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## **In vivo comparison of the proangiogenic properties of chlordecone and three of its dechlorinated derivatives formed by in situ chemical reduction**

Eid Alabed Alibrahim, Samuel Legeay, Pierre-André Billat, Emmanuelle Bichon, Ingrid Guiffard, Jean-Philippe Antignac, Pierre Legras, Jérôme Roux, Sébastien Bristeau, Nicolas Clere, et al.

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1 **IN VIVO COMPARISON OF THE PROANGIOGENIC PROPERTIES OF**  
2 **CHLORDECONE AND THREE OF ITS DECHLORINATED**  
3 **DERIVATIVES FORMED BY IN SITU CHEMICAL REDUCTION**

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5 Philippe Antignac<sup>2</sup>, Pierre Legras<sup>3</sup>, Jérôme Roux<sup>3</sup>, Sébastien Bristeau<sup>4,5</sup>, Nicolas Clere<sup>1</sup>, Sébastien Faure<sup>1</sup>,  
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7  
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9

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30 **KEYWORDS:** *In situ* chemical reduction, chlordecone, dechlorination, angiogenesis, prostate cancer

31

32

33 ABSTRACT

34 *In Situ* Chemical Reduction has been identified as a possible way for the remediation of soils  
35 contaminated by chlordecone (CLD). Evidences provided by the literature indicate an  
36 association between the development of prostate cancer and CLD exposure (Multigner et al.  
37 2010). In a previous study, we demonstrated that the two main CLD derivatives obtained by  
38 the reduction method: CLD-1Cl and CLD-3Cl have lower cytotoxicity and proangiogenic  
39 properties than CLD itself (Legeay et al. 2017). By contrast, nothing is known on the  
40 proangiogenic effect *in vivo* of these dechlorinated products. The aim of this study is to confirm  
41 the preliminary *in vitro* results on an *in vivo* model of human prostate cancer.

42 The proangiogenic effect of dechlorinated derivatives of chlordecone was evaluated on a  
43 murine model of human prostate tumor (PC-3) treated with CLD, CLD-1Cl, CLD-3Cl and  
44 CLD-4Cl at two exposure levels: 33 µg/kg and 1.7 µg/kg respectively reflecting acute and  
45 chronic toxic exposure in human (Adir et al. 1978); (Cohn et al. 1978).

46 The results of the serum concentration measurements shown that, for the same ingested dose,  
47 these three metabolites were significantly less present in the serum than CLD, meaning that  
48 dechlorination of CLD lead to molecules that are biologically faster absorbed or metabolized  
49 or both.

50 Evolution of prostate tumors growth was lower in the groups treated by the three metabolites  
51 compared to the one treated by CLD. The vascularization measured on the tumor sections was  
52 inversely proportional to the rate of dechlorination, the treatment with CLD-4Cl showing no  
53 difference with control animals treated with the vehicle oil used for all substances tested.

54 We can therefore conclude that pro-angiogenic effect of the CLD is significantly decreased  
55 following the ISCR-resulting dechlorination, and that the carcinogenic effect is reduced.  
56 Further investigations are now needed to elucidate the molecular mechanisms by which  
57 dechlorination of CLD reduces its carcinogenic and proangiogenic effects in prostate tumor.

58

59 **INTRODUCTION**

60 Chlordecone (CLD, a.k.a. Kepone, decachlorooctahydro-1,3,4-metheno-2H-  
61 cyclobuta[c,d]pentalen-2-one, CAS No: 143-50-0) is a chlorinated hydrocarbon insecticide,  
62 now classified as a persistent organic pollutant (POP). CLD has been intensively used in banana  
63 plantations of the French West Indies, as well as, to a lesser extent, in other countries (POPRC  
64 2007; Le Déault and Procaccia 2009; Joly 2010; Fintz 2010). This pesticide undergoes no  
65 significant biotic or abiotic degradation in the environment (Cabidoche et al. 2009), leading to  
66 chronic food contamination and accumulation in liver, muscles, and fat of animals (Clostre et  
67 al. 2014; Létondor et al. 2015; Lastel et al. 2016). These effects explain its high toxicity that  
68 lead to its global prohibition (Cannon et al. 1978; Landrigan et al. 1980). CLD pollution is  
69 bound to last for centuries if nothing is done to lower soil contamination (Cabidoche et al.  
70 2009). Such persistence in the environment is a major health problem. Indeed, several studies  
71 have previously reported that chronic exposure to CLD leads to hepatotoxicity and increases  
72 the risk of cancer in rats (Reuber 1979; Mehendale 1981; Guzelian 1982; Dalu and Mehendale  
73 1996; Murali et al. 2004). In Human, it significantly increases the risk of prostate and liver  
74 cancers, and pregnancy complications (Reuber 1979; Sirica et al. 1989; Faroon et al. 1995;  
75 Multigner et al. 2010; Dallaire et al. 2012; Kadhel et al. 2014; Multigner et al. 2016).  
76 Furthermore, previous study has reported that CLD is able to enhance *in vitro* angiogenesis  
77 through the activation of estrogen receptors, thus favoring both tumor growth and development  
78 (Clere et al. 2012).

79 *In situ* chemical reduction (ISCR) may be a solution for the remediation of soils contaminated  
80 by CLD (Clostre et al. 2010). ISCR (United States Patent 2000) is based on treatment cycles of  
81 alternated strongly reducing conditions with oxidizing conditions and has already been proven  
82 effective in remediation of soils contaminated by pesticides (Phillips et al. 2004, 2005, 2006;  
83 Kim et al. 2010). Among all the metabolites, only the major transformation products, *i.e.* a  
84 mono dechlorinated CLD: chlordecone - 1Cl (CLD-1Cl) and chlordecone - 3Cl (CLD-3Cl)  
85 were commercially available and tested. The CLD-1Cl and CLD-3Cl (the data available do not  
86 enable the position of dechlorination to be more precisely defined (Belghit et al. 2015))  
87 represents 50 % and 13% respectively of the cumulated relative peak areas of the eleven  
88 transformation products identified after the 27-day ISCR treatment of an alluvial soil  
89 concerning the hypothetic malignant effects of these metabolites (Mouvet et al.). A third

90 derivative even more dechlorinated, quadri-hydro CLD (CLD-4Cl) is also observed, with a  
91 much lower relative area than CLD-3Cl.

92 Recently, we have demonstrated from *in vitro* data, that CLD-1Cl and CLD-3Cl have lower  
93 proangiogenic properties compared to CLD (Legeay et al. 2017). While the *in vivo* effects of  
94 CLD-1Cl and CLD-3Cl and their properties on tumor growth remain unknown, the aims of this  
95 study were to evaluate the influence of CLD and its metabolites in the control of  
96 neovascularization in a mice model of prostate cancer.

97

98

## 99 **MATERIALS AND METHODS**

### 100 *Reagents and chemicals*

101 CLD, purity 99.5%, was purchased from Dr. Ehrenstorfer (reference n12291). CLD-1Cl, CLD-  
102 3Cl and CLD-4Cl formed by ISCR were synthesized by Alpha-Chimica (Alpha-Chimica,  
103 Châtenay-Malabry, FRANCE) with a purity of 97.2%, 97.4% and 95.8%, respectively. GC-  
104 MS/MS shows the impurities to be other CLD dechlorinated derivatives. PC-3 cells (CRL-  
105 1435) were obtained from ATCC (Manassas, VA). RPMI 1640 medium, F-12 nutrient mix,  
106 penicillin-streptomycin, fetal bovine serum (FBS), were purchased from Invitrogen (Carlsbad,  
107 CA). Anti-CD31 antibody was purchased from (BD Biosciences). Biotinylated second antibody  
108 IgG was purchased from (Vector BA-9401). ECM gel<sup>®</sup> was purchased from (Sigma-Aldrich).  
109 CYQUANT<sup>®</sup> direct cell proliferation kit was purchased from (Invitrogen<sup>®</sup>).

110

### 111 *Cell culture*

112 Human umbilical venous endothelial cells (HUVEC) obtained from male newborns were  
113 purchased from Lonza (CC2519) and grown in plastic flasks in EBM-2 medium (Lonza,  
114 CC3156), containing 1% streptomycin/penicillin (Lonza, DE17-602E) and 10% of heat-  
115 inactivated fetal bovine serum (FBS, Gibco, 10270-106). HUVEC were used between the  
116 second and fourth passage.

117 Prostate cancer cell line PC-3 were purchased from ATCC (CRL-1435) and grown in plastic  
118 flasks in complete RPMI 1640 medium (ThermoFisher, 11875-085), containing 2 mM of L-  
119 glutamine, 20 mM of HEPES, 1% streptomycin/penicillin (Lonza, DE17-602E) and 10% of  
120 heat-inactivated fetal bovine serum (FBS, Gibco, 10270-106). PC-3 were used between the first  
121 and fifth passage.

122

### 123 *Chlordecone and metabolite dose calculations for in vivo experiments*

124 Important points were considered when calculating the drug concentrations/doses for *in vivo*  
125 studies: i) dose calculations for oral administration of CLD and its metabolites *in vivo*, as  
126 compared with intravenous administration in mice, and ii) comparison of exposure to the  
127 molecules in mice serum with that observed in humans serum.

128 i) As bioavailability is about 90% in the rat (Egle et al. 1978), and the most common way of  
129 intoxication is the ingestion, the oral administration route has been chosen to administrate the  
130 molecules.

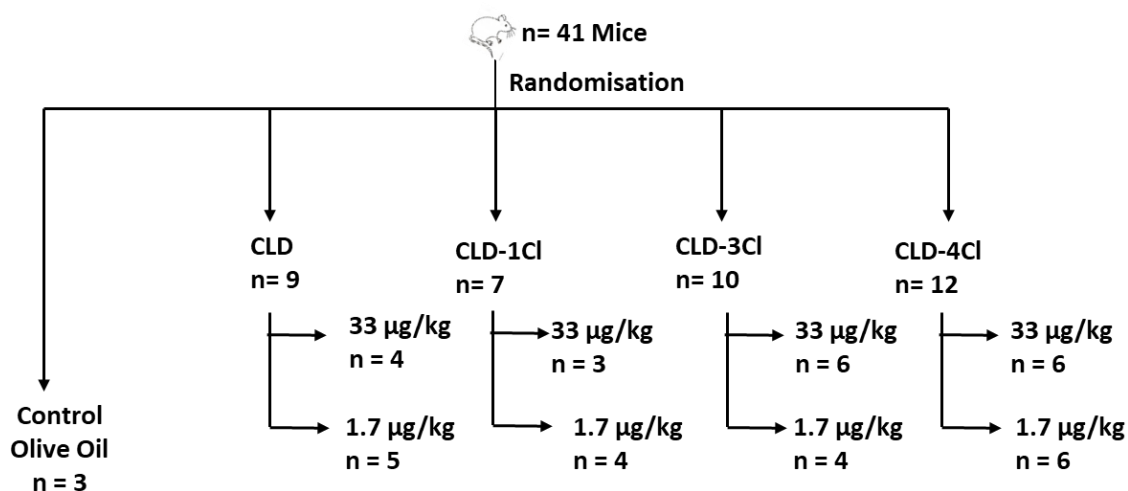
131 ii) Two targeted serum concentrations have been selected: 500 µg/L as the reflect of high  
 132 exposure in humans (Adir et al. 1978), and 25 µg/L as the exposure of industrial workers (Cohn  
 133 et al. 1978). As mice weights  $29.87 \pm 0.39$  g and have about 2 mL of blood, doses of 33 µg/kg  
 134 and 1.7 µg/kg have been chosen.

135 *Xenograft model*

136 PC-3 cell suspension ( $1.5 \cdot 10^6$  cells in 150 µL PBS w/o FBS) was xenografted subcutaneously  
 137 into the right flank of 5-weeks-old male *nude* mice (Envigo, Venray, N.L.). All animal  
 138 procedures listed in this article were performed as per the protocol approved by the local ethic  
 139 committees (Angers, France; protocol APAFIS #5872) and the number of animals was limited  
 140 regarding animal ethics.

141 The 41 mice were then divided randomly into nine groups. Eight groups were exposed to CLD  
 142 or its derivatives respectively at 33 µg/kg and at 1.7 µg/kg of body weight by daily gavage in  
 143 200 µL alimentary virgin olive oil for 8 weeks. The control group received daily gavage with  
 144 200 µL alimentary virgin olive oil for 8 weeks. The experimental plan followed is described in  
 145 figure 1.

146



147

148 **Figure 1: Distribution of animals according to the conditions of exposure to different**  
 149 **compounds.**

150

151 Tumor volume has been measured each 2 days according to the formula:

152 Tumor volume =  $[1/2(\text{length} \times \text{width}^2)]$  (Euhus et al. 1986).

153 Mice were sacrificed after 8 weeks and tumors were measured, dissected, weighed, and snap  
 154 frozen in Tissue-Tek® O.C.T. compound and stored at -80°C for further analysis.

155

156 *Serum concentrations measurement*

157 Whole blood was collected into a dry polypropylene microtube by direct intracardiac puncture  
158 under anesthesia 24 h after the last intake. Samples were left at room temperature for 30 min  
159 until blood clotting (Tuck et al. 2009). The clot was then removed by centrifugation at 1,000 g  
160 for 10 min at room temperature, and the supernatant has been stored at -80°C until analysis.

161 The molecules of interest: CLD, CLD-1Cl, CLD-3Cl, CLD-4Cl but also chlordecol (CLDOH)  
162 and 5b-hydro-chlordecone (5bCLD) were then measured in serum samples as previously  
163 described (Bichon et al. 2015). Briefly, internal standard (labeled CLD: 13C10-CLD at 10  
164 pg/μL) was added to 50 μL of mouse serum. Fat and proteins were then precipitated with 50μl  
165 formic acid by vortexing for 10 sec. Supernatants were loaded onto a C18 SPE cartridge,  
166 washed with hexane, eluted with acetonitrile and dried under a nitrogen stream. Dried extracts  
167 were reconstituted in 25 μL of a water/acetonitrile mix and injected into the LC-ESI-MS/MS  
168 instrument (Acquity-XevoTQS, Waters). Molecules were separated using an Accucor C30  
169 column (Thermo) with a water/acetonitrile gradient. Targeted compounds were analyzed in  
170 SRM mode, except for chlordecol in SIM mode. The calibration range was from 0.5 to 50 pg/μL  
171 of serum, and the limits of detection and quantification (LOQ) were from 0.02 to 3.83 pg/μL  
172 depending on the compound. Average recoveries into serum were between 63 to 91%.

173

174 *Immunochemistry and vessel area quantification*

175 Tumor sections (7μm) were fixed with paraformaldehyde 10 min at 4°C, then, blocked with  
176 4% bovine serum albumin for 1 h at room temperature and incubated at 4°C with the primary  
177 anti-CD31 antibody (10 μg/mL, Purified Rat Anti-Mouse CD31, BD Biosciences) at 1:500  
178 dilution. Then, tissues were incubated with a biotinylated second antibody IgG (Biotinylated  
179 Goat Anti-Rat IgG Antibody, mouse adsorbed, Vector BA-9401) at 1:200 dilution for 60min at  
180 room temperature and counterstained with DAPI (4',6-diamidino-2-phenylindole) at 1:10,000  
181 dilution for 3 min.

182 Vessels were quantified in the most representative areas, as identified by scanning tumor  
183 sections at low power (x40) with a confocal microscope. The total areas occupied by tumor  
184 vessels identified by CD31 staining in 3 spots per tumor, were calculated with ImageJ software.

185

186 *In vitro capillary network formation on ECM gel®*



187 HUVECs were seeded with a density of  $10^4$  cells per well previously covered with ECM gel<sup>®</sup>  
188 (Sigma-Aldrich). Briefly, 10  $\mu$ L of ECM<sup>®</sup> was added into a fifteen-well plate Ibidi<sup>®</sup> and allowed  
189 to solidify for 45 min at 37°C. Cells were then incubated with medium containing 10% FBS in  
190 presence of CLD or CLD-4Cl at concentrations  $5 \cdot 10^{-8}$  M,  $10^{-9}$  M or  $10^{-11}$  M and allowed to  
191 adhere for 24 h. Tube formation was examined by phase-contrast microscopy (MOTIC AE21)  
192 after 24 h and average length of capillaries was quantified using ImageJ software for evaluation  
193 of angiogenesis.

194

### 195 *Proliferation test on tumor cells PC-3*

196 The effect of CLD, CLD-1Cl, CLD-3 and CLD-4Cl on the proliferation of human prostate cells  
197 PC-3 (ATCC<sup>®</sup>, CRL-1435) was analyzed using CYQUANT<sup>®</sup> assay kit (Invitrogen<sup>®</sup>).  $5 \cdot 10^3$   
198 cells were seeded into a 96-well plate for 24 h and then were treated with  $10^{-6}$ M and  $5 \cdot 10^{-8}$ M  
199 CLD, CLD-1Cl, CLD-3 and CLD-4Cl at concentrations (these reflect the target serum  
200 concentrations at 33 $\mu$ g/kg and 1.7 $\mu$ g/kg respectively) for 24h. 100 $\mu$ L of detection reagent  
201 mixture was then added (Cyquant<sup>®</sup> direct nucleic acid stain and Cyquant<sup>®</sup> direct background  
202 suppressor 1) to 100 $\mu$ L of culture medium per well and the cells were incubated at 37°C for 60  
203 min. Fluorescence levels were read by a fluorescent microplate reader (Clariostar) with a filter  
204 at 508 nm for excitation and 527 nm for emission.

205

### 206 *Statistical analysis*

207 The evolution of tumor size, different concentrations in the serum and area occupied by the  
208 neo-vessels were compared between the different groups with ANOVA t-test.  $p < 0.05$  was  
209 considered statistically significant.

210

211 **RESULTS**

212 1. Evolution of tumor growth under chlordecone or its dechlorinated derivatives exposure  
213 in mice.

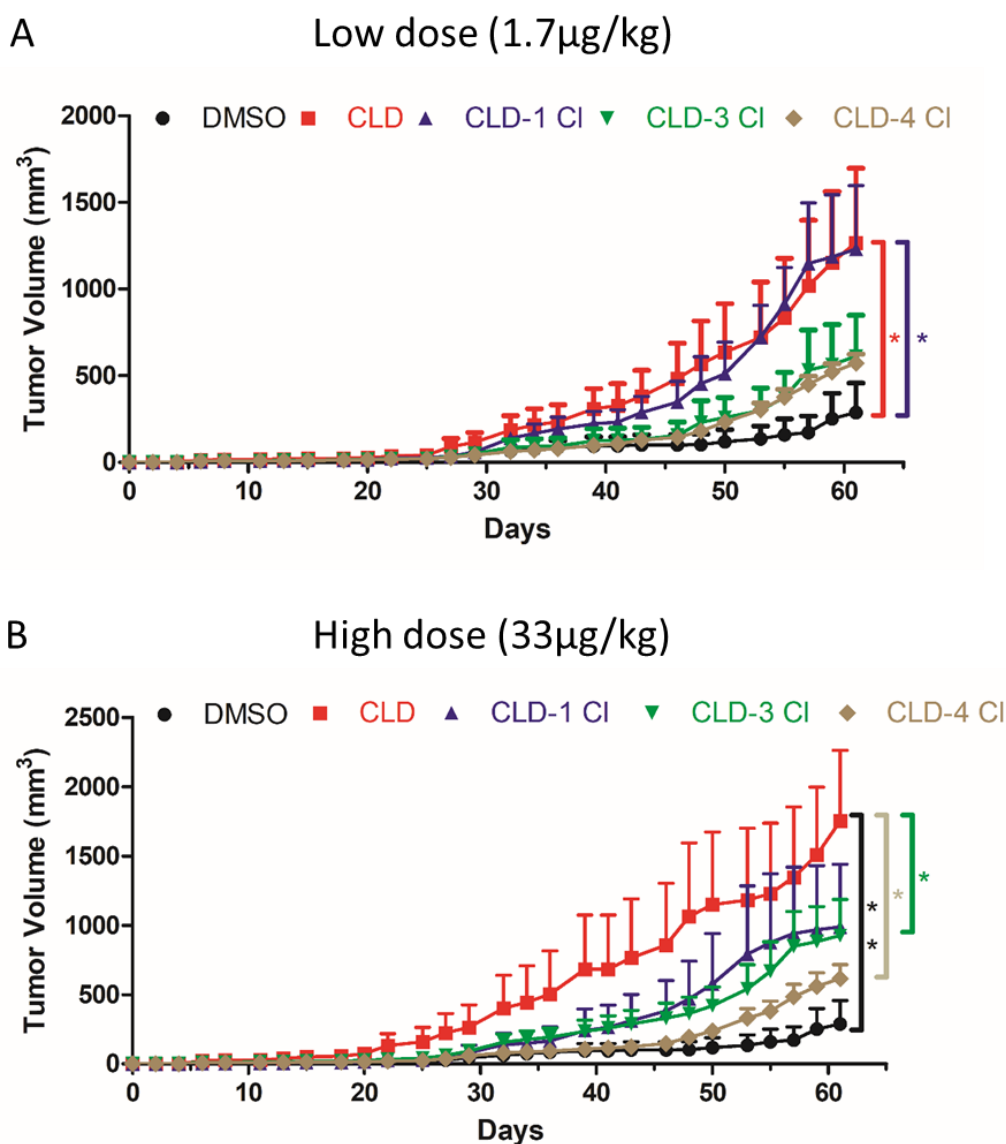
214 The objective of this analysis is to compare the evolution of tumor growth in mice treated with  
215 CLD or its derivatives.

216 In low dose groups (1.7 $\mu$ g/kg), a significant increase of tumor volume was found in mice treated  
217 with CLD and CLD-1Cl compared with controls (p=0.028 and p=0.035 respectively) (**Fig 2A**).

218 No significant difference of tumor growth was observed in mice treated with CLD-3Cl and  
219 CLD-4Cl compared to control mice.

220 In high dose groups (33  $\mu$ g/kg), a significant increase of tumor growth has been observed in  
221 mice treated with CLD in comparison with control animals (p=0.01). No significant difference  
222 was observed between mice treated with CLD and mice treated with CLD-1Cl while tumor  
223 growth was significantly lower for mice treated with CLD-3Cl and CLD-4Cl compared to mice  
224 treated with CLD (p=0.035 and p=0.025 respectively) (**Fig 2B**). In addition, no significant  
225 difference was observed between CLD-3Cl-treated mice and CLD-4Cl-treated mice compared  
226 to control mice.

227



228

229 **Figure 2: Daily treatments for 8 weeks at low (1.7 μg/kg) and high (33 μg/kg) dose with**  
 230 **CLD but not with CLD-3Cl and CLD-4Cl increase tumor growth formed by PC-3 cells**  
 231 **in mice compared to control mice. \*p<0.05; \*\*p<0.01. n=3-12.**

232

233 2. Serum dosage of chlordecone and its dechlorinated derivatives

234 After the low dose exposure of CLD (1.7 μg/kg), the mean serum concentration reached 26.3  
 235 μg/L and the mean serum concentration after high dose exposure of CLD (33 μg/kg) reached  
 236 192.8 μg/L. However, the same dose of dechlorinated CLD derivatives lead to very lower serum  
 237 concentrations with the following order: CLD > CLD-1Cl > CLD-3Cl > CLD-4Cl and values  
 238 ranging from 67.1±21.2 μg/L to under the limit of detection respectively for 33 μg/kg of CLD-  
 239 1Cl and 1.7 μg/kg of CLD-4Cl.

240 These serum dosages also reveal that for a same dose, chronicle ingestion of all dechlorinated  
 241 derivatives leads to much less serum concentrations compared to CLD following the order of  
 242 dechlorination: CLD > CLD-1Cl > CLD-3Cl > CLD-4Cl.

243 It is interesting to note that the treatment with a low dose of CLD-4Cl leads to a serum  
 244 concentration lower than the limit of detection. **In addition, the presence of impurities like CLD,**  
 245 **CLD-1Cl and CLD-3Cl have also been found but at concentrations 5 to 175 times lower than**  
 246 **the treatment in serum of mice (data not shown).**

247

248 **Table 1: Concentrations of CLD and its derivatives measured in mice serum samples.**

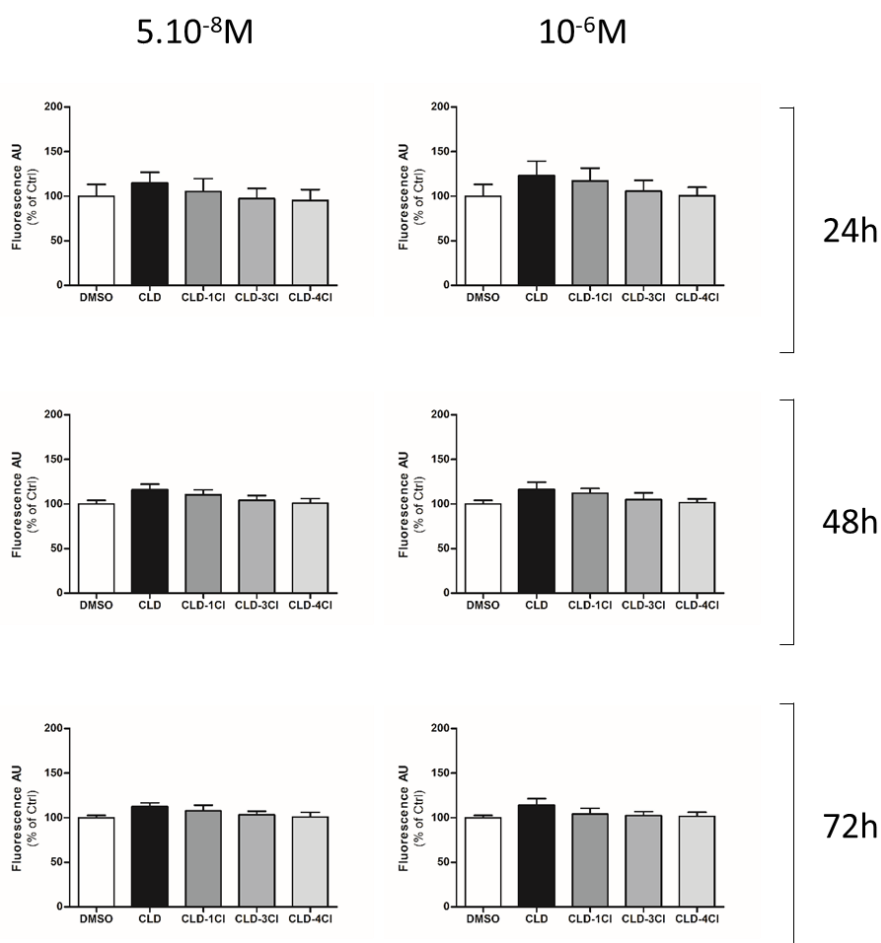
	Concentration, mean $\pm$ SD ( $\mu\text{g/L}$ )			
	CLD	CLD-1Cl	CLD-3Cl	CLD-4Cl
CLD 1.7 $\mu\text{g/kg}$	26.3 $\pm$ 12.7			
CLD 33 $\mu\text{g/kg}$	192.8 $\pm$ 73.6			
CLD-1Cl 1.7 $\mu\text{g/kg}$		11.9 $\pm$ 3.2		
CLD-1Cl 33 $\mu\text{g/kg}$		67.1 $\pm$ 21.2		
CLD-3Cl 1.7 $\mu\text{g/kg}$			4.0 $\pm$ 3.5	
CLD-3Cl 33 $\mu\text{g/kg}$			6.1 $\pm$ 4.3	
CLD-4Cl 1.7 $\mu\text{g/kg}$				< LOQ
CLD-4Cl 33 $\mu\text{g/kg}$				2.2 $\pm$ 0.9

249

250 3. Effect of chlordecone and its dechlorinated derivatives on the proliferation of PC-3 tumor  
 251 cells

252 No difference was found in PC-3 cell proliferation whatever the concentration and the type of  
 253 dechlorinated CLD derivative (**Fig 3**).

254

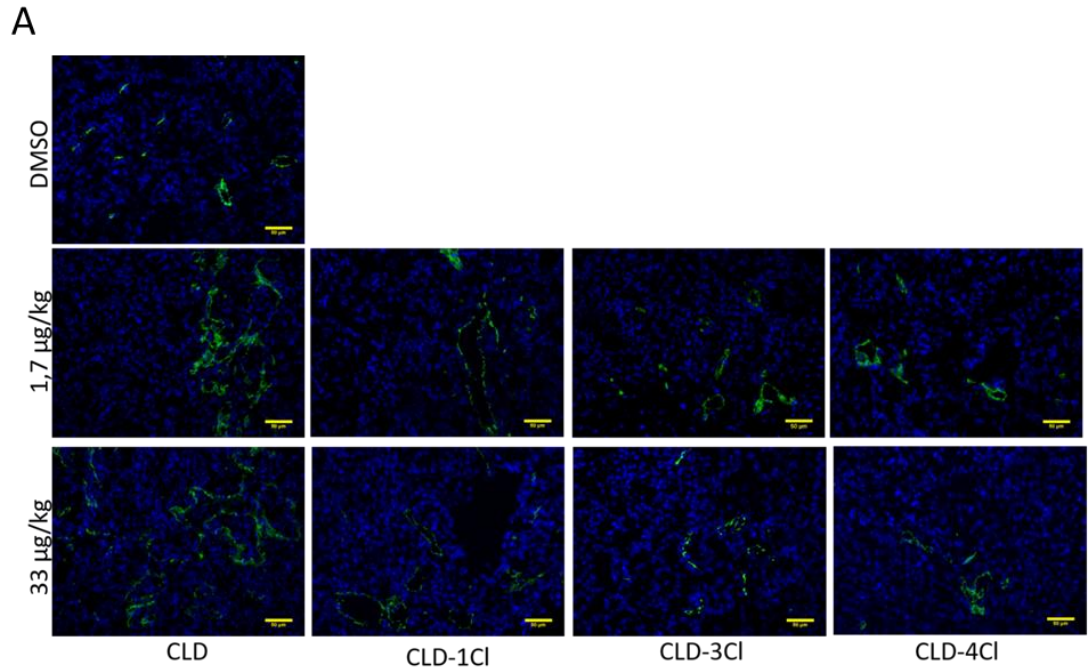


255

256 **Figure 3: Chlordecone or its derivatives even after 72h of exposure do not increase the**  
 257 **proliferation of PC-3 tumor cells. n=6.**

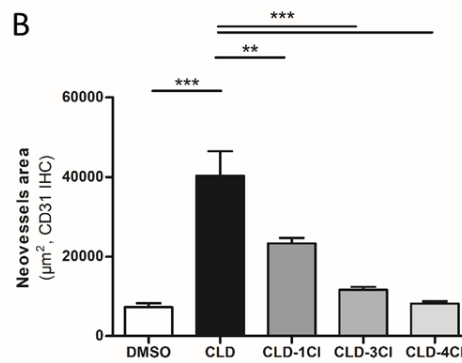
258

259 4. Effect of chlordecone and its dechlorinated derivatives on *in vivo* neovascularization  
 260 Treatments of mice with each dose of CLD significantly increased capillary area in tumor  
 261 compared to control condition (p=0.0007 for 1.7 μg/kg and p=0.0009 for 33 μg/kg) (**Fig 4**).  
 262 Furthermore, capillary areas were significantly reduced in tumors from mice treated with the  
 263 dechlorinated CLD derivatives (CLD vs CLD-1Cl, p=0.01 and p=0.0031; CLD vs CLD-3Cl,  
 264 p=0.0003 and p=0.0005; CLD vs CLD-4Cl, p=0.0007 and p=0.0009 respectively for the low  
 265 and the high dose) (Fig 3).

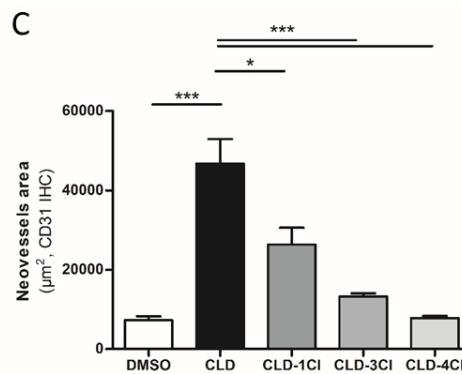


266

Low dose 1.7µg/kg



High dose 33µg/kg



267

268

269

**Figure 4: Dechlorination of CLD decreases the tumor neovascularization. A) Immunolabelling of tumor sections. In blue the labeling of cell nucleus with DAPI and in**

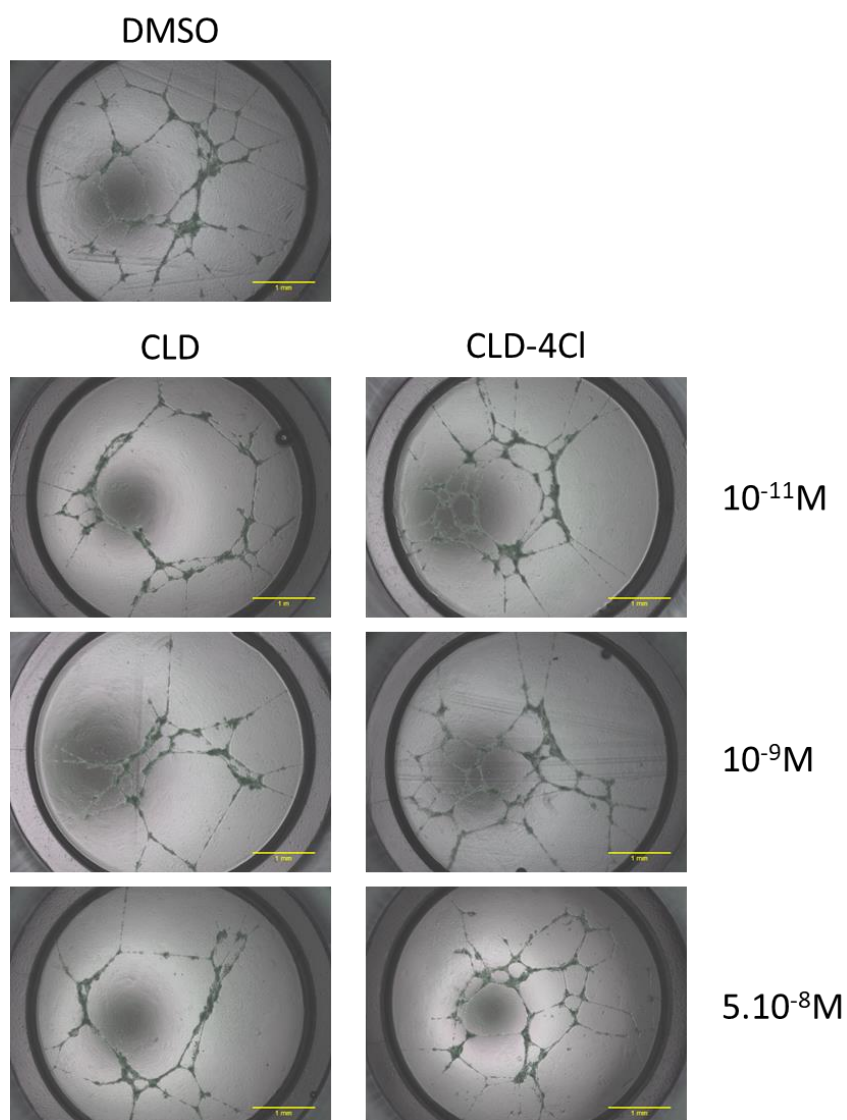
270 **green the labeling the endothelial marker CD31. (Scale 50  $\mu$ m). B and C) Quantification**  
271 **of the neovascularization. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. n=6**

272

273 5. Effect of chlordecone and its dechlorinated derivatives on *in vitro* angiogenesis

274 *In vitro* assays performed in a previous study suggested that CLD-1Cl and CLD-3Cl have lower  
275 proangiogenic properties than the CLD parent molecule on human primary endothelial cells  
276 (Legeay et al. 2017). Because the present study has an additional derivative, CLD-4Cl,  
277 compared to the above-mentioned work, the opportunity has been taken to complete *in vitro*  
278 data by treating HUVECs in the presence or absence of CLD or CLD-4Cl at the same three  
279 concentrations  $5 \cdot 10^{-8}$  M (concentration that has been found in plasma of patients exposed),  $10^{-11}$   
280 M (concentration that has been found in drinking water) and  $10^{-9}$  M (intermediate  
281 concentration) for 24h. It has been found that CLD-4Cl does not significantly increase capillary  
282 formation *versus* control cells for the three concentrations tested ( $5 \cdot 10^{-8}$  M,  $p = 0.064$ ;  $10^{-9}$  M,  
283  $p = 0.063$ ;  $10^{-11}$  M,  $p = 0.1$ ). Additionally, it has been observed that CLD-4Cl possesses a  
284 significantly lower proangiogenic effect than CLD ( $5 \cdot 10^{-8}$  M,  $p = 0.004$ ;  $10^{-9}$  M,  $p = 0.003$ ;  $10^{-11}$   
285 M,  $p = 0.009$ ) (**Fig 5 and Table 2**).

286



287

288 **Figure 5: CLD but not CLD-4Cl significantly increases capillary formation of HUVECs**  
 289 **in culture on ECMgel® after 24h of treatment. Scale=1mm.**

290

291

292 **Table 2: Quantification of the capillary area formed by HUVECs in percentage of**  
 293 **control**

	CLD	CLD-4Cl
$10^{-11}$ M	$196.00 \pm 20.85^a$	$123.00 \pm 10.39^{**}$
$10^{-9}$ M	$196.00 \pm 10.53^a$	$122.17 \pm 7.53^{**}$
$5.10^{-8}$ M	$198.67 \pm 13.77^a$	$122.83 \pm 8.08^{**}$

294 <sup>a</sup>p<0.001 vs ctrl



Author version

295 \*\*p<0.01 vs CLD

296 n=6

297

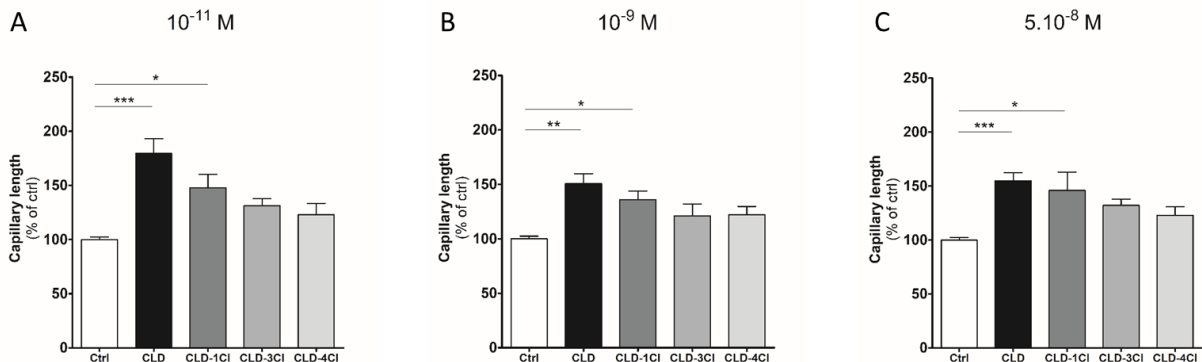
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299

300 **DISCUSSION**

301 In order to confirm our previous *in vitro* data (Legeay et al. 2017), this study aimed to evaluate  
 302 the proangiogenic properties of dechlorinated derivatives of CLD compared to CLD in a mouse  
 303 model of tumor-xenograft and *in vitro*. In line with the literature, CLD potentiates prostatic  
 304 tumor growth *in vivo* (Multigner et al. 2010) and this effect was associated with an increased  
 305 neovascularization (Clere et al. 2012; Legeay et al. 2017; Alabed Alibrahim et al. 2018),  
 306 confirming the proangiogenic effects of CLD. Prostate cancer is one of the most common  
 307 cancer diagnosed in men and higher incidence rates have been observed in regions highly  
 308 exposed to CLD like Caribbean (Center et al. 2012). A causal relationship between CLD  
 309 exposure and prostate cancer has been well described (Multigner et al. 2010; Nedellec et al.  
 310 2016a, b; Deloumeaux et al. 2017) and any possible way of lowering permanently the CLD soil  
 311 concentrations and the exposure levels of Caribbean inhabitants remains of major interest.  
 312 Decontamination of soils by ISCR leads to dechlorinated CLD derivatives which have been  
 313 previously identified with less or absence of proangiogenic effects (Legeay et al. 2017). From  
 314 *in vitro* and *in vivo* models, the present study confirms that the proangiogenic properties of CLD  
 315 derivatives decrease according to the level of dechlorination (Fig 6).

316



317

318 Figure 6: The levels of dechlorination leads to less or absence of pro-angiogenic effects  
 319 compared to CLD. Combined data from the present study and Legeay S. *et al* (2017). \*p<0.05;  
 320 \*\*p<0.01; \*\*\*p>0.001. n=6.

321

322 These results have been associated with both the absence of PC-3 cell proliferation and a  
 323 decreased tumor vascularization in mice treated with CLD-3Cl and CLD-4Cl compared to  
 324 CLD. These effects were also associated with a decreased capillary formation by human  
 325 primary endothelial cells.

326 The present study has been designed to obtain an *in vivo* murine model reflecting individual  
 327 prostate cancer patients. Thus, mice bearing PC-3 cells have been daily treated by gavage for 8

328 weeks with two doses: 1.7  $\mu\text{g}/\text{kg}$  and 33  $\mu\text{g}/\text{kg}$  in order to reach 25  $\mu\text{g}/\text{L}$  ( $5.10^{-8}$  M), the serum  
329 concentration in industrial workers, and 500  $\mu\text{g}/\text{L}$  ( $10^{-6}$  M), the serum concentration after high  
330 human exposure respectively described with CLD (Adir et al. 1978; Cohn et al. 1978). The  
331 serum concentrations of CLD measured after 8 weeks was  $26.7\pm 12.7$   $\mu\text{g}/\text{L}$  which is very close  
332 to the targeted serum concentration and confirms our extrapolations for the pharmacokinetics  
333 of CLD in mice. However, for the highest dose 33  $\mu\text{g}/\text{kg}$ , the serum concentration of CLD was  
334  $192.8\pm 7.6$   $\mu\text{g}/\text{L}$  while the targeted serum concentration was 500  $\mu\text{g}/\text{L}$ . This decrease of the  
335 observed serum concentration would be partly explained by a possible bioaccumulation in  
336 organs such as liver, fat or kidney, leading to a lower CLD concentration in the blood  
337 compartment as previously described after oral exposure to CLD (Cohn et al. 1978; Rochelle  
338 and Curtis 1994; Faroon et al. 1995). However, the same dose of dechlorinated CLD derivatives  
339 leads to very lower serum concentrations with the following order: CLD > CLD-1Cl > CLD-  
340 3Cl > CLD-4Cl and values ranging from  $67.1\pm 21.2$   $\mu\text{g}/\text{L}$  to under the limit of detection  
341 respectively for 33  $\mu\text{g}/\text{kg}$  of CLD-1Cl and 1.7  $\mu\text{g}/\text{kg}$  of CLD-4Cl. These data suggest a decrease  
342 of the oral bioavailability or an increase of the metabolism or both of the CLD derivatives  
343 compared to CLD. This hypothesis should be verified by deeper pharmacokinetic studies. The  
344 presence of CLD and some of its derivatives at very low concentration in serum of treated mice  
345 might be due to the presence of impurities in the original product and/or be explained by cross-  
346 contaminations. However, because their concentrations were 5 to 175 times lower, they can be  
347 considered as negligible.

348 According to the two targeted serum concentrations 25  $\mu\text{g}/\text{L}$  ( $5.10^{-8}$  M) and 500  $\mu\text{g}/\text{L}$  ( $10^{-6}$  M),  
349 PC-3 cell proliferation has been evaluated after exposure to CLD and its derivatives with these  
350 both concentrations for 24h to 72h. In addition, the evaluation of the angiogenic process has  
351 been performed in human primary endothelial cell after exposure to CLD or its derivatives for  
352 24h at concentrations from  $10^{-11}$  M to  $5.10^{-8}$  M, in order to reflect both the lowest serum  
353 concentration described in exposed human and the CLD concentration found in drinking water  
354 (Adir et al. 1978; Landrigan et al. 1980; Taylor 1982; Badach et al. 2000) according to our  
355 previous study (Legeay et al. 2017). Altogether, these *in vitro* data confirm that even at very  
356 low concentration, CLD and CLD-1Cl but not CLD-3Cl nether CLD-4Cl have proangiogenic  
357 effects while no proliferation of PC-3 cell are observed, certifying that the *in vivo* tumor growth  
358 after chronicle exposure is at least due to a stimulation of angiogenesis process.

359 It is interesting to note that the lower proangiogenic and tumorigenic properties of dechlorinated  
360 derivatives compared to CLD are correlated with the level of chlorination. From *in vivo* data,  
361 CLD effectively exhibits the most potentiation of tumor growth while CLD-3Cl and CLD-4Cl

362 have limited effect presenting no significant differences compared to control mice. This  
363 decrease of toxicity is also observed with the neovascularization immune staining of tumor  
364 sections and *in vitro* with de capillary length formed by primary endothelial cells. These *in vitro*  
365 data confirm that the decreased of the proangiogenic effect following the dechlorination of the  
366 CLD derivatives is well due to the dechlorination itself and not to a decrease of bioavailability  
367 or an increased metabolism suggested *in vivo*.

368 While no study has evaluated the impact of structural modifications of toxic molecules on the  
369 regulation of angiogenesis, a decrease of toxicity such as cell proliferation, cellular functions  
370 and mammalian cells alterations and death have been previously observed with polychlorinated  
371 biphenyls (Ganey and Boyd 2005; Beyer and Biziuk 2009; Gui et al. 2013) and CLD (Dolfing  
372 et al. 2012; Benoit et al. 2017). Interestingly, these cellular damages were correlated with the  
373 level of reductive dechlorination, suggesting that more the compound is dechlorinated and less  
374 its toxicity is. In endothelial cells, a reduced inflammation through VCAM, COX-2 and NF- $\kappa$ B  
375 expression and a reduced oxidative stress have been observed with biphenyl, the final  
376 dechlorination product of PCB77 (Venkatachalam et al. 2008; Eske et al. 2014). In these  
377 studies, it was clearly demonstrated that the cytotoxicity was decreased relatively to the degree  
378 of dechlorination. Furthermore, the dechlorination of highly chlorinated compounds was  
379 suggested to be beneficial for protecting vessels against vascular diseases (Venkatachalam et  
380 al. 2008; Eske et al. 2014).

381 A lot of research has been focused on process inducing reductive dechlorination of  
382 polychlorinated pollutants in order to increase their biodegradation and consequently to  
383 decrease their environmental toxicity. Bioremediation using microorganisms, plants or  
384 enzymes, has been wildly employed to detoxify the soil from chlorinated contaminants (Wiegel  
385 and Wu 2000; Borja et al. 2005; Jesus et al. 2016). However, these process did not last more  
386 than 6 months for CLD (Woignier et al. 2012, 2013). ISCR has already demonstrated its  
387 efficiency for the remediation of polychlorinated compounds as hexachlorocyclohexane (HCH)  
388 (Phillips et al. 2004, 2005, 2006; Kim et al. 2010) and was suggested as a future way to solve  
389 the soil pollution with CLD (Clostre et al. 2010).

390 Altogether, these data confirm the major interest of the dechlorination of polychlorinated  
391 compounds by ISCR for the limitation of CLD-induced endothelial dysfunction as angiogenesis  
392 and globally to prevent the CLD-induced human toxicity.

393

394 **CONCLUSION**

395 CLD dechlorinated derivatives CLD-3Cl and CLD-4Cl do not have pro-angiogenic effects *in*  
396 *vitro* and *in vivo* and do not significantly increase prostatic tumor growth in a mouse xenograft  
397 model. Consequently, the present study suggest, on one hand, the previous *in vitro* data  
398 concerning the prevention of the pro-angiogenesis effects by dechlorination of CLD (Legeay et  
399 al. 2017) and, on the other hand, that *ISCR* should be considered as promising method for the  
400 decontamination of soils polluted by CLD.

401

#### 402 **CONFLICTS OF INTEREST**

403 The authors declare no competing financial interests.

404

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