in the cell index (Atienza et al., 2006).

We selected for this study 6 environmental contaminants still found in water and/or food of the French population despite their prohibition (EAT2, ANSES, 2011). These were dioxin (TCDD) which is considered as a persistent organic pollutant (POP) and as a carcinogen (group 1 CIRC), the two organochlorine pesticides endosulfan and the DDT metabolite p,p′DDE, the two carbamates carbaryl and carbendazim developed in parallel of the ban of organochlorines, and finally hydroquinone which is used in several industries including pharmaceutical, cosmetics and agrochemical. The hepatocarcinoma cells HepG2 were chosen for their epithelial characteristics and their ability to acquire metastatic behavior in response to certain agents (Zucchini-Pascal et al., 2013).

The aim of this work was to evaluate *in vitro* the six xenobiotics selected using real-time cellular impedance and HCS technologies combined with EMT and HCC biomarkers, in order to provide new evidence for an effect of these chemicals on liver cancer progression that may lead to metastasis.

2. Materials and methods

2.1. Materials

The human hepatocellular carcinoma cell line HepG2 was obtained from ATCC (American Type Culture Collection, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin solution, sodium pyruvate and Eagle's non-essential amino acids were from BioWhittaker (Cambrex company, Walkersville, MD, USA). Dimethylsulfoxide (DMSO), endosulfan, carbaryl, carbendazim and hydroquinone were from Sigma–Aldrich (L'Isle d'Abeau Chesne, Saint Quentin Fallavier, France), p,p′DDE from Chem Service (West Chester, USA) and TCDD from AccuStandard, USA. Protein assay materials were from Bio-Rad, Hoechst 33342 and TMRE from Molecular Probes (Eugene, OR) and the antibodies used for western blotting experiments from Cell Signaling, Epitomics and Neomarkers (Table 1).

2.2. Cell culture and chemical treatments

HepG2 cells were maintained in DMEM with 1% penicillin/streptomycin, 1% nonessential amino acids, 1% sodium pyruvate and 10% FBS, in humidified atmosphere at 37 °C containing 95% O_2 and 5% CO_2. After washing with sterile phosphate buffer saline (PBS), cells were detached by trypsinization (trypsin/EDTA) and, depending on the experiment, plated at a concentration of 0.5–2×10^6 cells in 6-well plates or 1×10^4 in 96-well plates. For all experimental conditions, FBS was reduced to 5% in DMEM medium. The mitogen-activated protein kinase (MEK) 1/2 inhibitor U0126 was added at a concentration of 5 μM and the control cyclosporine A was used at 30 μM. All chemicals and pharmacological inhibitors were prepared as stock solutions in DMSO. The final concentration of DMSO in the medium was 0.25% in all conditions.

2.3. Viability test

Viable cells were determined by measuring the conversion of the tetrazolium salt MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma–Aldrich (St. Louis, MO) to formazan, as previously described (Fautrel et al., 1991). Briefly, cells were seeded in 96-well plates and treated at 50% confluency with a concentration of 20 μM endosulfan during 48 h. The cells were

Table 1
Primary antibodies used for western blot.

Antigen	Phosphorylation site	Source/type	Manufacturer	Dilution
pErk1/2	thr202/tyr204	Rabbit mAb1	Cell signaling	1:2000
Erk2		Rabbit pAb2	Cell signaling	1:5000
pAkt	Thr308	Rabbit pAb	Cell signaling	1:2000
Akt		Rabbit pAb	Cell signaling	1:2000
pSmad1/5	Ser463/465	Rabbit mAb	Cell signaling	1:2000
pSmad2	Ser465/467	Rabbit mAb	Cell signaling	1:2000
Smad5		Rabbit pAb	Cell signaling	1:2000
pStat3	Tyr705	Rabbit pAb	Cell signaling	1:2000
Cyclin D1		Rabbit pAb	NeoMarkers	1:2000
P21waf1		Rabbit mAb	NeoMarkers	1:2000
Bax		Rabbit pAb	Cell signaling	1:2000
Bcl-xl		Rabbit mAb	Cell signaling	1:2000
pFak	Tyr925	Rabbit pAb	Cell signaling	1:2000
Fak		Rabbit pAb	Cell signaling	1:2000
Gapdh		Rabbit mAb	Cell signaling	1:15000
E-cadherin		Rabbit mAb	Epitomics	1:5000
β-catenin		Mouse mAb	Santa Cruz	1:2000
Fibronectin		Rabbit mAb	Epitomics	1:1000
Snail1		Rabbit pAb	Santa Cruz	1:1000

1:mAb.monoclonal antibody.
2:pAb.polyclonal antibody.