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# Molecular investigation of the effects of lindane in rat hepatocytes: Microarray and mechanistic studies

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ABSTRACT

Although many studies of lindane toxicity have been carried out, we still know little about the underlying molecular mechanisms. We used a microarray specifically designed for studies of the hepatotoxic effects of xenobiotics to evaluate the effects of lindane on specific gene expression in primary cultured rat hepatocytes. These genes were assigned to detoxication processes (CYP3A4, Gsta2, CYP4A1), cell signalling pathways and apoptosis (Eif2b3, Eif2b4, PKC). In this study, we demonstrate that lindane up-regulates PKC by increasing oxidative stress. TEMPO (a well known free radical scavenger) and Ro 31-8220 (an inhibitor of classical PKCs) prevented the inhibition of spontaneous and intrinsic apoptosis pathway (characterised by Bcl-xL induction, Bax down-regulation, caspases inhibition) and the induction of necrosis by lindane in rat hepatocytes. Thus, these findings indicate that several dependent key signalling pathways, including detoxification, apoptosis, PKC activity and redox status maintenance, contribute to lindane-induced toxicity in primary cultured rat hepatocytes. This may account more clearly for the acute and chronic effects of lindane *in vivo*, with the induction of cell death and tumour promotion, respectively.



## 1. Introduction

Lindane, the γ isomer of hexachlorocyclohexane (γ-HCH), is a broad-spectrum pesticide widely used to control arthropod pests in agriculture, veterinary and human medicine. Like many organochlorine insecticides, lindane persists in the environment and this persistence associated with its potential biomagnification remain a matter of public health concern in many countries, because it can store in fats where it persists for a long time. Lindane disrupts the immune response and reproductive functions in experimental animals and is known to be neurotoxic, hepatotoxic and hepatocarcinogenic (Meera et al., 1992; Cooper et al., 1989; Parmar et al., 2003; Videla et al., 1991).

We recently provided further insight into the cytotoxic action of lindane in the liver, demonstrating that the disruptions of autophagy, apoptosis and necrosis processes are responsible for lindane toxicity in primary cultured rat hepatocytes (Zucchini-Pascal et al., 2009). However, the events mediating these dysregulations of cell death remain to be determined. It has been suggested that lindane stimulates the generation of reactive oxygen species (ROS) and that this might be responsible for hepatotoxicity (Videla et al., 1990). In particular, metabolic studies in rodents have suggested that biotransformation processes may be involved in the toxicity of lindane, as indicated by the relationship between induced cytochrome P450 levels and the oxidative stress generated (Videla et al., 2000; Fernández et al., 2003). Indeed, during substrate oxidation, electron transport system from CYP has been demonstrated to produce ROS, such as superoxide radicals, formed by the univalent reduction of molecular oxygen (White and Coon, 1980; Chat et al., 1998). Hence, several CYPs isoforms have been implicated in causing liver injury by generating oxidative stress. For instance, lindane administration in rats causes enhancement of CYP2E1 expression related to superoxide radical generation. Moreover, CYP3A, which accounts for a large amount of drug metabolism in mammals, has been involved in hepatotoxicity in rats, a mechanism related to ROS generation (Minamiyama et al., 2004).

Oxidative stress may damage tissues and cells by altering lipid peroxidation and protein or nucleic acid structure and function. Indeed, ROS generation influences many signalling proteins interfering with the molecular and biochemical processes responsible for cell differentiation, proliferation and death (Allen and Tresini, 2000). Proteins kinase C (PKCs) are considered to be "oxidative

*Abbreviations:* Ab, antibody; BCA, bicinchonic acid; BSA, bovine serum albumin; CYP, cytochrome P450; DTT, dithiothreitol; FBS, foetal bovine serum; L, lindane; PKC, protein kinase C; PMSF, phenylmethylsulfonylfluoride; ROS, reactive oxygen species; TEMPO, 2,2,6,6-tetramethylpiperidine 1-oxyl; UT, untreated cells.

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stress sensors'' because they are activated by ROS and involved in various pathways regulating stress responsiveness (Stabel and Parker, 1991). These enzymes constitute a superfamily of serine–threonine kinases that are classified into conventional or classical (α, β1, β2, and γ), novel (δ, ε, η, θ, and μ), and atypical (ζ, λ/ι) subfamilies on the basis of their molecular structure and cofactor requirements. PKCs members are crucial signalling proteins that are subjected to redox regulation and may control these responses in liver (Nitti et al., 2008).

The aim of this study was to explore the mechanisms underlying lindane-induced cytotoxicity in cultured rat hepatocytes. For this purpose, we developed dedicated microarrays for studies of hepatotoxicity in rats. Approximately 270 genes from several categories (listed in Supplementary Table 1) were selected based on our knowledge of the ways in which known toxic substances operate (Pennie, 2000). Our ''Hepatotoxic rat microarray'' was used to identify genes deregulated by lindane treatment in primary cultured rat hepatocytes. Based on the results obtained with this technology and our previous works, we hypothesised that the signalling pathways deregulated by lindane were dependent on oxidative stress generation and PKC activation. We therefore analysed the effects of the antioxidant molecule TEMPO and the PKC inhibitor Ro 31-8220 on the inhibition of apoptosis by lindane in primary cultured rat hepatocytes. Overall, this study provided an opportunity to identify the genes and signal transduction pathways involved in the hepatotoxic effect of lindane.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Chemicals

Williams' medium E and foetal bovine serum (FBS) were obtained from Invitrogen (Rockville, USA) and penicillin/streptomycin solution from Bio-Whittaker (CAMBREX Company, Walkersville, USA). Collagenase was from Boehringer Mannheim (Mannheim Corp; Sydney, Australia), insulin from Nova Nordisk (Nova Nordisk A/S, Bagsvaerd, DENMARK) and RiboGreen from Molecular Probes (Eugene, Oregon, USA). All other chemicals were from Sigma–Aldrich (L'Isle d'Abeau Chesne, Saint Quentin Fallavier, France) unless otherwise specified.

#### 2.1.2. Antibodies (Abs)

Polyclonal Ab to Bcl-xL was from Transduction Laboratories (Lexington, KY, USA); monoclonal Ab to rat CYP3A1 (clone 2.13.1), was from Oxford Biomedical Research (Oxford, MI), polyclonal Abs to PKCα, δ, and η were from Santa Cruz. Blots were reprobed with monoclonal Ab to human and rat β Tubulin (clone 2.1) from Sigma.

### 2.2. Rat hepatocytes culture and treatments

Hepatocytes were isolated from male Sprague–Dawley rats (IFFA Credo, France) weighing 150–250 g. All animals received humane care in compliance with institutional guidelines. Hepatocytes were obtained by reverse two-step collagenase perfusion as previously described (Zucchini et al., 2005). Isolated cells were resuspended in medium consisting of William's medium E with 10% FBS, penicillin (50 UI/ml), streptomycin (50 μg/ml) and insulin (0.1 UI/ml). Cell viability was determined as at least 80% by the Erythrosin B exclusion test. Hepatocytes were seeded in collagen type 1-coated dishes and then incubated for four hours to permit adhesion in a humidified atmosphere with 95% air and 5% $CO_2$ at 37 °C. Then, cultures were washed to remove dead or unattached cells and the first medium was replaced by a similar one that did not contain serum and was supplemented with hydrocortisone hemisuccinate (1 μM) and bovine serum albumin (240 μg/ml).

Rat hepatocytes were treated with graded concentrations of lindane: 10 μM, 25 μM, 50 μM and 75 μM for 24, 48 or 72 h. To examine oxidative stress and PKCs involvement in the lindane signalling pathway, hepatocytes were treated with lindane in the presence or not of 100 μM TEMPO (2,2,6,6-Tetramethylpiperidine 1-oxyl) or 100 nM Ro 31-8220 for 48 h. Lindane, TEMPO and Ro 31-8220 were prepared as a dimethylsulphoxide (DMSO) stock solution and were directly added to cultures. The final DMSO concentration was 0.25% (v/v).

### 2.3. RNA isolation

After treatment, the medium was removed and cells were washed once with cold PBS. Total RNAs were isolated by acidic phenol extraction from control or treated cells. The purity of the RNAs obtained was evaluated by agarose gel electrophoresis and by the calculated A260/A280 (up to 1.9) and A260/A230 nm absorbance ratios. Quantification of RNAs was done using Molecular Probes' Ribogreen RNA Quantification kit according to the manufacturer's instructions.

### 2.4. Preparation of probes and spotting of cDNA microarray

Microarrays were produced on the INRA/CNRS Genomics Platform (Sophia-Antipolis, France; Feyereisen and Barbry teams). The cDNA microarrays contained ~265 distinct rat cDNA probes. This set of cDNAs represents toxicologically relevant genes in liver such as drug-metabolizing enzymes (e.g., cytochromes P450), apoptosis (e.g., Bcl-2 family), cell proliferation (e.g., cyclin) and signal transduction (Supplementary Table 1). The cDNA probes were PCR-amplified from cDNA derived from RNA freshly isolated. They were amplified with specific primers (Sigma–Genosys, Pampisford, UK) designed in the 3' end of coding region of rat genes using Primer 3 calculation software. Probes ***1***) had a normalised length of 300 ± 50 bp; ***2***) were specific for a unique rat gene and ***3***) were controlled by DNA sequencing. Following PCR amplification, products were analysed by gel electrophoresis to estimate product size and confirm amplification specificity. PCR products were purified by using QIAquick 96 PCR Purification Kit (Qiagen) and resuspended in 3X SSC at a concentration of 200 ng/μl. Microarrays were printed with amine-labelled cDNA probes using a SDDC-2 chipwriter (Bio-Rad, Virtek) on homemade glutaraldehyde-coated glass microscope slides. Each glass slide contained four copies of all hepatocytes probes and included negative control cDNAs (Spikes ArrayControls, Ambion) to confirm hybridisation specificity along with several housekeeping cDNAs.

### 2.5. cDNA microarray hybridisation

Cy3 and Cy5 labelled cDNA were synthesized from 10 μg of total RNA using the CyScribe First Strand cDNA Labelling Kit (Amersham-Pharmacia Biotech), in the presence of *spike* mRNA (Spikes ArrayControls, Ambion). Template RNA was then removed by incubation with RNase A and single-stranded cDNA was purified using QIaquick Nucleotide Removal Kit (Qiagen USA, Valencia, CA), dried under vacuum, and finally dissolved in 20 μL of DigEasy Hyb buffer (Boehringer Mannheim/Roche, Basel). This hybridisation mix was added to the home-made rat cDNA microarray surface and covered with a standard coverslip. Slides were placed in hybridisation chambers (Corning, Corning, NY), and 20 μL of 3X SSC was placed inside each chamber before sealing. Slides were incubated for 18 h in a water bath at 42 °C and then were sequentially washed in the following solutions: 1X SSC, 0.03% (w/v) SDS for 5 min, 0.2X SSC for 5 min, and 0.05X SSC for 5 min. Slides were dried by centrifugation at 600*g* for 2 min before they were scanned. Fluorescence intensities of Cy3 and Cy5 were measured separately at 532 and 635 nm with a laser scanner (Axon Genepix4000A) at a 10-μm resolution. The resulting 16-bit data files were imported into an image analysis programme (Genepix 2.0, Axon Instruments). To reduce systematic bias, dye swap replication was performed using two total RNA samples form the same extraction, in which dye assignment was reversed in the second hybridisation. Moreover, to ensure the reproducibility of the microarray results, three biological replicates of this design were performed.

**Table 1**
Sequence of primers used for real-time RT-PCR analysis.

| Accession No. | Symbol | Forward primer | Reverse primer |
|---|---|---|---|
| NM_031144 | Actb | AACTGGGACGATATGGAGA | GTCCATCACAATGCCAGTG |
| NM_153307 | Cyp4a1 | TCCAAGTCACACTCTCCATT | CCACAATCACCTTCATCTCA |
| NM_173144 | Cyp3a23/3a1 | TTCAGCAAGGAGAACAAGG | ACAAGGCTGGAAGGAGAA |
| NM_033539 | Eef1a1 | AGTGGAAACTGGTGTTCTC | CTTCCAGCTTCTTACCAGA |
| NM_053950 | Eif2b4 | TGGCAGAACAACTCATCAC | AGGACAACAGGCACAGAAC |
| NM_133609 | Eif2b3 | ACTGTTACCAACTGCCTTCTC | CTTCAATCCTCTGACCACTTC |
| NM_017013 | Gsta2 | GTACTTGCCTGCCTTTGAA | CAGAGGGAAAGAGGTCAGAA |
| NN1_031085 | Prkch | AAGATCGCAGAGCAACG | AGTCCTTGTCGCATTATCC |