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**Chlordecone potentiates hepatic fibrosis in chronic liver injury induced by carbon tetrachloride in mice**

Running title: Chlordecone potentiates hepatic fibrosis

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**Highlights**

- Hepatic impact of chronic co-exposure to chlordecone and CCl<sub>4</sub> investigated in mouse model.
- Chlordecone enhances the liver damage in CCl<sub>4</sub> murine chronic hepatitis.
- Chlordecone enhances the progression of liver fibrosis in mice.

## Abstract

Chronic liver damage due to viral or chemical agents leads to a repair process resulting in hepatic fibrosis. Fibrosis may lead to cirrhosis, which may progress to liver cancer or a loss of liver function, with an associated risk of liver failure and death. Chlordecone is a chlorinated pesticide used in the 1990s. It is not itself hepatotoxic, but its metabolism in the liver triggers hepatomegaly and potentiates hepatotoxic agents. Chlordecone is now banned, but it persists in soil and water, resulting in an ongoing public health problem in the Caribbean area. We assessed the probable impact of chlordecone on the progression of liver fibrosis in the population of contaminated areas, by developing a mouse model of chronic co-exposure to chlordecone and a hepatotoxic agent, carbon tetrachloride (CCl<sub>4</sub>). After repeated administrations of chlordecone and CCl<sub>4</sub> by gavage over a 12-week period, we checked for liver damage in the exposed mice, by determining serum liver transaminase (AST, ALT) levels, histological examinations of the liver and measuring the expression of genes encoding extracellular matrix components. The co-exposure of mice to CCl<sub>4</sub> and chlordecone resulted in significant increases in ALT and AST levels. Chlordecone also increased expression of the Col1A2, MMP-2, TIMP-1 and PAI-1 genes in CCl<sub>4</sub>-treated mice. Finally, we demonstrated, by quantifying areas of collagen deposition and alpha-SMA gene expression, that chlordecone potentiated the hepatic fibrosis induced by CCl<sub>4</sub>. In conclusion, our data suggest that chlordecone potentiates hepatic fibrosis in mice with CCl<sub>4</sub>-induced chronic liver injury.

## Abbreviations

AST: Aspartate aminotransferase

ALT: Alanine aminotransferase

CD: Chlordecone

CCl<sub>4</sub>: Carbon tetrachloride

$\alpha$ -SMA:  $\alpha$ -smooth muscle actin

ECM: Extracellular matrix

**Keywords:** Liver; Fibrosis; Chlordecone; CCl<sub>4</sub>; Collagen; Kepone

## Introduction

Fibrogenesis is a wound-healing response to repeated liver injury. It is triggered in response to chronic liver damage, regardless of the cause of the damage: viral infections, alcohol abuse, drug exposure, metabolic dysfunctions or autoimmune disorders. It manifests as a progressive replacement of the liver parenchyma with scar tissue. This process takes place over many years and can lead to other complications, such as cirrhosis, leading to liver failure or an increase in the risk of hepatocellular carcinoma. Hepatic fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM) (Friedman, 2003). At advanced stages of the disease, there may be six times more ECM in fibrotic livers than in normal livers (Benyon and Iredale, 2000). This accumulation results from an imbalance between the synthesis and degradation of ECM components (Arthur, 2000), such as fibrillar collagen, essentially collagen type I (Tsukada et al., 2006), matrix metalloproteinases (MMPs) (Takahara et al., 1995) and their tissue inhibitors (TIMPs) (Benyon and Iredale, 2000).

Humans are exposed, on a daily basis, to various toxic agents, chemicals and drugs in the environment. The liver is the major site of detoxification for these compounds in the body. This organ may therefore be considered a target of their toxicity. In humans, hepatotoxicity due to exposure to alcohol, drugs such as acetaminophen, imidazole fungicidal agents and other chemicals has also been studied in a context of exposure to mixtures of these agents. It has been shown that exposure to alcohol + acetaminophen, or alcohol + imidazole fungicidal agent mixtures results in worse damage to the human liver than exposure to any of these agents alone (Zimmerman and Maddrey, 1995).

Many animal models for studying the molecular processes underlying the pathophysiology of the liver during exposure to hepatotoxic compounds administered alone or in combination have been developed, to improve our understanding of the molecular mechanisms of action of these hepatotoxic compounds and the host response, and to identify biological markers of liver damage. As in humans, mixtures of chemical agents were found to aggravate liver damage in most of the animal models studied (Brautbar and Williams, 2002).

In animals, diverse causes of fibrogenesis have been studied, but the principal *in vivo* model of fibrosis is based on the repeated administration of carbon tetrachloride (CCl<sub>4</sub>) over a period of several weeks (Brautbar and Williams, 2002; Chang et al., 2005). CCl<sub>4</sub> is a hepatotoxic agent that can induce liver fibrosis, and even cirrhosis, after long-term exposure. CCl<sub>4</sub> is known to trigger centrilobular hepatic necrosis and the first event in its hepatotoxicity

is its metabolic activation in the liver (Weber et al., 2003). CCl<sub>4</sub> is increased by other agents, such as alcohol, in situations of co-exposure in animals (Benyon et al., 1996; Chobert et al., 2012; McLean and McLean, 1966). In particular, the hepatotoxicity of this compound has been shown to be increased in models of co-exposure to pesticides, such as mirex and chlordecone.

Chlordecone, also known as kepone, is an organochlorine pesticide that was produced essentially from 1958 through 1975 in the United States, for export to Latin America, Africa and Europe for use as an ant and cockroach poison. Kepone was also used to control banana root borer, primarily in the Caribbean area. Kepone production was banned in 1976 after it was discovered that the Life Science Products Company factory in Hopewell, Virginia, which was responsible for producing this chemical, had caused serious contamination of the environment and many of its workers were found to have medical problems. All workers at this factory presented symptoms of chlordecone poisoning, including headaches, anxiety, irritability and memory disturbance, abnormal hepatic function and defective spermatogenesis. Studies of chlordecone toxicity in humans and animal models implicated this agent in a broad array of syndromes, affecting the nervous and reproductive systems in particular. Chlordecone is known to accumulate mostly in the liver (Egle et al., 1978) triggering hepatomegaly (Guzelian, 1985). Chlordecone is not itself hepatotoxic, but several studies have shown that it can strongly potentiate the hepatotoxic effects of CCl<sub>4</sub> in models of acute co-exposure (Curtis et al., 1979; Klingensmith et al., 1983; Mehendale, 1989).

Chlordecone is no longer used, but it persists in the soils and water of the Caribbean area. It thus remains a public health problem, due to its potential contribution to many chronic diseases, including liver diseases.

In this study, we developed a mouse model of chronic co-exposure to chlordecone and CCl<sub>4</sub>, to assess the impact of chlordecone on the development and progression of liver fibrosis. We evaluated the liver damage caused by the interaction between chlordecone and CCl<sub>4</sub>, the expression of genes involved in matrix remodeling and the amount of collagen deposited in mouse livers.

## **Materials and Methods**

### **Animals and experimental protocol**

Eight-week-old male C57Bl/6 mice were purchased from Janvier (Le Genest-sur-Isle, France) and reared in specific pathogen-free (SPF) conditions in the local animal house, in accordance with French laws and institutional guidelines for the handling and care of experimental animals (accreditation of M. Samson #3596).

Animals were assigned to four groups at random. In the first group, the mice received olive oil daily, by gavage, as a control treatment. In the second group, mice were treated, once weekly, with CCl<sub>4</sub> (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 0.1 g/kg. In the third group, the mice received chlordecone (Santa Cruz Biotechnology) daily, at a dose of 5 mg/kg. In the fourth group, the mice received chlordecone at a dose of 5 mg/kg daily for two weeks and then 0.1 g/kg CCl<sub>4</sub> once weekly, in addition to their daily dose of chlordecone. CCl<sub>4</sub> and chlordecone were diluted in olive oil and administered by oral gavage. During the 12 weeks of the study, the mice were observed and their body weight was measured once weekly. The doses of the chemicals used were well tolerated and no deaths occurred. Blood was collected from the mice 48 h and 72 h after the administration of CCl<sub>4</sub> in weeks 5, 7, 9 and 12 of the study. The mice were killed 72 h after the administration of the last dose of CCl<sub>4</sub>. The liver was removed and fragments of this organ were fixed in 4% paraformaldehyde and embedded in paraffin or frozen in liquid nitrogen in the presence of the cryoprotectant isopentane. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined according to IFCC primary reference procedures, with an Olympus AU2700 Autoanalyser® (Olympus Optical Optical Co. Ltd., Tokyo, Japan).

### **RNA isolation and RT-qPCR**

We homogenized liver tissue samples weighing approximated 100 mg and extracted total RNA in Trizol reagent (Invitrogen). The first-strand cDNA was produced with SuperScript™ II Reverse Transcriptase (Invitrogen). The cDNA was then amplified, with the GAPDH housekeeping gene as a control. Quantitative PCR was performed with the fluorescent dye SYBR Green and the double strand-specific SYBR® Green system (Applied Biosystems), with an ABI 7900 HT Prism sequence analyzer (Applied Biosystems). Total cDNA (30 ng) was used as a template for amplification, with the specific primer pair (Table 1) used at a final concentration of 300 nM. Each measurement was performed in triplicate. We determined 18S mRNA levels as a control, and the level of expression of each gene studied is expressed relative to that of the 18S gene. The primers used were, for collagen 1A2 (forward: 5'AGGCTGACACGAACTGAGGTA3', reverse:

5'ATGCACATCAATGTGGAGGA3'), for MMP-2 (forward: 5'TTGAGAAGGATGGCAAGTAT3', reverse: 5'ATGGTAAACAAGGCTTCATG3'), for TIMP-1 (forward: 5'TTCCAGTAAGGCCTGTAGC3', reverse: 5'TTATGACCAGGTCCGAGTT3'), for PAI-1 (forward: 5'TCCAGAAGCAGAGAGGGAAA3', reverse: 5'CAGTGCAGCTCTGACTCACC3'), for 18S (forward: 5'CGCCGCTAGAGGTGAAATTC3', reverse: 5'ATGCACATCAATGTGGAGGA3').

### **Immunocytochemistry and histology**

For the assessment of liver injury, 4  $\mu$ m-thick sections of paraffin-embedded liver were cut, placed on microscope slides and stained with hematoxylin and eosin (H&E). Slides were scanned with a Nanozoomer NDP and images were acquired with NDP viewer software.

Collagen deposition in treated mouse livers was assessed by Sirius red staining on 4  $\mu$ m-thick paraffin-embedded liver sections. The stained area was measured, over the total liver surface, with NIS element software. The percentage of the total liver area displaying fibrosis was calculated.

Liver sections (10  $\mu$ m thick) from frozen samples, were fixed on slides, by incubation in 4% formaldehyde for 10 minutes. They were then washed and immunostained for  $\alpha$ -smooth muscle actin (Cy3-conjugated monoclonal anti-actin  $\alpha$ -smooth muscle antibody, Clone 1A4, Sigma) for 1 hour. The liver sections were then washed in 1x PBS, mounted in fluorescence mounting medium (Dako) and scanned with the Nanozoomer NDP for image acquisition. Four mice per set of treatment conditions were analysed and the  $\alpha$ -SMA stained was quantified with Image J software, using a common threshold for all samples.

### **Multiphoton SHG microscopy**

Multiphoton SHG images were acquired on a photonic imaging platform (PIXEL facility of GIS EUROPIA, University of Rennes 1, France, <http://pixel.univ-rennes1.fr>). The imaging setup consisted of a confocal Leica TCS SP2 scanning head (Leica Microsystems, Mannheim, Germany) mounted on a Leica DMIRE2 inverted microscope and equipped with a MAITAI Spectra Physics femtosecond laser (Spectra Physics, Santa Clara, CA, USA). A Leica dry objective HCPLAPO 10X (NA=0.4) and Leica condenser S23 (NA=0.53) were used to apply a constant 12 mW of excitation at 820 nm to the sample, and for collection, respectively. BG39 bandpass and 410 nm IR (10 nm FWHM) filters were placed before the



trans-detection photomultiplier tube (PMT) detector. Constant gains were achieved by setting 650 V and 750 V at PMTs for the trans-detection of the forward SHG signal and the epi-detection of the autofluorescence signal, respectively. Images were acquired with 20  $\mu$ m-thick deparaffinized liver slices. Four random fields of view per liver section were imaged. The SHG signal was analyzed and quantified with open-source ImageJ software. Briefly, background pixels were set to NaN (not-a-number) values and a common threshold was set for all samples. The SHG signal was quantified by determining the percentage of pixels with a signal above the threshold.

### Statistical analysis

Kruskal–Wallis one-way analysis of variance (ANOVA) was performed, and mean differences between experimental groups were assessed in nonparametric Mann–Whitney U-test, with GraphPad Prism5 software. For all statistical analyses, \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.01$  and \*\*\* denotes  $p < 0.001$ .

## Results

### Chlordecone administration potentiates the chronic liver injury induced by CCl<sub>4</sub>

We investigated the impact of chlordecone on chronic liver injury, by treating mice with CCl<sub>4</sub> and chlordecone by gavage, according to the experimental design presented in figure 1S. Measurements of body weight over a period of 12 weeks revealed no significant difference between the four treatments (Fig. 2S), liver-to-body weight ratio was significantly higher in mice treated with chlordecone alone or in combination with CCl<sub>4</sub> than in mice treated with CCl<sub>4</sub> alone or oil (negative control) (Fig. 1A). Levels of the transaminases ALT and AST were measured in the collected serum after 5, 7, 9 and 12 weeks of treatment (Fig. 1B and 3S). Chlordecone gavage had no effect on ALT and AST levels, whereas treatment with CCl<sub>4</sub> alone caused a significant increase of the levels of these enzymes in the serum. The exposure of mice to both CCl<sub>4</sub> and chlordecone resulted in significantly higher levels of ALT and AST than exposure to CCl<sub>4</sub> alone or oil, indicating that hepatic injury was more severe in conditions of co-exposure. The staining of liver sections with hematoxylin and eosin showed the necrotic areas to be larger after combined treatment with CCl<sub>4</sub> and chlordecone than after exposure to CCl<sub>4</sub> exposure alone, whereas liver sections from mice treated with chlordecone alone or with oil contained no zones of hepatic necrosis (Fig. 1C). Moreover, the areas of

necrosis increased over time for all treatments. These measurements demonstrate that chlordecone administration potentiates the chronic liver injury caused by CCl<sub>4</sub>.

### **Chlordecone enhances expression of the Col1A2, MMP-2, TIMP-1 and PAI-1 genes in the CCl<sub>4</sub>-treated mouse model**

Chronic liver injury is usually associated with changes in the extracellular matrix. Molecular changes in the livers of mice treated with oil, chlordecone, CCl<sub>4</sub> or with both chlordecone and CCl<sub>4</sub> were investigated by studying the expression of a number of genes encoding components of the extracellular matrix. Expression of the gene encoding type 1 collagen, the most abundant collagen in fibrotic tissues, increased significantly over time in mice exposed to both chlordecone and CCl<sub>4</sub> and the level of expression of this gene was higher in these mice than in CCl<sub>4</sub>-treated mice (Fig. 2A). Expression of the genes encoding matrix metalloproteinase-2 and its inhibitor (TIMP-1) was upregulated in the livers of mice treated with both chlordecone and CCl<sub>4</sub> (Fig. 2B, 2C). Expression of the gene encoding PAI-1, which is known to enhance collagen deposition in injured livers, was slightly stronger in mice exposed to both chlordecone and CCl<sub>4</sub> than in mice exposed to CCl<sub>4</sub> alone (Fig. 2D). These changes in gene expression suggest that there is an imbalance in the synthesis and degradation of extracellular matrix components in mice treated with both chlordecone and CCl<sub>4</sub> and that this imbalance results in greater liver injury.

### **Chlordecone potentiates the hepatic fibrosis induced by CCl<sub>4</sub>**

As repeated liver injury leads to liver fibrosis, we assessed the impact of chlordecone on chronic CCl<sub>4</sub>-induced liver fibrosis development and progression, by assessing collagen deposition in mouse liver. Sirius red staining was performed on mouse liver sections, for all treatments. Control and chlordecone-treated mice had normal collagen distributions, whereas CCl<sub>4</sub>-treated mice presented excessive collagen deposition, increasing with treatment duration. Collagen deposition levels were highest in liver sections from mice exposed to both chlordecone and CCl<sub>4</sub> (Fig. 3A). Quantification of the areas of collagen deposition on sections stained with Sirius red showed that a significantly larger area of the liver was covered with collagen in mice treated with both chlordecone and CCl<sub>4</sub> than in mice treated with CCl<sub>4</sub> alone (Fig. 3B).

We also assessed collagen deposition by second harmonic generation (SHG), to provide further evidence for a role of chlordecone in potentiating CCl<sub>4</sub>-induced hepatic fibrosis.

Representative liver sections were imaged by multiphoton microscopy, and we found that the SHG signal was stronger on imaged liver samples obtained from mice treated with both chlordecone and CCl<sub>4</sub> for 12 weeks than in mice treated with CCl<sub>4</sub> alone (Fig. 4A). The quantification of SHG signal images showed that significantly more collagen was deposited in mice exposed to both CCl<sub>4</sub> and chlordecone than in mice treated with CCl<sub>4</sub> alone (Fig. 4B). The imaging results obtained for Sirius red staining and SHG analysis clearly demonstrate that treatment with both chlordecone and CCl<sub>4</sub> increases the size of the fibrotic area in mouse liver.

### **Chlordecone upregulates the expression of $\alpha$ -SMA in CCl<sub>4</sub>-treated mice**

The excessive production of extracellular matrix during the development and the progression of liver fibrosis is mostly due to hepatic stellate cells (HSCs). These cells undergo a process of activation in chronically damaged livers, characterized by the gain of a myofibroblastic phenotype and by the production of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (Geerts, 2001). We assessed the impact of chlordecone on the production of  $\alpha$ -SMA protein in the livers of mice treated with oil, chlordecone, CCl<sub>4</sub>, or chlordecone+CCl<sub>4</sub> for 5 or 12 weeks. Immunostaining for  $\alpha$ -SMA on liver sections yielded a positive signal when mice were treated with CCl<sub>4</sub> for five weeks and a stronger signal when they were treated for 12 weeks. However, the exposure of mice to both chlordecone and CCl<sub>4</sub> clearly enhanced the  $\alpha$ -SMA signal on liver sections (Fig. 5A). The quantification of  $\alpha$ -SMA staining revealed that the area displaying such staining was significantly larger in the livers of mice treated with both chlordecone and CCl<sub>4</sub> than in those of mice exposed to CCl<sub>4</sub> only (Fig. 5B)

### **Discussion**

Chlordecone is a persistent environmental contaminant that its resistance to biodegradation and its high potential for bioaccumulation have hindered its elimination since the prohibition of its use. Thus, even though this toxic pesticide is no longer used, studies on the impact of chlordecone on human health will remain relevant for a long time to come. Hepatomegaly is the most frequent symptom observed in people exposed to chlordecone (Cohn et al., 1978). Indeed, as chlordecone accumulates in the liver and is eliminated slowly, the liver is a major target organ for studies of the toxicity of chlordecone (Belfiore et al., 2007; Egle et al., 1978). Studies in rodent models have shown that chlordecone can induce several molecular changes

in the liver, including the induction of cytochrome P450 enzymes (Mehendale et al., 1977, 1978) and changes to cholesterol homeostasis (Carpenter and Curtis, 1991; Gilroy et al., 1994).

In this study, we investigated the impact of chlordecone on the development and progression of chronic liver diseases, such as hepatic fibrosis. We developed a mouse model of chronic exposure to chlordecone and to the hepatotoxic chemical CCl<sub>4</sub>. The interactions between chlordecone and several other chemicals have been studied in mouse and rat models, in acute exposure protocols involving the administration of only one or a few doses of chlordecone. For instance, chlordecone has been shown to potentiate the hepatotoxic and lethal effects of acetaminophen and bromotrichloromethane (Agarwal and Mehendale, 1982; Fouse and Hodgson, 1987). The impact of chlordecone on CCl<sub>4</sub> hepatotoxicity has also been studied in models of acute co-exposure. Rodents subjected to chlordecone treatment and then challenged with CCl<sub>4</sub> display enhanced bioactivation of CCl<sub>4</sub> and a lack of hepatocyte regeneration (Klingensmith et al., 1983; Lockard et al., 1983).

In our model of chronic co-exposure, mice were treated by gavage for 10 days with a dose of 5 mg/kg chlordecone, to ensure that the animals received the whole dose and was saturated with chlordecone (Lagarrigue et al., 2014). After this pretreatment, the mice were challenged by gavage with a moderate dose of CCl<sub>4</sub> (0.1 g/kg) once per week for 5, 7, 9 and 12 weeks in addition to their daily dose of chlordecone (5 mg/kg). As previously reported, treatment with chlordecone induced hepatomegaly. Co-exposure to chlordecone and CCl<sub>4</sub> significantly increased this hepatomegaly, in a time-dependent manner. Determinations of serum hepatic enzymes (AST and ALT), which are considered to be the principal indicators of liver injury (Pratt and Kaplan, 2000), showed the levels of these enzymes to be significantly higher in mice exposed to both chlordecone and CCl<sub>4</sub>, with the levels of these enzymes increasing with the duration of treatment. Further evidence for the impact of co-exposure to chlordecone and CCl<sub>4</sub> on liver injuries was provided by the histological examination of livers, which showed larger areas of necrosis for mice exposed to both chemicals and for longer durations of treatment. We used the CCl<sub>4</sub>-induced chronic liver injury model to assess hepatic fibrosis. With the experimental strategy used, we were able to detect molecular changes due to the process of fibrogenesis and to observe typical liver fibrosis after CCl<sub>4</sub> application between weeks 5 and 12 of the experiment. During fibrosis progression, changes in the composition of the ECM were observed, with an increase in fibrillar collagen production (Tsukada et al., 2006). We found that expression of the collagen type I gene was stronger in mice exposed to

both chlordecone and CCl<sub>4</sub> than in those exposed to CCl<sub>4</sub> alone. Similarly, co-exposure to chlordecone and CCl<sub>4</sub> led to an upregulation of MMP-2 and its inhibitor, TIMP-1 (Herbst et al., 1997), contributing to ECM remodeling. Our study thus reveals that pretreatment with chlordecone aggravates the molecular changes underlying fibrogenesis in the mouse model of CCl<sub>4</sub>-induced liver fibrosis.

From the fifth week of chronic treatment with CCl<sub>4</sub> alone, we observed an increase in collagen deposition in mouse livers. Furthermore, treatment with both chlordecone and CCl<sub>4</sub> resulted in significantly higher levels of collagen deposition, as shown by both Sirius red staining and multiphoton SHG analysis. Immunostaining for  $\alpha$ -SMA and its quantification in mouse liver showed that chlordecone clearly upregulated the production of this protein during fibrogenesis.

In conclusion, this study demonstrates that hepatic fibrosis can be influenced by chronic co-exposure to chlordecone, which aggravates liver disease. In our mouse model, chlordecone potentiated hepatic fibrosis in animals with chronic liver injury induced by carbon tetrachloride. As chlordecone has been shown to potentiate the effects of hepatotoxic drugs, such as acetaminophen (Fouse and Hodgson, 1987), studies should be carried out on human populations living in chlordecone-contaminated areas, to check the effects of exposure to this chemical on chronic liver diseases.

### **Conflict of interest**

The authors have no financial or commercial conflict of interest to declare.

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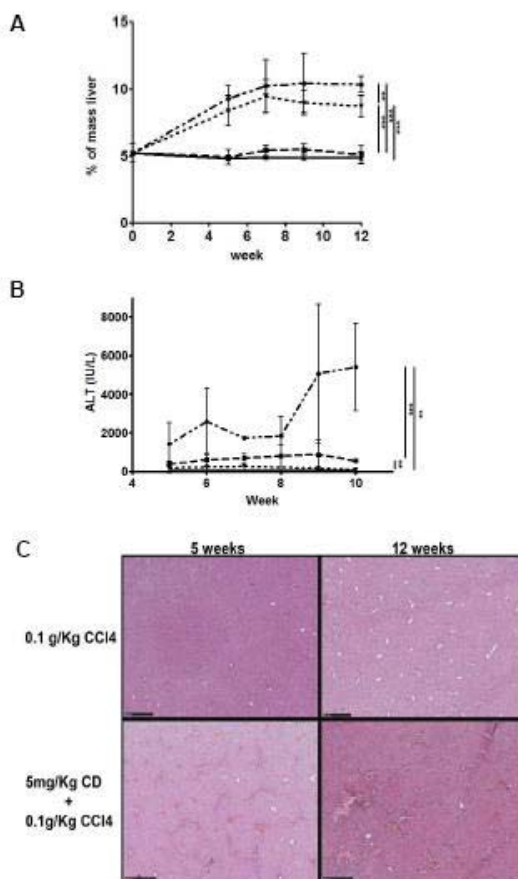
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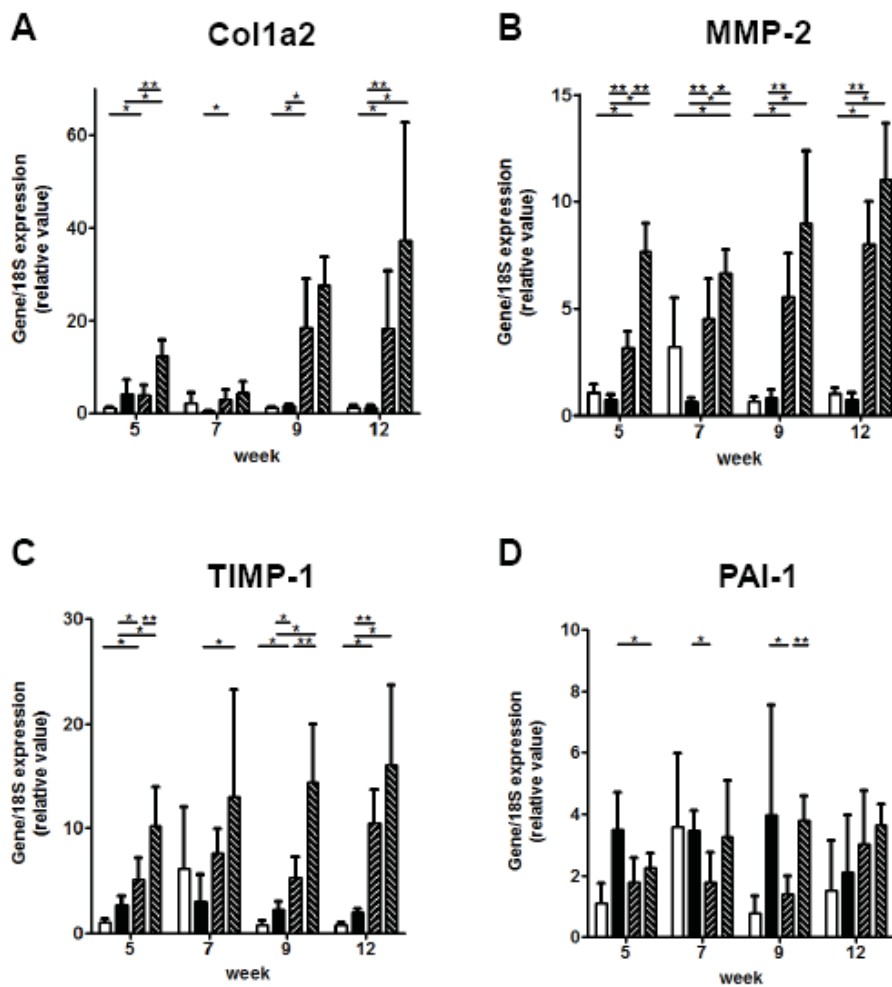


## Figure Captions

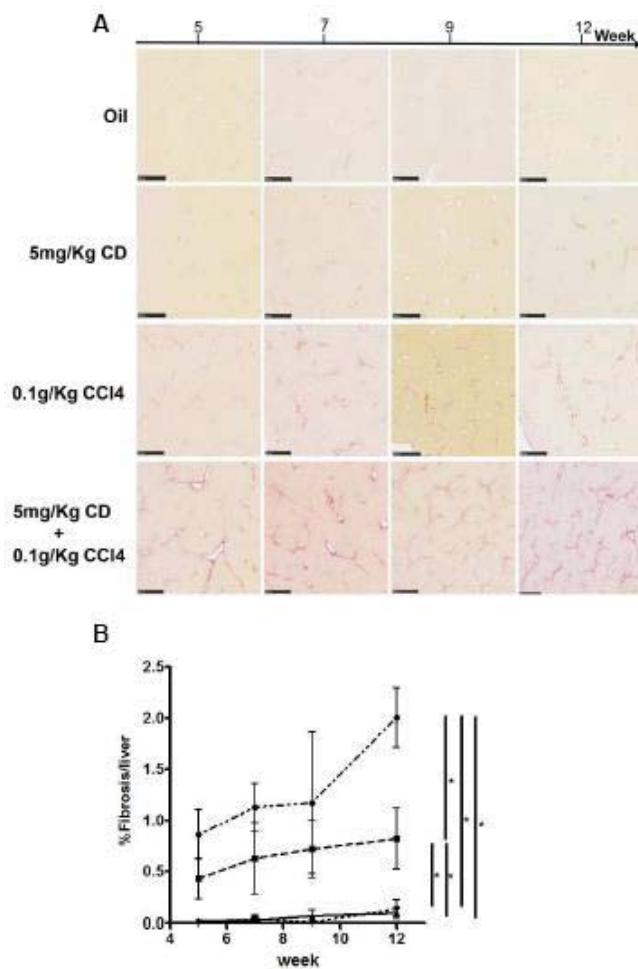
**Figure 1: Chronic liver injury caused by CCl<sub>4</sub> and chlordecone in C57BL/6 mice.** Mice received oral gavage with olive oil, chlordecone (5 mg/kg body weight), CCl<sub>4</sub> (0.1 g/kg body weight) or chlordecone+CCl<sub>4</sub>. They were killed at 5, 7, 9 and 12 weeks of the study. (A) Liver-to-body weight ratio, demonstrating the occurrence of hepatomegaly in the treated mice. (B) Serum levels of ALT (IU/L) in mice at the four time points studied. (C) Liver sections stained with hematoxylin and eosin, showing the necrotic area in mice at 5 and 12 weeks of study. Scale bars = 500  $\mu$ m. The different treatment groups are shown in (A) and (B) as  for oil,  for chlordecone,  for CCl<sub>4</sub> and  for chlordecone+CCl<sub>4</sub>. The data shown are means  $\pm$  SD for four animals per group and the *p*-values for Mann-Whitney tests, at the right margin of the graphs, indicate the significance of differences between groups (\*\**P*<0.01; \*\*\**P*<0.001).



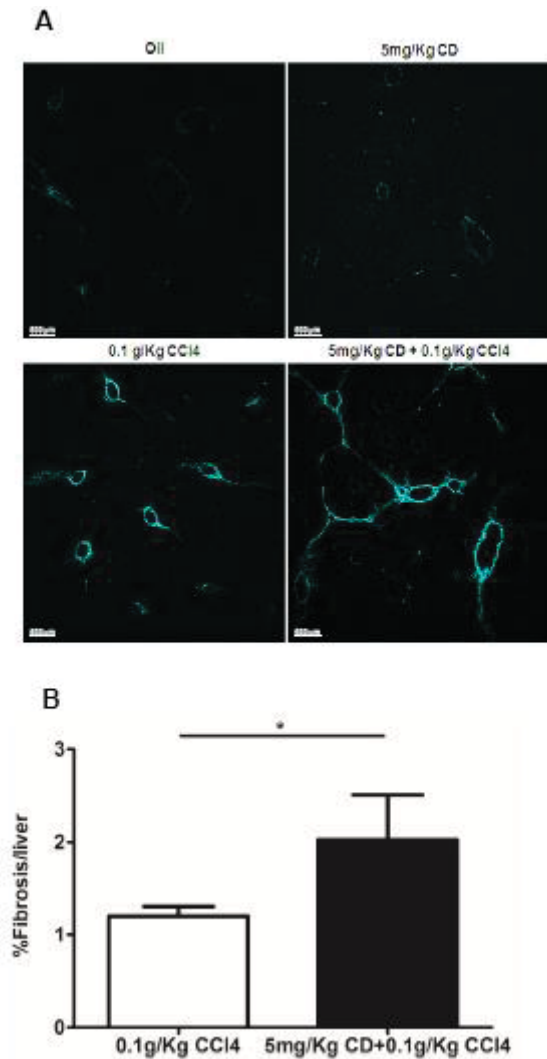
**Figure 2: Reverse transcription-PCR analysis of collagen 1a2 mRNA levels.** (A) MMP-2 (B), TIMP-1 (C) and PAI-1 (D). Liver total RNA was extracted from mice treated with oil, chlordecone, CCl<sub>4</sub> or chlordecone+CCl<sub>4</sub> for 5, 7, 9, or 12 weeks, as described in the materials and methods section. After RT-PCR analysis, mRNA levels were expressed as ratios relative to 18S levels. The histogram bars corresponding to the different treatment groups are shown as  for oil,  for chlordecone,  for CCl<sub>4</sub> and  for chlordecone+CCl<sub>4</sub>. The data shown are the means  $\pm$  SD for four animals in each group and the *p*-values for Mann-Whitney tests and one-way ANOVA test indicate the significance of differences between groups (\**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001).



**Figure 3: Effect of chlordecone on CCl<sub>4</sub>-induced liver fibrosis.** Photomicrographs of liver sections stained with Sirius red from mice treated with oil, chlordecone (5 mg/kg body weight), CCl<sub>4</sub> (0.1 g/kg body weight) or chlordecone+CCl<sub>4</sub> for 5, 7, 9 and 12 weeks (Scale bars = 500  $\mu$ m) (A). Sirius red staining for collagen was quantified with NIS element software and the percentage of the total liver area examined displaying fibrosis was calculated. The data shown are the means  $\pm$  SD for four animals in each group and *p*-values for Mann-Whitney tests, at the right margin of the graphs, indicate the significance of differences between groups (*\*P*<0.05).



**Figure 4: Imaging of liver fibrosis by second harmonic generation microscopy.** (A) SHG images illustrating collagen deposition in the livers of mice treated with CCl<sub>4</sub> only or chlordecone+CCl<sub>4</sub> for 12 weeks. Scale bars = 500  $\mu$ m. (B) The SHG signal was quantified with Image J software, as indicated in the methods section, and the percentage of the liver area displaying fibrosis was calculated for each mouse. The data shown are the means  $\pm$  SD for four animals per group and the *p*-values for Mann-Whitney tests indicate the significance of differences between groups (\**P*<0.05).



**Figure 5: Immunostaining of liver sections for  $\alpha$ -SMA.** (A) Immunofluorescence images showing  $\alpha$ -SMA staining in the livers of mice treated with oil, chlordecone, CCl<sub>4</sub> or chlordecone+CCl<sub>4</sub> for 5 and 12 weeks. Scale bars = 500  $\mu$ m. (B) Stained areas were quantified with Image J software and the data shown are means  $\pm$  SD for four animals in each group and the  $p$ -values for Mann-Whitney tests indicate the significance of differences between groups ( $*P<0.05$ ).

