

Cellular impact of combinations of endosulfan, atrazine, and chlorpyrifos on human primary hepatocytes and HepaRG cells after short and chronic exposures

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Received: 4 July 2013 / Accepted: 2 December 2013 / Published online: 17 December 2013
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Abstract Chronic exposure to low doses of pesticides present in the environment is increasingly suspected to cause major health issues to humans. Toxicological evaluations become more complex when the exposure concerns chemical combinations. Atrazine, chlorpyrifos, and endosulfan are pesticides used worldwide in agriculture and are therefore currently found at residual levels in food and the environment, even in countries in which they are now banned. Our study aimed to use Real-Time Cell Impedance Analyzer to investigate changes in phenotypical status of primary human hepatocytes and differentiated HepaRG cells induced by short and chronic exposures to these three chemicals. In contrast to the traditionally used endpoint cytotoxicity test, this technology allows kinetic measurements in real-time throughout the entire experiment. Our data show significantly higher cytotoxic effects of mixtures as compared to individual pesticides and a greater

susceptibility of human hepatocytes as compared to HepaRG to short-term exposure (24 h). Repeated exposure over 2 weeks to endosulfan and endosulfan-containing mixture induced HepaRG cell death in a time- and dose-dependent manner. Of the typical genes involved in metabolism and cell-response to xenobiotics, we found an exposure time- and condition-dependent deregulation of the expression of *CYP3A4* and *UGT1A* in HepaRG cells exposed to low doses of pesticides and mixtures. Our data demonstrate the usefulness of real-time cell monitoring in long-term toxicological evaluations of co-exposure to xenobiotics. In addition, they support but at the same time highlight certain limitations in the use of HepaRG cells as the gold standard liver cell model in toxicity studies.

Keywords Pesticides · Chronic exposure · Human hepatocytes · HepaRG · Real-time cell monitoring · Gene expression

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Introduction

Pesticides, widely used chemicals in sanitary and phytosanitary treatments, are an important health concern due to their ability to persist in the environment. With a wide array of target organisms, they display a great variety of structures and mechanisms of action (Cairns and Sherma 1992). Humans are continuously exposed by residential or occupational use to low doses of pesticides via oral ingestion, inhalation, or dermal absorption. Many studies have reported adverse effects

of such chemical compounds including their mutagenicity, carcinogenicity, teratogenicity, and immunotoxicity (Blair and Zahm 1995; Garry et al. 1996; LaFiura et al. 2007; Meinert et al. 2000; Thrasher et al. 2002), as well as their involvement in neurodegenerative diseases such as Parkinson's disease (Chade et al. 2006) and abnormalities in cerebral development (Mendola et al. 2002; Wirdefeldt et al. 2011). Pesticides rarely occur in the environment as single compounds but rather as mixtures of different chemicals. Thus, while interactions between certain chemical compounds are still a matter of debate, it is important to consider the effect of potential mixtures when evaluating the impact of pesticides on human health. Evidence does exist that mixtures can cause lower or higher toxic effects than would be expected from single compounds (Larsen et al. 2003). In this study, we have selected three compounds belonging to different classes of pesticides, namely atrazine (triazine herbicide), chlorpyrifos (organophosphorous insecticide), and endosulfan (organochlorine insecticide), with different modes of action. These pesticides were found to be persistent in the environment and also present in food.

Atrazine is a triazine compound that is used as a selective pre-emergence and post-emergence herbicide and also in aquariums as an algacide. Although it was banned in the EU in 2004 because of its persistence in groundwater (Ackerman 2007), atrazine remains a drinking water contaminant in several countries. It is a known endocrine disrupter affecting normal reproductive function and development in mammals (Fan et al. 2007; McMullin et al. 2004; Roy et al. 2009), fish, and amphibians (Bisson and Hontela 2002). Chlorpyrifos is a broad spectrum conventional organophosphorous insecticide which principally acts by inhibiting acetylcholinesterase (Cometa et al. 2007) thereby disrupting the nervous system. Endocrine disruption properties have also been described for chlorpyrifos and other organophosphate pesticides (Haviland et al. 2010). Endosulfan is a broad-spectrum chlorinated cyclodiene insecticide with clearly demonstrated toxicity in the environment. By 2009, it had been banned in almost 62 countries (EJF 2009; Warren et al. 2003) but still remains intensively used in Asia (i.e., India and China). It acts as a metabolic and endocrine disruptor (Lemaire et al. 2004; Silva et al. 2009) and is suspected to induce neurodegenerative disease even at low doses in humans (Scremin et al. 2011). The induction of intracellular oxidative stress leading to abnormal cellular development and

differentiation by low doses of chlorpyrifos and endosulfan has also been described (Bebe and Panemangalore 2003; Heistad 2006). In our recent work, we showed that low doses of endosulfan modulated some phases I and II metabolic and detoxification enzymes (namely CYPs and GSTs) and deregulated the apoptotic process in primary cultured human and mouse hepatocytes. Our previous results have revealed the anti-apoptotic tendency of endosulfan, at low dose, by increasing the Bcl-XL/Bax ratio (Rouimi et al. 2012).

Human cell models are essential in investigating the potential effects of pesticides *in vitro*. While primary cultures of human hepatocytes remain the gold standard model for such screenings, their rapid rate of dedifferentiation make them suitable only for short-term investigations targeting interspecies biotransformation patterns, enzyme inductions, and inhibitions or acute toxicity and drug–drug interactions. They are not however suitable for experiments measuring temporal alterations in response to repeated long-term exposures requiring cell cultivation over long periods. The recently described HepaRG cell line may be a promising alternative to primary hepatocytes in toxicological studies. The differentiated state of these cells exhibits a metabolic pattern close to normal liver hepatocytes, with stable and inducible enzyme expression over long periods, making them a unique model for testing chronic toxicity (Guillouzo et al. 2007).

We have previously demonstrated that exposure to low doses of atrazine, chlorpyrifos, and endosulfan were able to modulate the expression of phase I and phase II drug metabolizing enzymes in mice hepatocytes in a short-term experiment (Rouimi et al. 2012). Here, we aimed to compare the effects of the three pesticides at low and medium concentrations, alone and in a mixture under both short- and long-term exposure conditions. To this end, we continuously monitored the behavior of HepaRG cells using a real-time impedance cell analyzer (Xi et al. 2008), for up to 2 weeks. In parallel, we evaluated changes at the mRNA level of some key metabolic genes (i.e., *CYP3A4*, *GST1A1*, and *UGT1A1*) by quantitative RT-PCR. *CYP3A4* metabolizes drugs as well as xenobiotics and is implicated in the formation of carcinogenic bioactive metabolites (Xie et al. 2000). Induction of *CYP3A4* expression and activity, after pregnane X receptor (PXR) activation, can alter the metabolism of drugs that may affect their duration of action, efficacy, and toxicity, and produce side effects due to toxic metabolites (Gao and Xie 2010; Nakata et al. 2006).

Depending on the intracellular content of cofactors, these reactive metabolites can be then be inactivated by the enzymes GST or UGT.

By comparing the modifications in integrity and metabolic capacities induced on liver cells by the pesticides individually or combined in mixtures, our data should provide new insights with regards to the early evaluation of a chemical's safety to reduce the risk to human health.

Materials and methods

Materials

Atrazine (A), chlorpyrifos (C), and endosulfan (E) were purchased above 98 % purity from Sigma-Aldrich (Saint Quentin Fallavier, France) and used either individually (A, C, and E), as binary mixtures (AC, AE, and CE), or as a ternary combination (ACE). Mixtures contained pesticides at equimolar concentrations (e.g., 75 μM mixture of AC was composed of 75 μM A and 75 μM C). Each treatment was freshly prepared in DMSO as a 400 \times stock solution for human hepatocytes and as a 100 \times stock solution for HepaRG cells, stored at 4 °C for the duration of each experiment. DMSO and all other chemicals and reagents were purchased from Sigma-Aldrich and were of analytical grade.

Cellular models and cell culture

Two different types of hepatic cells were used in our experiments: primary cultured human hepatocytes and the immortalized human hepatic cell line, HepaRG. All experiments performed on human tissue were in accordance with ethical standards of the responsible committee on human experimentation and with the Helsinki declaration.

Hepatocytes were isolated from human liver surgical biopsies, cryopreserved and thawed as already described (de Sousa et al. 1996) before use. Culture conditions were as follows. In brief, an erythrosine B exclusion test was performed after thawing to ensure at least 80 % cell viability. Hepatocytes were then seeded in type-1 collagen-coated 12-well culture plates (Corning, USA) at 2.5×10^4 cells/cm² and cultured in William's E medium with 1 % L-glutamine (Invitrogen, France), 10 % fetal bovine serum (PAA, Austria), 50 UI/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, and 0.1 UI/mL insulin from Novo Nordisk (Bagsvaerd, Denmark) for 24 h. The

medium was then replaced by a similar serum-free medium complemented with hydrocortisone hemisuccinate (1 μM) and bovine serum albumin (240 $\mu\text{g}/\text{mL}$). Twenty-four hours after plating the culture medium was renewed prior to another 24-h incubation with pesticides. Treatments were performed using 1, 10, or 25 μM of pesticide and mixtures dissolved in DMSO (final concentration in the medium=0.25 %). Three different human hepatocyte batches were used.

HepaRG cells were from Biopredic International (Rennes, France) and were maintained as already described (Gripon et al. 2002). Briefly, cells were seeded in 12-well culture plates at low density (2.6×10^4 cells/cm²) in William's E medium supplemented with 1 % L-glutamine (Invitrogen, USA), 10 % fetal bovine serum (PAA, Austria), 0.1 U/mL insulin and 5 μM hydrocortisone hemisuccinate, and grown for 2 weeks at 37 °C in 5 % CO₂ atmosphere. The medium was then supplemented with 1 % DMSO and renewed every 2 or 3 days for another 2 weeks until complete differentiation of the cells. Differentiated HepaRG cells were then exposed to pesticides either once for 24 h, or repeatedly every 2 to 3 days for 2 weeks. Rifampicin (RIF), benzo[a]pyrene (BaP), and 3-methylcholanthrene (3-MC) were used as specific activators of the PXR and aryl hydrocarbon receptor (AhR).

RNA isolation, reverse transcription, and gene expression analysis

Immediately following incubation, total RNA was extracted from primary hepatocytes and HepaRG cells, utilizing 0.5 mL of TRIzol[®] Reagent (Invitrogen, USA). The concentration and purity of the isolated RNA were assessed on a NanoDrop 2000 spectrometer (Thermo Scientific, USA), and RNA integrity checked by agarose gel electrophoresis. Total RNA (1 μg) was reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, USA). All reactions were performed according to manufacturer protocols unless otherwise stated.

Relative quantification of *CYP3A4* and *UGT1A1* gene expressions were determined by quantitative RT-PCR (qPCR) using the LightCycler[®]480 Probes Master (Roche, France) together with the FAM-labeled hydrolysis probes from the Universal ProbeLibrary Set, Human (Roche, France). Intron-spanning primers were designed using the Universal ProbeLibrary Assay

Design Center (<http://www.universalprobelibrary.com>). For quantitative PCR, the program comprised a pre-denaturing step at 95 °C for 5 min followed by 10 s at 95 °C, 40 cycles of 30 s at 60 °C, and a cooling step at 40 °C for 30 s. Calculations were performed by the LightCycler®480 v1.5.0 SP3 Software utilizing the efficiency-based E-method, with crossing-point (C_p) values being calculated based on the second derivative maximum method. GAPDH was used as the endogenous control reference for samples obtained from primary human hepatocytes and TBP was used as control for samples from HepaRG cells.

Impedance, cell index measurements

Cell changes were monitored in real-time using the xCELLigence system (Roche Diagnostics, France) which measures electrical impedance across microelectrodes integrated into the bottom of specialized 96-well plates (E-plates). A parameter known as the cell index (CI) is calculated in order to quantify cell status based on the measured cell-electrode impedance, according to the following equation:

$$CI = \max_{i=1,\dots,N} \left(\frac{R_{\text{cell}}(f)}{R_0(f)} - 1 \right)$$

where $R_{\text{cell}}(f)$ and $R_0(f)$ are the frequency-dependent electrode resistances (a component of impedance) with or without the presence of cells, respectively, and N the number of frequency points at which the impedance is measured (Solly et al. 2004). Therefore, CI quantitatively measures the overall status of the cells in an electrode-containing well. Thus, under the same physiological conditions, more cells attaching onto the electrodes lead to a larger $R_{\text{cell}}(f)$ value and a larger value for cell index. However, for the same number of cells present in the well, a change in the cell status such as mortality, motility, membrane receptor activation, etc., will lead to a change in the cell index. This system has been applied to numerous cell-based assays measuring cytotoxicity (Solly et al. 2004), cell adhesion, and spreading (Atienza et al. 2005). We calculated a normalized cell index (NCI) at a given time point by dividing the CI at that time point by the CI at a reference time point. Thus, at the reference time point, the NCI was 1. Normalization was performed from the last time point before compound addition. To evaluate differences among treatment conditions, we normalized the data

by expressing at each time point the CI of treated cells (human hepatocytes) as a ratio of the CI of DMSO (vehicle)-treated cells the value of which was 1 over the entire treatment period (NC).

E-plates used for plating primary human hepatocytes, not those for HepaRG cells, were coated with 2 µg of collagen/well. Hepatocytes were seeded at 2.5×10^4 cells/cm² and left for 24 h before exposure to pesticides. HepaRG cells were seeded at 1.3×10^5 cells/cm² and maintained in the aforementioned medium until the CI stopped increasing (approximately 1 week). At this point, the medium was supplemented with 1 % DMSO and renewed every 2–3 days during 2 weeks until the cells were fully differentiated. Cells were then continuously exposed to 50, 75, or 100 µM final concentrations of pesticides for another 2 weeks in the same medium which was renewed every day. The NCI, the CI normalized to DMSO at $t=0$ (CIN), and NCI values presented here were calculated from three independent experiments in triplicate (technical replicates), except when otherwise mentioned. Data represent the average ± standard deviation (SD).

Statistical analysis

The values obtained are expressed as the mean ± SD. Statistical analysis of differences between groups was carried out using a one-way ANOVA or two-way factorial ANOVA. Homogeneous groups were determined by a two-way factorial ANOVA followed by Tukey's HSD *post hoc* test. A probability of 0.05 was taken as the level of statistical significance. For two-way factorial ANOVA, homogeneous groups provided grouping for the means. Means within the same subset were not significantly different from each other at a probability of 0.05. Analysis was performed using Statistica Software (Statsoft, France).

Results

Real-time impedance measurements on primary human hepatocytes exposed to pesticides

After cell seeding, we renewed the culture medium twice in the first 4 h, as seen by the abrupt changes in the CI records in Fig. 1a, before allowing the hepatocytes to stabilize for 20 h. A control recording at 24 h showed a CI value around 1.2, reflecting the formation of a stable monolayer of hepatocytes in interaction with

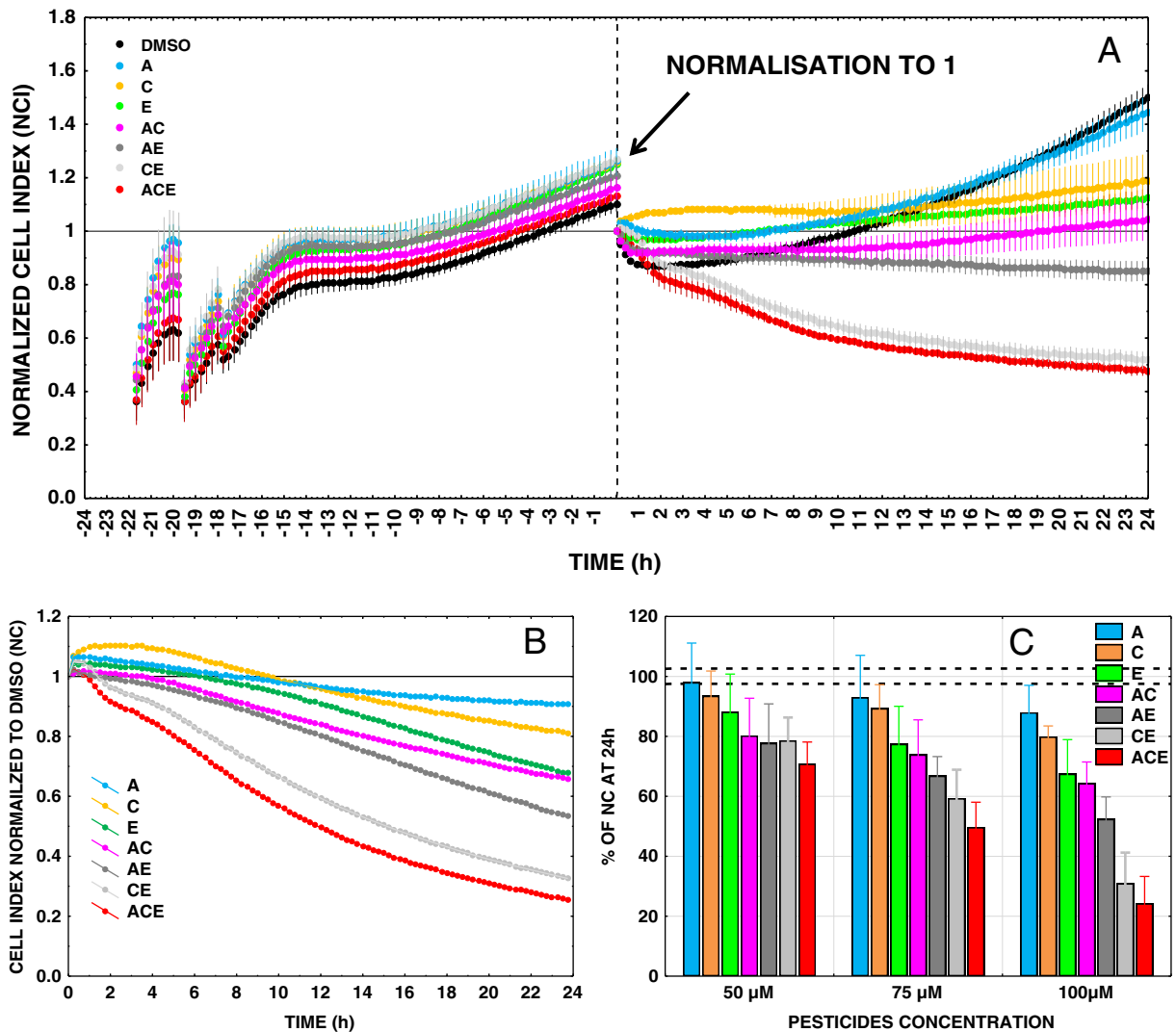


Fig. 1 a Dynamic monitoring of human hepatocyte adhesion and response to pesticide exposure at 100 μM. The figure shows one typical experiment out of three experiments performed. The average of three independent wells is shown. Cell index curve values were monitored every 15 min for 48 h, left panel 24 h for the attachment periods and right panel: 24 h after pesticide addition. All value was normalized to the cell index measure at time = 0 (addition of the pesticides). b Comparative kinetic curves of the cytotoxicity of pesticides and their mixtures after NC (vehicle-treated cells) normalization in human hepatocytes. Data were normalized by expressing at each time point the Cell Index (CI) of treated cells as a ratio of the CI of control (DMSO-treated cells)

for which the value was 1 over the entire treatment period Each point represents the mean of three independent experiments performed in triplicate, on human hepatocytes treated with 100 μM of compound. For clarification, *error bars* have been omitted from graphs though standard deviation values at 24 h can be seen in (c). c Comparison of the cytotoxicity of three pesticides and their binary and ternary mixtures after 24 h of treatment. Data points are the mean±SD of three experiments performed in triplicate. The *horizontal dashed lines* on the plot represent the confidence interval of DMSO-treated cells. NC=CI of treated cells/CI of DMSO (vehicle)-treated cells. Significant differences are shown in Table 1. DMSO was fixed at 0.25 % for each treatment and control

microelectrodes on the bottom of the culture plates. Following the stabilization period, we exposed the cells to pesticides, normalized the CI index to 1 and allowed the experiments to run for another 24 h (Time=0 on the Fig. 1a, one measure every 15 min). Since CI is

dependent on the cell number, their morphology and degree of adhesion to the substrate, variations in CI reflect any alterations in cell spreading, cellular death or cytotoxicity. Figure 1a shows a typical experiment in which human hepatocytes were treated with individual

pesticides and a mixture, each at a concentration of 100 μM. After normalization, we plotted the time–response curve as in Fig. 1b to represent the variation of NC over the 24 h of exposure to the individual pesticides and the mixture (100 μM). Curve profile analysis revealed a continuous decrease in NC, the slope of which depended on the treatment condition. On comparing NCIs corresponding to the different 24-h treatments at the different concentrations, only those associated with the ternary mixture at 100 and 75 μM and the binary mixture of CE at 100 μM were below 0.5NC (Fig. 1b, c).

In order to classify the effects of the different treatments, we performed a multifactorial ANOVA test. Statistical analysis allowed classifying the different treatments into 10 homogenous groups which could then be simplified into 5 (represented as a Venn diagram: Table 1) groups classifying from the lowest to the highest toxicity. The two first groups included 12 conditions and concerned all individual pesticide treatments, except E, at 100 μM. Within the three groups containing the most toxic concentrations of individual pesticides and

mixtures, endosulfan was represented 8 times within 9 treatment conditions (Table 1). Our results enabled the classification based on toxic strength giving ACE>CE, AE>AC>E >>C, A which was supported by other used concentrations. Thus among the individual pesticides tested, we found endosulfan to be the most toxic. Moreover, the toxic action of endosulfan on primary cultures of human hepatocytes was enhanced when combined with other pesticides, even though the accompanying pesticides exhibited no apparent strong toxic effects at the given concentrations.

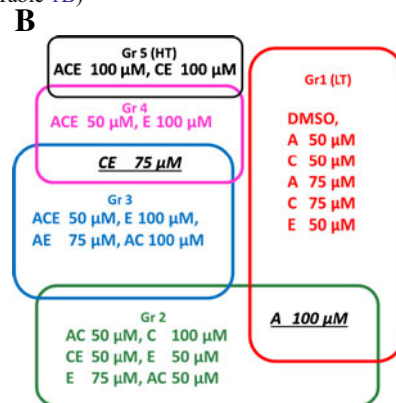
Real-time impedance measurements on HepaRG cells exposed to pesticides

Immediately following HepaRG cells seeding in E-plate wells, the CI quickly started to increase due to attachment of the cells to the bottom of the wells. Coating the wells with collagen aided neither attachment nor growth of the cells and we thus considered it unnecessary. CI rose continuously until it stabilized at a value around 7 to 10

Table 1 Effect of treatment and concentration factors on human hepatocytes: a factorial ANOVA was performed, which created 10 homogeneous groups (the appartenance to a group is represented

Treatment	Conc (μM)	Cell Index (%)	Homogenous groups					
			5	4	3	2	1	
ACE	100	24.2	■					
CE	100	30.9		■				
ACE	75	49.7		■				
AE	100	52.4			■			
CE	75	59.3			■			
AC	100	64.3				■		
AE	75	66.8				■		
E	100	67.5					■	
ACE	50	70.8					■	
AC	75	73.9					■	
E	75	77.4						■
AE	50	77.7						■
CE	50	78.6						■
C	100	79.9						■
AC	50	80.2						■
A	100	87.8						■
E	50	88.1						■
C	75	89.3						■
A	75	93.5						■
C	50	93.5						■
A	50	98.1						■
DMSO		100						■

by a hash key within a column or by a color, $p < 0.05$), from which five groups were then extracted as summarized in a Venn Diagram (Table 1B)



Homogenous subsets, represented in color frame, provided groupings for the mean. Means within the same frame were not significantly different from one another ($p < 0.05$). Pesticides or mixtures are classified from the lowest (Gr 1, LT) to the highest toxicity in terms of their potency (Gr.5, HT) according to the color of the frame: red the lowest toxicity, then green, blue, violet, and black. Mixtures and pesticides belonging to two groups are indicated at the intersection of two sets and underlined in black font

(Fig. 2a) with a rate depending on the number of cells seeded; 1 week was needed to reach stabilization after 1.3×10^5 cells/cm² seeding. After this point, the CI remained predominantly stable when the DMSO-free medium was replaced by the medium containing 1 % DMSO and until the end of the experiment, typically around 3 weeks.

Differentiated HepaRG cells were exposed to 50, 75, and 100 μ M of individual pesticides and mixtures dissolved in DMSO (final concentration in the medium = 1.0 %) for 2 weeks (Fig. 2b). Medium was renewed every 2–3 days. In a preliminary experiment, concentrations from 10 to 100 μ M were tested and 50 μ M was found to be the highest concentration having no effect in our conditions (see Table 1).

In order to classify the effects of single pesticides and mixtures, we calculated the time to reach 0.5 NCI in real-time impedance measurements, as compared to DMSO controls (Fig. 2c). Addition of atrazine did not alter the NCI values at any of the three concentrations tested. Chlorpyrifos either alone or combined to atrazine (AC) caused a slight concentration-dependent increase of the NCI to around 1.1–1.2. Endosulfan, alone or in combination, was the only pesticide that caused marked disruption of cell integrity and cell death. This was reflected by the steady decrease in the NCI values in wells exposed to the pesticide. After 6 days of exposure to the different compounds (6 medium renewals), the most toxic combination showed to induce an abrupt decrease in the NCI value concomitant with detachment of large patches of cells observed at time of medium renewal, which were due to treatment-induced weakening of cell adhesion to the support. This free space was not colonized by new dividing cells. With repeated exposures, endosulfan began to be toxic after 9 medium renewals. Surprisingly, combination of endosulfan with other pesticides enhanced its toxic action on the HepaRG cells. This potentiating effect was especially marked at the lowest 50 μ M concentration which could still induce cell death (Fig. 2b). Following statistical analysis, 4 homogenous groups distinguished the effect of endosulfan and pesticide mixtures (Table 2). The most toxic group included endosulfan at 100 μ M and all binary and ternary combinations of pesticides at 75 and 100 μ M that included this pesticide. The second most toxic group was composed of E at 75 μ M and ACE and CE at 50 μ M. The last two groups included AE at 50 μ M and E at 50 μ M. It should be noted that atrazine and chlorpyrifos needed to be co-incubated with endosulfan to induce HepaRG cell death.

Modulation of gene expression caused by pesticides

Using quantitative PCR, we evaluated changes in mRNA expression levels of three genes involved in cell metabolism and detoxification, the prototypical PXR-activated gene CYP3A4 and the phase II metabolizing enzymes GSTA1 and UGT1A1 genes, in primary hepatocytes and HepaRG cells exposed to pesticides for either 24 h or 2 weeks.

We tested different concentrations of the three individual pesticides (A, C, and E) ranging from low to medium (1, 10, and 25 μ M), three binary mixtures (AC, AE, and CE), and a ternary mixture (ACE) at equimolar concentrations. These concentrations did not induce modification of the xCELLigence curve profiles. To assess the effects of the pesticides, we expressed their induction (Fig. 3a) as a ratio to that of the real PXR agonist rifampicin (25 μ M), a prototypical pregnane X receptor (PXR)-dependent inducer of CYP3A4. Phenobarbital (1 mM), a CAR/PXR activator, reached 60 % of the inducing capacity of rifampicin.

Endosulfan alone or mixed with the two other pesticides was found to modulate the expression of CYP3A4 in both primary hepatocytes and HepaRG cells following short- or long-term exposures. The expression level of UDP-glucuronosyl transferase 1A1 (*UGT1A1*) following short-term (24 h) exposure to all pesticides, was unaffected in primary hepatocytes and remained low in HepaRG cells. In contrast, the 2-week treatments were found to significantly increase the expression of this enzyme in HepaRG cells (Fig. 3b). Enzyme induction was concentration-dependant, reaching up to sixfold for the highest concentration of 25 μ M for AE, CE, and ACE, as compared to DMSO control. It is noteworthy that despite these combined-pesticide treatments containing equimolar concentrations of the different molecules, endosulfan by itself markedly increased the expression of UGT1A1 by about fourfold. The expression of GSTA1, another phase II metabolic enzyme previously described to be induced by chlorpyrifos in liver cells (Medina-Díaz et al. 2011), remained unchanged in our study.

Discussion

In order to reflect real life situations, toxicological evaluations of chemical compounds with regards to their risk to human health require real-time examination of

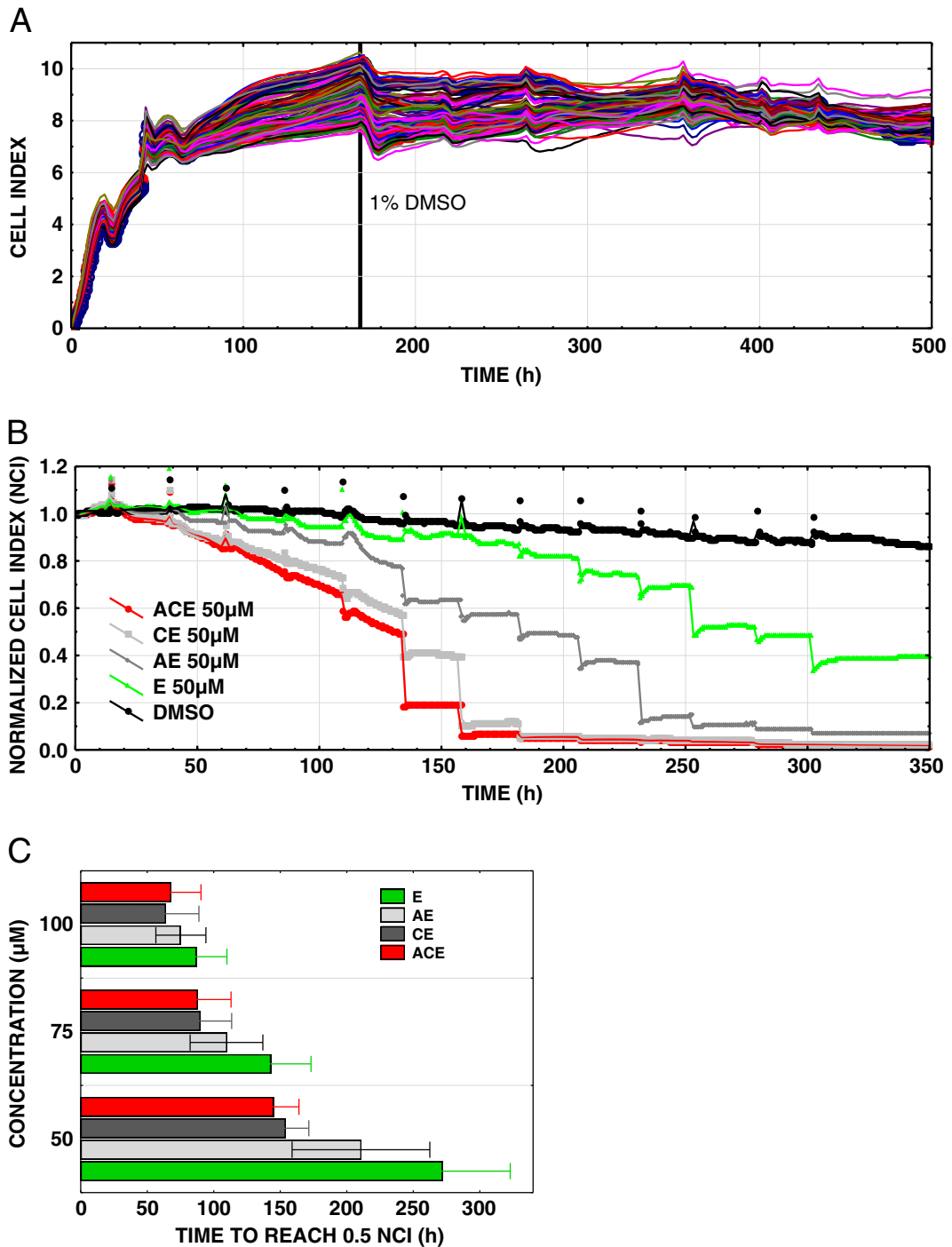
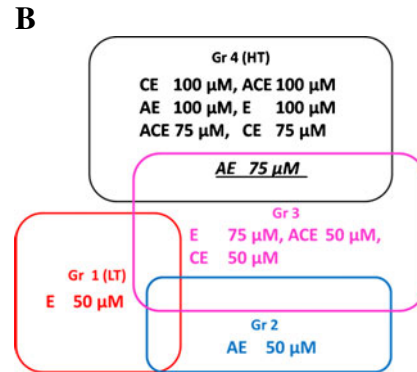


Fig. 2 a Dynamic monitoring of HepaRG cell adhesion and differentiation. The figure shows the CI of individual wells of a 96-well plate. Cell index curve values were monitored every 30 min before the addition of pesticides. The concentration of DMSO was maintained at 1 %. b Comparative kinetic cytotoxicity curves over 2 weeks of treatment with pesticides and their mixtures after CI normalization in

HepaRG cells. c Comparison of the cytotoxicity of the three pesticides and their binary and ternary mixtures after 2 weeks of daily treatment. The concentration of DMSO was maintained at 1 % in all conditions. Data points are the mean±SD of three experiments performed in triplicate. Significant differences are shown in Table 2

Table 2 Effect of treatment and concentration factors on time to reach NCI 0.5 on HepaRg cells

Treatment	Conc (μM)	Mean (hours)	Homogenous Groups Number
CE	100	64.3	4
ACE	100	68.4	4
AE	100	75.3	4
E	100	86.7	4
ACE	75	88.0	4
CE	75	89.6	4
AE	75	109.5	4,3
E	75	143.3	3
ACE	50	145.3	3
CE	50	154.3	3
AE	50	210.6	2
E	50	271.9	1



A factorial ANOVA was performed, which created 4 homogeneous groups (numbered from 1 to 4, $p < 0.05$, Table 1A), which were extracted and summarized in a Venn Diagram (Table 2B). Homogenous subsets, represented in color frame, provided groupings for the mean. Means within the same frame were not significantly different from one another ($p < 0.05$). Pesticides or mixtures are classified from the lowest (Gr 1, LT) to the highest toxicity in terms of their potency (Gr.4, HT) according to the color of the frame: red the lowest toxicity, then blue, violet, and black. Mixtures and pesticides belonging to two groups are indicated at the intersection of two sets and underlined in black font

cellular and metabolic disturbances induced by chronic exposure to mixtures of these compounds. To date, no in vitro model has been validated for use in assessing

chronic toxic endpoint. As liver injury is related to long-term treatments, there is a need to predict chronic toxicity caused by multiple exposures to single xenobiotics

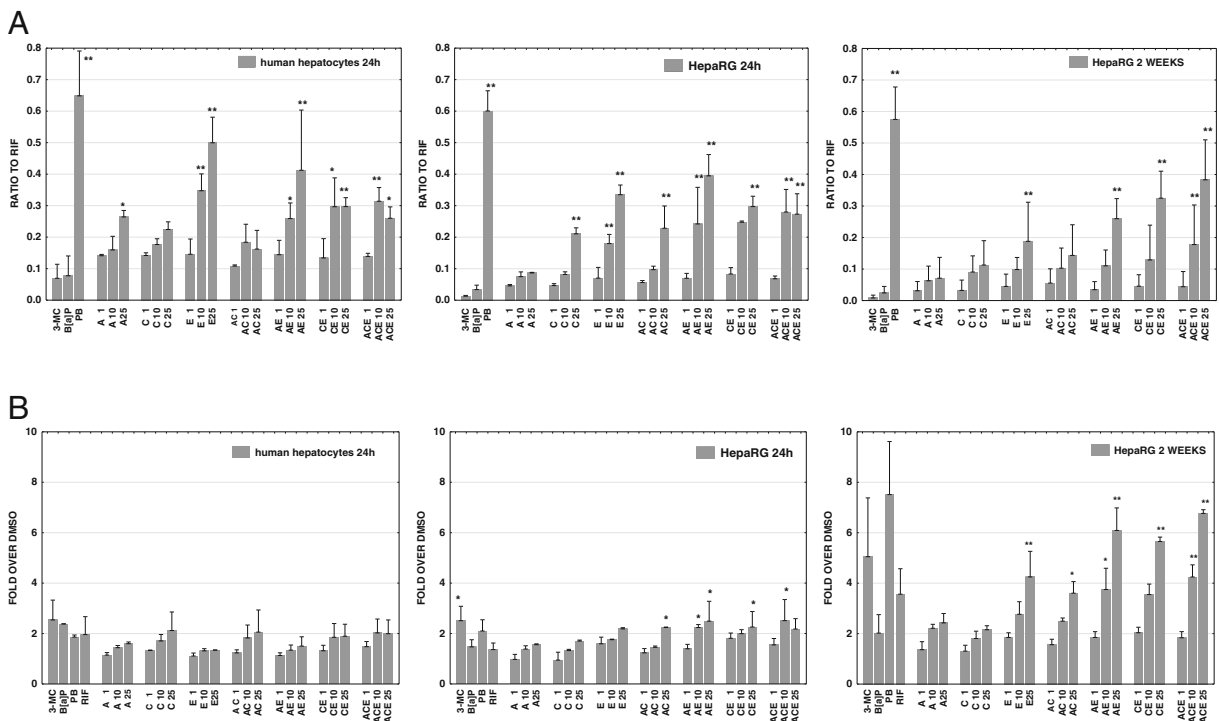


Fig. 3 Impact of short- and long-term exposure to pesticides and their mixtures on CYP3A4 and UGT1A1 mRNA expression in human hepatocytes and HepaRG cells. **a** CYP3A4 expression: values are expressed as a ratio of observed mRNA levels versus levels after rifampicin treatment and represent the mean±S.D. of

technical replicates (see “Materials and methods” section). **b** UGT1A1 expression: values are expressed relative to control (DMSO) levels and represent the mean±S.D. as described above. Each value represents the mean±S.D. of triplicate treatments. *, $p < 0.05$ versus B[a]P; **, $p < 0.01$ versus B[a]P

or to mixtures of different molecules, at concentrations far below those corresponding to acute toxicity.

In the present study, we analyzed *in vitro* cytotoxicity and gene expression in pertinent liver cellular models after short- or long-term exposure to the three pesticides atrazine (A), chlorpyrifos (C), and endosulfan (E) either individually or as binary and ternary combinations. For the short-term assays, we used human hepatocytes which are considered to be the gold standard for studying metabolism and toxicity of xenobiotics (Gomez-Lechon et al. 2003). However, in order to overcome the limitation encountered with hepatocytes (rapid de-differentiation *in vitro*), we exploited the improved stability of the HepaRG cell-line model (Guillouzo et al. 2007) for our long-term assays, since it is able to differentiate and maintain this differentiation for several weeks.

Much research on pesticide toxicity has focused on testing selected single compounds. An originality of our work is the use of a real-time cell analyzer (xCELLigence®, Roche) to evaluate and to compare by continuous real-time measurement the effects of pesticides and their mixtures after both short and long-term exposures. The use of this technique in toxicity screening is fairly recent. The main advantage relates to the continuous measurement of cellular impedance variations which reflect the inherent morphological and adhesive changes occurring within the cells. Such real-time kinetic measurements require no cellular labeling, are non-invasive and reflect the impact of the toxic compounds tested at the cellular level.

Short-term exposure (24 h) of the differentiated HepaRG cells to the pesticides alone or as mixtures resulted in no modification of cell status whatever the treatment condition. Human hepatocytes on the other hand were affected by some treatments. This showed that despite similar metabolic capacities, freshly differentiated HepaRg cells are less sensitive as compared to human hepatocytes. These results correlate well with recent data obtained by Gerets et al. which reveal a reduced sensitivity of HepaRG cells versus human hepatocytes in detecting chemical hepatotoxicity, even after 48 and 72 h exposure (Gerets et al. 2012). One possible reason to explain this could be a protective effect induced by the high concentration of DMSO present in the differentiating medium (Gilot et al. 2002; LeCluyse et al. 2000).

No sign of toxicity was observed when differentiated HepaRG cells were exposed for 24 h to single doses of

up to 100 μM pesticides either individually or as mixtures. To test whether the cytotoxicity induced by pesticides is concentration-dependent and cumulative, the cells were then treated every 2–3 days for 2 weeks. The toxicity was demonstrated to be cumulative over the 2-week period. Among the three pesticides, only E affected impedance in a dose-dependent manner in our test. Interestingly, the effect of E was also potentiated when combined with A and even more intensively with C. Nevertheless, the effects observed on cells treated with the ternary ACE mixture were not amplified as compared to those of the binary EC combination. Our data therefore appear to exclude any combined effect of the three pesticides in a mixture. In addition, they are in accordance with the non-toxic effects of atrazine described in other studies on various cell systems including HepG2 cells, rainbow trout adrenocortical steroidogenic cells, PC12 adrenal medulla cells, and rat hepatocytes (Bisson and Hontela 2002; Das et al. 2000; Jondeau et al. 2006; Pistl et al. 2003; Sawicki et al. 1998; Tchounwou et al. 2001). On the other hand, our results confirm previous data showing that chlorpyrifos is hepatotoxic only at 100 μM (Das et al. 2008). Despite previous studies mentioning atrazine as nontoxic at similar doses for rat hepatocytes, our data show that the AC binary combination is significantly hepatotoxic to primary hepatocytes regardless of the concentration tested. These data support one proposal stating that the mixture of atrazine and chlorpyrifos may result in greater than additive effects on vertebrate liver cells (Tyler Mehler et al. 2008). Altogether, our data from real-time cellular toxicity measurements highlight the potential harmful effects that can be exerted by pesticides with chronic exposure.

In the present short-term investigations, we used one batch of freshly isolated human primary hepatocytes and two different cryopreserved batches. Interestingly, despite differences between batches of human hepatocytes in terms of expression of metabolic enzymes, their response to pesticides was quite similar. Thus the high sensitivity of this method allows the discrimination between the effects of pesticides and their respective mixtures with statistical significance.

These above observations prompted us to investigate the cytotoxicity of the compounds tested by examining the expression of some key metabolic genes (CYP3A4, GSTA1, and UGT1A1) by quantitative RT-PCR under exposure to non-toxic concentrations (1, 10, and 25 μM). CYP3A4 is a prototypical gene activated by

hPXR induction (Bertz and Granneman 1997; Urquhart et al. 2007) that has particular importance in phase I metabolism during which it is responsible for the metabolism of an estimated 50 % of xenobiotics in humans. The hepatocytes displayed a significant rise in CYP3A4 gene expression levels with almost all the treatments, as did HepaRG cells even after 2 weeks exposure to single pesticides and their mixtures. Both reached the significant level of at least 20 % the inducing potency of rifampicin. These data confirm endosulfan to be a strong hPXR activator (Lemaire et al. 2004; Rouimi et al. 2012) and demonstrate the maintenance of this modulation with long-term exposure. Although both atrazine and chlorpyrifos were recently demonstrated to equally increase CYP3A4 mRNA levels in HepaRG cells (Abass et al. 2012), only chlorpyrifos increased the expression of CYP3A4 in the HepaRG cells in our study with atrazine inducing an increase only after short-time exposure in hepatocytes.

UDP-glucuronosyltransferases (UGTs) are major enzymes among the phase II metabolizing enzymes and are mainly expressed in the liver where their expression is regulated by AhR, PXR and CAR (Xie et al. 2003). They have been proposed to be clinically relevant in drug–drug interactions toxicity in humans (Hanioka et al. 2006). UGT1A1 is a critical enzyme responsible for detoxification and metabolism of endogenous and exogenous lipophilic compounds such as bilirubin (Sugatani et al. 2001). We found no change in the expression of the UGT1A1 gene in hepatocytes after 24 h of treatment suggesting that expression of this gene is insensitive to the pesticides and their mixtures in these cells. In contrast to that previously reported (Soars et al. 2004; Sugatani et al. 2012), this enzyme was not modulated by the well-known PXR/CAR activator, phenobarbital either. In addition, the above-mentioned activation of the prototypical hPXR-regulated CYP3A4 by phenobarbital suggests additional complexity in the mechanism involved in UGT1A1 expression. This is supported by the fact that longer exposures resulted in the induction of UGT1A1 in HepaRG cells. Our results are in agreement with previous work showing that UGT1A1 is highly inducible in the HepG2 hepatocarcinoma cell line but much less in primary human hepatocytes (Hanioka et al. 2006). With long-term exposure, HepaRG cells show a significant increase in UGT1A1 expression which becomes highly amplified after 2 weeks. These results are in agreement with previous dose- and time-dependent gene

expression studies (Lambert et al. 2009). The differences observed between short- and long-term exposures indicate that the gene modulations induced by pesticides (especially endosulfan) in our models correlate with the observed modifications of cell behavior and cell death. While GSTA1 has been reported to be downregulated in other human cell lines after a 24-h exposure to phenobarbital (Romero et al. 2006), we found no change in GSTA1 expression by phenobarbital or any other treatments in HepaRG cells. This may be evidence in favor of speculations made by some authors that HepaRG cells may be derived from a GSTA1/A2 non-responsive individual (Lambert et al. 2009). The differences we observed between hepatocytes and HepaRG responses to the pesticides tested both support and highlight some slight limitations in the use of HepaRG as the gold standard model of liver cells in toxicity studies.

Our study was not designed to estimate the precise additive or synergic effects of the pesticides tested (concentration, addition or independent action) which should include prototypical estimation of mixtures using equitoxic exposures. Nevertheless, our work does provide some interesting data on the topic of repeated long-term exposures using the HepaRG model such as: (a) the mixing of two or three pesticides at doses which are devoid of measurable toxicity alone may lead to significant effects and (b) addition of non-toxic pesticides to endosulfan appears to potentiate its effect. In conclusion, this study has described the first measurement in real-time of the cytotoxic impact of the three pesticides, atrazine, chlorpyrifos, and endosulfan, and their binary and ternary combinations. It demonstrates the usefulness of real-time cell monitoring in evaluating the toxicity of long-term and/or co-exposure to multiple environmental contaminants. The occurrence of changes in the expression of well-known PXR/CAR-regulated key genes involved in procarcinogen activation and detoxication (i.e., CYP3A4 and UGT1A1, respectively), alongside the observed phenotypical changes in our tested cells provide evidence in favor of cellular homeostatic disruption occurring under medium/low dose long-term exposures to residual contaminants. Such modifications could potentially modify a cell's capacity to metabolize drugs and/or endogenous substances and detoxify xenobiotics with a detrimental effect on human health. Overall, the use of real-time measurements may improve the quality of early evaluations of product safety to humans.

Fundings The authors received a Public Institutional Funding from INRA, the French National Research Program 07-PNRA-006, and the ANSES (ES-021).

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