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Two dechlorinated chlordecone derivatives formed by in situ chemical reduction are devoid of genotoxicity and mutagenicity and have lower proangiogenic properties compared to the parent compound

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Abstract Chlordecone (CLD) is a chlorinated hydrocarbon insecticide, now classified as a persistent organic pollutant. Several studies have previously reported that chronic exposure to CLD leads to hepatotoxicity, neurotoxicity, raises early child development and pregnancy complications, and increases the risk of liver and prostate cancer. In situ chemical reduction (ISCR) has been identified as a possible way for the remediation of soils contaminated by CLD. In the present study, the objectives were (i) to evaluate the genotoxicity and the mutagenicity of two CLD metabolites formed by ISCR, CLD-5a-hydro, or CLD-5-hydro (5a- or 5- according to CAS nomenclature; CLD-1Cl) and tri-hydroCLD (CLD-3Cl), and (ii) to explore the angiogenic properties of these molecules. Mutagenicity and genotoxicity were investigated using the Ames's technique on *Salmonella typhimurium* and the in vitro micronucleus micromethod with TK6 human lymphoblastoid cells. The proangiogenic properties were evaluated on the in vitro capillary network formation of human primary endothelial cells. Like CLD, the dechlorinated derivatives of CLD studied were devoid of genotoxic and mutagenic activity. In the assay targeting angiogenic properties, significantly lower microvessel lengths formed by endothelial cells were observed for the CLD-3Cl-treated cells compared to the CLD-treated cells for two of the three tested concentrations. These results suggest that dechlorinated CLD derivatives are devoid of mutagenicity and genotoxicity and have lower proangiogenic properties than CLD.

Keywords In situ chemical reduction · Chlordecone · Dechlorination · Genotoxicity · Mutagenicity · Angiogenesis

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Introduction

Chlordecone (CLD, decachlorooctahydro-1,3,4-metheno-2H-cyclobuta[c,d]pentalen-2-one, CAS No: 143–50-0), also named Kepone, is an organochlorine insecticide that has been intensively used in banana plantations of the French West Indies, and to a lesser extent in Cameroon, Ivory Coast, Equator, Honduras, Nicaragua, and Panama (Fintz 2009; OPECST 2009; Joly 2010) and in the USA until 1976 as well as in Asia (UNEP/POPS/POPRC 2007). CLD is highly persistent in the environment and considered by the Stockholm Convention as a persistent organic pollutant (POP). From 1973 to 1993, approximately 300 t of CLD has been applied in the French West Indies (FWI) (PNAC 2008–2010). As a result, 20 to 30,000 ha have been contaminated, representing up to 25% of the total agricultural surface of the FWI (PNAC 2008–2010). This pollution is bound to last for centuries if nothing is done to lower soil contamination (Cabidoche et al. 2009). Indeed, despite its use being banned since 1993 in France, CLD is still present in soil, water, and consequently in food in FWI. As a consequence, inhabitants of FWI can be chronically exposed to CLD through local food and drinking

water. This is a major health issue because CLD is classified as possibly carcinogenic for human (group 2B) by the international agency for research on cancer (IARC). Moreover, it has been reported that exposure to CLD leads to hepatotoxicity and increases the risk of cancer in rats (Reuber 1979; Sirica et al. 1989; Faroon et al. 1995). In addition, long-term exposure to CLD has been reported to be associated with a neurotoxicity, a raise of early child development and pregnancy complications, and an increased risk of liver and prostate cancer in Human (Multigner et al. 2010; Dallaire et al. 2012; Kadhel et al. 2014; Multigner et al. 2016).

CLD is considered as an endocrine disruptor notably by interacting with the estrogen receptor α (ERα) (Hammond et al. 1979; Yang et al. 2016). Even if the targets of CLD are not fully identified, this mechanism could explain in part its carcinogenicity and its developmental toxicity (Multigner et al. 2010; Clere et al. 2012). Indeed, pharmacological blockade of ERα has been identified to prevent CLD-induced angiogenesis, an endothelial process involved in tumor growth (Clere et al. 2012).

In situ chemical reduction (ISCR) has been identified as a promising way for the remediation of soils contaminated by CLD (Clostre et al. 2010). ISCR (Bryant and Wilson 2003) is based on treatment cycles of alternated strongly reducing conditions with oxidizing conditions. The efficiency of ISCR has already been proven in remediation of soils contaminated by various pesticides (Phillips et al. 2004, 2005, 2006; Kim et al. 2010).

The potential of chemical reduction for remediation of CLD-contaminated soil has been investigated in laboratory pilot-scale 80 kg mesocosms for andosol, ferralsol, and nitisol from FWI banana plantations. After a 6-month treatment, 11 CLD-dechlorinated transformation products, from mono- to penta-dechlorinated, were identified in all three soil types. The CLD soil concentration was lowered by 74% in nitisol, 71% in ferralsol, and 22% in andosol (Mouvet et al. 2016a).

The efficiency of ISCR has been confirmed at field scale (Mouvet et al. 2016b) leading to perspectives for large-scale application of the process. The two main transformation products resulting from ISCR applied to CLD-contaminated soils have been made available as analytical standards by dedicated synthesis (Mouvet et al. 2016c). The major transformation product has been established to be a mono-dechlorinated CLD (CLD-1Cl), the CLD-5a-hydro or CLD-5-hydro (5a- or 5- according to CAS nomenclature, the data available do not enable the position of dechlorination to be more precisely defined) (Belghit et al. 2015). This single CLD-1Cl represents 50% of the cumulated relative peak areas (measured by GC/MS) of the 11 transformation products identified after the 27-day ISCR treatment of an alluvial soil (Mouvet et al. 2016b). The second most important degradation product is known to be a tri-dechlorinated CLD (CLD-3Cl, the position on the carbon skeleton of the 3 Cl replaced by H remains unknown Belghit et al. 2015) that represents 13% of the cumulated relative peak areas of all transformation products (Mouvet et al. 2016b). Studying two degradation products representing 63% of the cumulated relative peak areas of the 11 transformation products was considered appropriate to determine the efficiency (from a toxicological standpoint) of the ISCR relative to the initial CLD concentration.

Based on (i) the proven potential of ISCR for soil remediation, (ii) the availability of analytical standards of the two main CLD derivatives formed by ISCR, and (iii) the lack of data on the toxicity of these compounds, the aim of the present study was to compare the genotoxicity and the mutagenicity of CLD, CLD-1Cl, and CLD-3Cl, and to evaluate their proangiogenic properties on endothelial cells.

Material and methods

Chemicals and reagents

Chlordecone, purity 93.5%, was purchased from Dr. Ehrenstorfer (reference C11220000). CLD-5a-hydro or CLD-5-hydro (5a- or 5- according to CAS nomenclature; CLD-1Cl) and the main tri-hydroCLD (CLD-3Cl) formed by ISCR were synthesized by Alpha-Chimica (Alpha-Chimica, Châtenay-Malabry. FRANCE) with a purity of 92.6 and 88.6%, respectively. GC/MS/MS show the impurities to be other CLD dechlorinated derivatives. The purity of the tested compounds was taken into account for the preparation of each concentration investigated.

Penicillin, sodium pyruvate, streptomycin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromid (MTT), biotin, histidine, and NaCl were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), dimethylsulfoxide, ethanol, L-glutamine, KCl, and NaOH were purchased from Merck (Darmstadt, Germany), glucose-6-phosphate and NADP were purchased from Roche (Mannheim, Germany), and top agar from Oxoid (Basingstoke, UK).

Mitomycin C (CAS Registry Number: 50–07-7), benzo[a]pyrene (CAS Registry Number: 50–32-8), sodium azide (CAS Registry Number: 26,628–22-8), and 9-aminoacridine (CAS Registry Number: 90–45-9) were provided by Sigma-Aldrich (Sigma-Aldrich Chemical Co.; L'Isle d'Abeau Chesnes, France). 2-Anthramine (CAS Registry Number: 613–13-8) was supplied by Sigma-Aldrich GmbH (Steinheim, Germany) and 2-nitrofluorene (CAS Registry Number: 607–57-8) was provided by Merck (Darmstadt, Germany).

Cell culture

TK6 human lymphoblastoid cells obtained from ECACC (Porton Down, Salisbury, UK) were maintained in RPMI

1640 medium supplemented with 200 units/mL penicillin, 50 μg/mL streptomycin, 2.5 μg/mL amphotericin B, 200 μg/mL L-glutamine, 200 μg/mL sodium pyruvate, and 10% (*v*/*v*) heat-inactivated horse serum (RPMI 10 medium) at 37°C in humidified atmosphere containing 5% CO_2. They have a stable caryotype (47, XY, 13+) and have a 16–18-h doubling time. Each new batch of cells was tested to confirm the absence of mycoplasma contamination (Mycoalert mycoplasma detection kit, Cambrex Bio Science Rockland, Inc.).

Human umbilical venous endothelial cells (HUVEC) obtained from male newborns were purchased from Lonza (CC2517) and grown in plastic flasks in EBM-2 medium (Lonza, CC3162) containing 10% of heat-inactivated fetal bovine serum (FBS, Gibco, 10,270–106) at 37°C in humidified atmosphere containing 5% CO_2. HUVEC were used between the second and fourth passage.

Metabolic activation system used for genotoxicity

After enzymatic induction with Arochlor 1254, rat liver S9 was prepared according to Ames et al. (1975) and Maron and Ames (1983).

In the Ames test, S9-mix contained per milliliter: 0.1 mL of S9, 0.02 mL of $MgCl_2$ 0.4 M, 0.02 mL of KCl 1.65 M, 0.5 mL of phosphate buffer 0.2 M (pH 7.4), 0.04 mL of NADP 0.1 M, 0.005 mL of glucose-6-phosphate 1 M, and 0.315 mL of H_2O and was used at 50% during treatment, i.e., 5% of S9 in final concentration.

In the in vitro micronucleus assay, S9-mix contained per milliliter: 0.4 mL of S9, 0.2 mL of 150 mM KCl, 0.2 mL of 25 mg/mL NADP and 0.2 mL of 180 mg/mL glucose-6-phosphate and was used at 5%, giving 2% of S9 in final concentration.

Except S9, all cofactors were filtered through a sterilizing membrane before use.

Bacterial mutagenicity test on *Salmonella typhimurium* His- using Ames' technique

CLD, CLD-1Cl, and CLD-3Cl were tested in strains TA1537, TA98, TA100, and TA102 at the highest final dose of 200 μg/plate expressed as pure compound (using an initial concentrated solution in DMSO at 2 mg/mL), as well as 5 lower doses (from 100 to 1 μg/plate). The test was performed using the standard plate incorporation method (Ames et al. 1975) both with and without S9 by microsomal liver fractions. A triplicate test for each experimental point was made, and the plates were then kept at 37°C for 48 h, after which the number of revertant colonies was determined for each plate.

Appropriate positive reference controls were also performed (without S9: TA1537: 9-amino-acridine 50 μg/plate, TA98 2-nitro fluorene 2 μg/plate, TA100 sodium azide 1 μg/plate and TA102 mitomycin C 0.125 μg/plate; with liver S9: TA1537, TA98 and TA100 2-anthramine 2 μg/plate and TA102 benzo[a]pyrene 2 μg/plate).

A response is considered positive in the assay if a dose-response relationship is observed on three consecutive doses with, for the highest increase, an induction ratio greater than or equal to 3 (strain TA1537) or 2 (strains TA98, TA100, and TA102). In parallel, data were analyzed by means of Dunnett's method (Mahon et al. 1989) allowing the comparison of the mean value for each dose to the mean value for the corresponding solvent control.

In vitro micronucleus assay in micromethod with TK6 human lymphoblastoid cells

The procedure for this micromethod for in vitro micronucleus assay has been previously detailed (Nesslany et al. 2009). Main points are reminded below:

The assay was carried out with and without S9 microsomal liver. CLD, CLD-1Cl, and CLD-3Cl were dissolved in DMSO at the initial concentration of 100 mg/mL, giving the final concentration of 1000 μg/mL when added at 1% in culture medium.

Exponentially growing TK6 cells were treated either for 3 h (with or without S9-mix) followed by a 24-h recovery time before harvesting or during 27 h, corresponding to ca 1.5 cell cycles, immediately followed by the harvest (without S9-mix only). Each treatment was performed in duplicate and was coupled to a cytotoxicity assessment using a MTT (3-[4,5-dimethylthiazol-2-gl]-2,5 diphenyl tetrazolium bromid, Sigma) colorimetric method (Borenfreund et al. 1988).

At each step, microplates were centrifugated 6 min at 1000 rpm and the supernatant discarded by gentle pouring off.

The cells were first washed (0.2-mL culture medium RPMI +1% pluronic acid 10% solution) and gently resuspended before hypotonic treatment (4 min with 0.15 mL RPMI 0 diluted 1 vol/1 vol in distilled water +1% pluronic acid). Then, a volume of 40 μL ethanol/acetic acid (3 vol/1 vol) was added in each well for prefixation step. Afterward, the cells were fixed by addition of 0.15 mL ethanol/acetic acid (3 vol/1 vol) for at least 10 min. The cells were finally resuspended by drawing and expelling with a Pasteur pipette, dropped onto wet clean glass slides, and allowed to dry at room temperature. After at least 12 h, the air-dried slides were stained 10 min with 2% Giemsa water solution, rinsed, coded, and analyzed.

In the case of cytotoxicity, the highest concentration was fixed according to the reduction of the MTT incorporation ($\leq 55 \pm 5\%$). At least two lower concentrations were also retained for the genotoxic analysis. Micronuclei, identified according to criteria described by Fenech et al., were analyzed in at least 2000 mononucleated cells for the three retained concentrations. The statistical significance of differences between groups was determined using the CHI2 test (Fenech et al. 2003). Positive controls were included in each

corresponding treatment schedule: mitomycin C without S9-mix at 0.5 μg/mL (3-h treatment) or 0.2 μg/mL (27-h treatment without S9-mix), griseofulvin (aneugenic mode of action) at 5 μg/mL (27-h treatment without S9-mix), and cyclophosphamide at 5 μg/ml with liver S9-mix.

In vitro capillary network formation on ECM gel

HUVEC were seeded at a density of 10,000 cells per well. Each well was precoated with ECM gel (Sigma–Aldrich, E1270). Briefly, 10 μL of ECM gel was added into a 15-well angiogenesis plate (Ibidi®, Biovalley® 81,506) and allowed to solidify for approximately 45 min at 37°C. Then, cells were incubated with medium containing 10% of FBS and either in the absence or presence of CLD, CLD-1Cl, or CLD-3Cl used at the three relevant concentrations 10^{-11} M (a plasma concentration common in exposed humans) (Heath 1978; Taylor 1982) (3×10^{-8} M (a concentration found in drinking water) (Badach et al. 2000) and 10^{-9} M (intermediary concentration). Tube formation was examined by phase-contrast microscopy (MOTIC AE21) after 24 h of incubation, and the average length of capillaries was quantified using ImageJ software. Data are presented as mean ± standard error of the mean; *n* represents the number of independent experiments. Statistical analyses were performed by a Student's *t* test. $p < 0.05$ was considered to be statistically significant.

Results

Ames test on *Salmonella typhimurium* strains TA1537, TA98, TA100, and TA102

CLD and its dechlorinated derivatives, i.e., CLD-1Cl and CLD-3Cl, induced neither biologically nor statistically significant changes in the number of revertants in all four *Salmonella typhimurium* strains TA1537, TA98, TA100, and TA102 tested either in the absence or in the presence of the metabolic activation system. Thus, CLD mono- or tri-dechlorination does not modify the mutagenic potential of CLD (Tables 1, 2, and 3). In return, noteworthy, each of the substances had distinct behaviors in terms of bacteriostatic activity. Indeed, while the highest interpretable doses of CLD were set at 30, 10, and even 3 μg/plate, CLD-1Cl was not or moderately toxic up to the doses ranging from 30 to 200 μg/plate. Finally, no bacteriostatic activity was observed in any of the four strains of *Salmonella typhimurium* treated with CLD-3Cl up to the highest dose tested of 200 μg/plate. Even if the cytotoxicity assessment performed during the genotoxicity assays aimed at providing information about the reliability of these tests and at setting the range of doses to be actually tested, as they were obtained under the same experimental conditions, it was deemed interesting to compare the cytotoxicity of CLD and both its dechlorinated derivatives, as determined in these tests. Indeed, the data highlight a clear difference in terms of cytotoxicity in bacteria during the Ames test (with for instance, during the assays without metabolic activation, the highest analyzable doses of 10, 30, and >200 μg/plate for CLD, CLD-1Cl, and CLD-3Cl, respectively). The following order of cytotoxic potential on *Salmonella typhimurium* was therefore observed: CLD > CLD-1Cl > CLD-3Cl.

In vitro micronucleus assay with TK6 human lymphoblastoid cells

The positive controls (mitomycin C in both treatments without rat liver S9-mix, griseofulvin in the continuous treatment without S9-mix, and cyclophosphamide in presence of S9-mix) induced statistically and biologically significant increases in the number of micronucleated TK6 cells as expected, with values comparable to the ones usually observed in the laboratory.

First, it is interesting to note that the three substances, i.e., CLD and its dechlorinated derivatives, did not have similar cytotoxicity (Tables 4, 5, and 6). Indeed, in the short-term treatment without metabolic activation, the highest concentrations leading to acceptable level of toxicity were of 31.25 μg/mL (6.37×10^{-5} M), 62.5 μg/mL (1.37×10^{-4} M), and 125 μg/mL (3.23×10^{-4} M) for CLD, CLD-1Cl, and CLD-3Cl, respectively. In the long-term treatment, the highest concentrations reached 31.25 μg/mL for both CLD and CLD-1Cl (6.37×10^{-5} and 6.85×10^{-5} M, respectively), and 62.5 μg/mL (1.61×10^{-4} M) for CLD-3Cl. In the treatment with metabolic activation, the top concentrations were set at 62.5 μg/mL ($1.27.10^{-4}$ and $1.37.10^{-4}$ M) for both CLD and CLD-1Cl and 125 μg/mL ($3.23.10^{-4}$ M) for CLD-3Cl.

Furthermore, no genotoxic activity was noted in the assays with and without rat liver S9-mix using both a short- and a long-term treatment (Table 4). Indeed, no biologically significant increases in the incidence of TK6 human lymphoblastoid micronucleated cells were observed on the whole range of concentrations analyzed of CLD and its derivatives: CLD induced 2 to 11.5 micronucleates/2000 mononucleated TK6 cells in the short treatment without metabolic activation, 1 to 7 micronucleates/2000 mononucleated TK6 cells in the continuous treatment without metabolic activation and 3 to 7 micronucleates/2000 mononucleated TK6 cells in the short treatment with rat liver S9-mix compared to 7, 9, and 2 micronucleates/2000 mononucleated TK6 cells in their respective solvent controls. CLD-1Cl induced in the short-term treatment without metabolic activation 3 to 4 micronucleated/2000 mononucleated TK6 cells vs. 7 in the solvent control, 5 to 8 micronucleated/2000 mononucleated TK6 cells vs. 9 in the continuous treatment and 0 to 5 micronucleates/2000 mononucleated TK6 cells vs. 2 for the

Table 1 Mutagenic activity of CLD evaluated with Ames's technique

	Strain	TA1537		TA98		TA100		TA102	
	DOSES in µg/plate	Revertants /plate	Induction Ratio	Revertants /plate	Induction Ratio	Revertants /plate	Induction Ratio	Revertants /plate	Induction Ratio
Positive control	(a)[c]	*1360.0*	**302.2**	*480.0*	**24.6**	*701.3*	**5.7**	*1232.0*	**6.3**
CLD without S9-mix	0	*4.5*	–	*19.5*	–	*122.0*	–	*194.3*	–
	1	*7.3*	**1.6**	*21.3*	**1.1**	*118.3*	**1.0**	*197.3*	**1.0**
	3	*5.0*	**1.1**	*15.3*	**0.8**	*115.7*	**0.9**	*164.0*	**0.8**
	10	*3.7*	**0.8**	*10.0*	**0.5**	*37.3*	**0.3**	*53.7*	**0.3**
	30	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
	100	[b]	[b]	[a]	[a]	[b]	[b]	[b]	[b]
	200	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]
Positive control	(b)[d]	*261.3*	**52.3**	*1616.0*	**78.1**	*2032.0*	**17.2**	*1573.3*	**5.1**
CLD with S9-mix	0	*5.0*	–	*20.7*	–	*118.0*	–	*307.3*	–
	1	*6.3*	**1.3**	*17.0*	**0.8**	*117.0*	**1.0**	*234.7*	**0.8**
	3	*7.7*	**1.5**	*22.3*	**1.1**	*114.0*	**1.0**	*256.7*	**0.8**
	10	*4.7*	**0.9**	*15.3*	**0.7**	*87.3*	**0.7**	*201.3*	**0.7**
	30	*3.0*	**0.6**	*9.7*	**0.5**	[a]	[a]	[a]	[a]
	100	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
	200	[b]	[b]	[a]	[a]	[b]	[b]	[b]	[b]

Induction ratio = number of revertants in the treated condition / number of revertants in the control condition. In italic: number of revertants *per* plate, in bold: ratio compared to the control condition

[a] Presence of microcolonies of toxicity

[b] No bacterial growth

Reference positive compounds (µg/plate):

[c] TA1535 and TA100: Sodium azide; TA1537: 9-amino-acridine; TA98: 2-nitrofluorene; TA102: Mitomycin C

[d] TA1535, TA1537, TA98, TA100: 2-anthramine; TA102: benzo[a]pyrene

solvent control. Finally, in the short-term and continuous treatments without metabolic activation and in the short-term treatment with rat liver S9, CLD-3Cl induced 1 to 7, 4 to 13, and 2 to 4 micronucleates /2000 mononucleates TK6 cells vs. 8, 7, and 2 in the corresponding negative controls. Dechlorinated derivatives CLD-1Cl and CLD-3Cl, like CLD, were clearly devoid of genotoxicity.

Evaluation of the proangiogenic properties in vitro

After 24-h incubation, CLD was able to increase capillary length formed by HUVEC compared to non-treated cells for the three tested concentrations (at 10^{-11} M, 179.51 ± 13.47%, $p < 0.001$; at 10^{-9} M, 150.53 ± 8.41%, $p < 0.01$; and at 5×10^{-8} M, 154.88 ± 7.37%, $p < 0.001$; Fig. 1a–c). The mono-dechlorinated CLD derivative CLD-1Cl also exerted proangiogenic properties on HUVECs in culture on ECM gel for the three concentrations compared to control cells (at 10^{-11} M, 147.68 ± 12.42%, $p < 0.05$; at 10^{-9} M, 136.08 ± 7.84% $p < 0.05$; and at 5.10^{-8} M, 145.94 ± 16.74%, $p < 0.05$; Fig. 1a–c). However, the tri-dechlorinated CLD derivative CLD-3Cl did not significantly increase endothelial capillary formation compared to control cells (at 10^{-11} M, 131.10 ± 5.96%, $p = 0.071$; at 10^{-9} M 121.06 ± 10.84%, $p = 0.169$; and at 5×10^{-8} M, 132.10 ± 5.73%, $p = 0.068$, Fig. 1a–c). Moreover, the mean capillary length formed by HUVEC was significantly lower after CLD-3Cl treatment compared to CLD treatment with two of the three tested concentrations (at 10^{-11} M, 179.51 ± 13.47% vs 131.10 ± 5.96%, $p < 0.05$; at 10^{-9} M, 150.53 ± 8.41% vs 121.06 ± 10.84%, $p = 0.113$; and at 5.10^{-8} M, 154.88 ± 7.37% vs 132.10 ± 5.73%, $p < 0.05$, Fig. 1a, c). No significant statistical difference was found between all the other conditions.

Discussion

In order to compare the mutagenicity of CLD-1Cl and CLD-3Cl with CLD, the Ames test has been performed both with and without metabolic activation, on four different *Salmonella typhimurium* strains TA1537, TA98, TA100, and TA102 able of demonstrating genetic events leading to mutagenicity such as frameshift or base pair substitution. This test did not evidence any mutagenic effect of CLD up to the highest dose tested of

Table 2 Mutagenic activity of CLD-1Cl evaluated with Ames's technique

	Strain	TA1537		TA98		TA100		TA102	
	DOSES in μg/plate	Revertants /plate	Induction Ratio	Revertants /plate	Induction Ratio	Revertants /plate	Induction Ratio	Revertants /plate	Induction Ratio
Positive control	(a)[c]	*1360.0*	**302.2**	*480.0*	**24.6**	*701.3*	**5.7**	*1232.0*	**6.3**
CLD-1CL without S9-mix	0	*4.5*	–	*19.5*	–	*122.0*	–	*194.3*	–
	1	*4.7*	**1.0**	*16.0*	**0.8**	*117.3*	**1.0**	*221.3*	**1.1**
	3	*3.7*	**0.8**	*13.3*	**0.7**	*119.3*	**1.0**	*188.7*	**1.0**
	10	*5.7*	**1.3**	*12.7*	**0.7**	*109.0*	**0.9**	*184.7*	**1.0**
	30	*6.0*	**1.3**	*15.0*	**0.8**	*61.3*	**0.5**	*101.3*	**0.5**
	100	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
	200	[b]	[b]	[b]	[b]	[b]	[b]	[a]	[a]
Positive control	(b)[d]	*261.3*	**52.3**	*1616.0*	**78.1**	*2032.0*	**17.2**	*1573.3*	**5.1**
CLD-1CL with S9-mix	0	*5.0*	–	*20.7*	–	*118.0*	–	*307.3*	–
	1	*4.3*	**0.9**	*14.3*	**0.7**	*113.7*	**1.0**	*217.3*	**0.7**
	3	*5.3*	**1.1**	*19.7*	**1.0**	*109.3*	**0.9**	*262.0*	**0.9**
	10	*6.7*	**1.3**	*13.7*	**0.7**	*112.7*	**1.0**	*220.7*	**0.7**
	30	*4.0*	**0.8**	*19.7*	**1.0**	*88.3*	**0.7**	*198.7*	**0.6**
	100	*2.7*	**0.5**	*9.3*	**0.4**	[a]	[a]	[a]	[a]
	200	[a]	[a]	*9.3*	**0.4**	[a]	[a]	[a]	[a]

Induction ratio = number of revertants in the treated condition / number of revertants in the control condition. In italic: number of revertants *per* plate, in bold: ratio compared to the control condition

[a] Presence of microcolonies of toxicity

[b] No bacterial growth

Reference positive compounds (μg/plate):

[c] TA1535 and TA100: Sodium azide; TA1537: 9-amino-acridined 50; TA98: 2-nitrofluorened 2; TA102: Mitomycin C

[d] TA1535, TA1537, TA98, TA100: 2-anthramined 2; TA102: benzo[a]pyrened 2

200 μg/plate. These results are in agreement with those from Schoeny et al. (1979) who also concluded to the lack of mutagenicity of CLD on *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100 (Schoeny et al. 1979). In the same way, both CLD derivatives, i.e., CLD-1Cl and CLD-3Cl, did not demonstrate any mutagenic activity in this bacterial test system.

Furthermore, CLD induced no chromosomal aberration as demonstrated in the in vitro micronucleus test performed on human lymphoblastoid TK6 cells. These first results are rather in accordance with those generally observed with CLD which is considered to be a non-genotoxic carcinogen (Reuber 1979); for instance, following Ikegwuonu and Mehendale (1991), CLD induced a low level of unscheduled DNA synthesis in the hepatocytes of rats pretreated with 10 mg/kg b.w. CLD (Ikegwuonu and Mehendale 1991). Only DNA strand breaks were reported in this study. Chlordecone was also found to increase induced ssDNA breaks in isolated human and rat testicular cells as measured by alkaline filter elution but exclusively at high concentrations (300 to 1000 μM), an exposure that was associated with some toxicity in both cell lines (Bjørge et al. 1996).

Dechlorinated derivatives CLD-1Cl and CLD-3Cl were also devoid of genotoxicity, meaning that the ISCR process does not lead to degradation products of concern regarding these endpoints.

Most interestingly, a noteworthy difference in terms of toxicity was clearly shown in mutagenicity tests. The following order of toxicity to cells in Ames/micronucleus tests can be established: CLD > CLD-1Cl > CLD-3Cl that may suggest that dechlorinated CLD derivatives are probably less toxic than CLD. Toxicity mechanisms of CLD have been well established on in vitro eukaryotic cultured cells (End et al. 1981). Briefly, these authors reported that CLD was able to inhibit mitochondrial oxidative phosphorylation and associated Ca^{2+} transport in neuronal cells. Thus, oxidative stress would be, in part, responsible for the toxicity of CLD, but studies evaluating the CLD-induced oxidative stress and the dechlorinated CLD derivatives mechanism of toxicity are now needed.

Angiogenesis is a critical step for tumor growth and is considered as a prerequisite for the rapid expansion of macroscopic tumors (Carmeliet and Jain 2011). Furthermore, Clere et al. have already evidenced a CLD-induced proangiogenic effect for two concentrations, 5×10^{-8} M, a plasmatic value of CLD which is associated with an increased risk of cancer and 2×10^{-11} M, a concentration of CLD approximately found in

Table 3 Mutagenic activity of CLD-3Cl evaluated with Ames's technique

	Strain	TA1537		TA98		TA100		TA102	
	DOSES in μg/plate	Revertants /plate	Induction Ratio	Revertants /plate	Induction Ratio	Revertants /plate	Induction Ratio	Revertants /plate	Induction Ratio
Positive control	(a)[a]	*1360.0*	**302.2**	*480.0*	**24.6**	*701.3*	**5.7**	*1232.0*	**6.3**
CLD-3CL without S9-mix	0	*4.5*	–	*19.5*	–	*122.0*	–	*194.3*	–
	1	*8.3*	**1.8**	*15.0*	**0.8**	*111.7*	**0.9**	*191.3*	**1.0**
	3	*6.7*	**1.5**	*12.7*	**0.7**	*128.3*	**1.1**	*215.3*	**1.1**
	10	*4.3*	**1.0**	*16.7*	**0.9**	*113.7*	**0.9**	*208.7*	**1.1**
	30	*5.3*	**1.2**	*11.7*	**0.6**	*102.7*	**0.8**	*199.3*	**1.0**
	100	*3.7*	**0.8**	*16.7*	**0.9**	*87.3*	**0.7**	*172.0*	**0.9**
	200	*5.3*	**1.2**	*16.3*	**0.8**	*101.0*	**0.8**	*150.0*	**0.8**
Positive control	(b)[b]	*261.3*	**52.3**	*1616.0*	**78.1**	*2032.0*	**17.2**	*1573.3*	**5.1**
CLD-3CL with S9-mix	0	*5.0*	–	*20.7*	–	*118.0*	–	*307.3*	–
	1	*7.3*	**1.5**	*22.7*	**1.1**	*100.7*	**0.9**	*326.0*	**1.1**
	3	*5.0*	**1.0**	*24.3*	**1.2**	*110.0*	**0.9**	*366.0*	**1.2**
	10	*5.3*	**1.1**	*21.0*	**1.0**	*109.3*	**0.9**	*360.0*	**1.2**
	30	*5.7*	**1.1**	*19.0*	**0.9**	*94.7*	**0.8**	*356.0*	**1.2**
	100	*3.7*	**0.7**	*19.3*	**0.9**	*116.0*	**1.0**	*344.7*	**1.1**
	200	*7.7*	**1.5**	*14.7*	**0.7**	*93.7*	**0.8**	*285.3*	**0.9**

Induction ratio = number of revertants in the treated condition / number of revertants in the control condition. In italic: number of revertants *per* plate, in bold: ratio compared to the control condition

Reference positive compounds (μg/plate):

[a] TA1535 and TA100: Sodium azide; TA1537: 9-amino-acridined 50; TA98: 2-nitrofluorened 2; TA102: Mitomycin C

[b] TA1535, TA1537, TA98, TA100: 2-anthramined 2; TA102: benzo[a]pyrened 2

drinking water (Clere et al. 2012). Our data indicating that CLD and CLD-1Cl have in vitro proangiogenic effects for the concentrations 10^{-11}, 10^{-9}, and 5.10^{-8} M confirm the previous published data for CLD and extend the observation to CLD-1Cl. Most interestingly, the tri-dechlorinated derivative CLD-3Cl did not exhibit proangiogenic effects and exert a capillary formation significantly lower than CLD for 10^{-11} and 5.10^{-8} M.

Table 4 In vitro micronucleus test for CLD in micromethod on TK6 human lymphoblastoid cells

		Assay without metabolic activation						Assay with metabolic activation		
		3-h treatment followed by 24 h of recovery			27-h continuous treatment			3-h treatment followed by 24 h of recovery		
Compound	Conc. in μg/ml	% relative survival	Mean number of MMNC /2000 MNC	*p*	% relative survival	Mean number MMNC /2000 MNC	*p*	% relative survival	Mean number of MMNC /2000 MNC	*p*
Negative control	***0***	–	**7.0**	–	–	**9.0**	–	–	**2.0**	–
Mitomycin C	***0.5***	*98.5*	**95.0**	**<0.001**				–	–	–
	0.2	–	–	–	*90.9*	**116.0**	**<0.001**	–	–	–
Griseofulvine	***5.0***	–	–	–	*86.2*	**41.0**	**<0.001**	–	–	–
Cyclophosphamide	***5.0***	–	–	–	–	–	–	*100.6*	**42.0**	**<0.001**
CLD	**31.25**	*99.7*	**5.0**	**N.S.**	*112.4*	**1.0**	**<0.05**	*76.9*	**7.0**	**<0.05**
	15.63	*104.1*	**11.5**	**N.S.**	*124.4*	**1.0**	**<0.05**	*86.5*	**3.0**	**N.S.**
	7.81	*102.4*	**2.0**	**N.S.**	*118.3*	**7.0**	**N.S.**	*92.5*	**4.0**	**N.S.**

In italic: the percentage of relative survival, in bold: the mean number of MMNC / 2000 MNC

MMNC micronucleated mononucleated cells, *MNC* mononucleated cells, *N.S.* not statistically significant with $p < 0.05$

Table 5 In vitro micronucleus test for CLD-1Cl in micromethod on TK6 human lymphoblastoid cells

		Assay without metabolic activation						Assay with metabolic activation		
		3-h treatment followed by 24 h of recovery			27-h continuous treatment			3-h treatment followed by 24 h of recovery		
Compound	Conc. in μg/mL	% relative survival	Mean number of MMNC /2000 MNC	*p*	% relative survival	Mean number of MMNC /2000 MNC	*p*	% relative survival	Mean number of MMNC /2000 MNC	*p*
Negative control	***0***	–	**7.0**	–	–	**9.0**	–	–	**2.0**	–
Mitomycin C	***0.5***	*98.5*	**95.0**	**<0.001**				–	–	–
	0.2	–	–	–	*90.9*	**116.0**	**<0.001**	–	–	–
Griseofulvine	***5.0***	–	–	–	*86.2*	**41.0**	**<0.001**	–	–	–
Cyclophosphamide	***5.0***	–	–	–	–	–	–	*100.6*	**42.0**	**<0.001**
CLD-1Cl	**62.5**	*66.9*	**1**	**<0.05**	–	–	–	*73.4*	**0**	**N.S.**
	31.25	*100.0*	**4**	**N.S.**	*129.7*	**7**	**N.S.**	*81.9*	**5**	**N.S.**
	15.63	*96.2*	**3**	**N.S.**	*125.4*	**8**	**N.S.**	*84.5*	**2**	**N.S.**
	7.81	–	–	–	*118.0*	**5**	**N.S.**	–	–	–

In italic: the percentage of relative survival, in bold: the mean number of MMNC / 2000 MNC

MMNC micronucleated mononucleated cells, *MNC* mononucleated cells, *N.S.* not statistically significant with $p < 0.05$

These results indicate that CLD dechlorination reduces the CLD proangiogenic properties once 3 Cl have been replaced by H. The replacement of 1 Cl by H does not have a statistically significant effect. The replacement of 2 Cl by H might have an effect, but an analytical standard of didechlorinated CLD was not available for this study. Mechanisms responsible for the decrease of the proangiogenic effect by CLD dechlorination remain unknown. It has been demonstrated that pharmacological blockade of ERα or a silencing of ERα in endothelial cells prevents the CLD-induced angiogenesis both in vitro with HUVECs in culture on ECM gel® and in vivo with an ECM gel® plug used in mice (Clere et al. 2012). Regarding these data, it would be interesting to estimate the presence or the absence of possible interactions between these two CLD dechlorinated derivatives and ERα in order to evaluate their estrogenicity.

As the implementation of the standard step-wise approach using a basic battery of in vitro genotoxicity tests led to clear negative results, it can be concluded that the three substances

Table 6 In vitro micronucleus test for CLD-3Cl in micromethod on TK6 human lymphoblastoid cells

		Assay without metabolic activation						Assay with metabolic activation		
		3-h treatment followed by 24 h of recovery			27-h continuous treatment			3-h treatment followed by 24 h of recovery		
Compound	Conc. in μg/mL	% relative survival	Mean number of MMNC /2000 MNC	*p*	% relative survival	Mean number of MMNC /2000 MNC	*p*	% relative survival	Mean number of MMNC /2000 MNC	*p*
Negative control	***0***	–	**7.0**	–	–	**9.0**	–	–	**2.0**	–
Mitomycin C	***0.5***	*98.5*	**95.0**	**<0.001**				–	–	–
	0.2	–	–	–	*90.9*	**116.0**	**<0.001**	–	–	–
Griseofulvine	***5.0***	–	–	–	*86.2*	**41.0**	**<0.001**	–	–	–
Cyclophosphamide	***5.0***	–	–	–	–	–	–	*100.6*	**42.0**	**<0.001**
CLD-3Cl	**125,0**	*70.1*	**1**	**<0.05**	–	–	–	*66.6*	**2**	**N.S.**
	62.5	*89.9*	**7**	**N.S.**	*41.9*	**13**	**N.S.**	*78.2*	**3**	**N.S.**
	31.25	*103.8*	**2**	**N.S.**	*112.7*	**5**	**N.S.**	*86.0*	**4**	**N.S.**
	15.63	–	–	–	*114.9*	**4**	**N.S.**	–	–	–

In italic: the percentage of relative survival, in bold: the mean number of MMNC / 2000 MNC

MMNC micronucleated mononucleated cells, *MNC* mononucleated cells, *N.S.* not statistically significant with $p < 0.05$

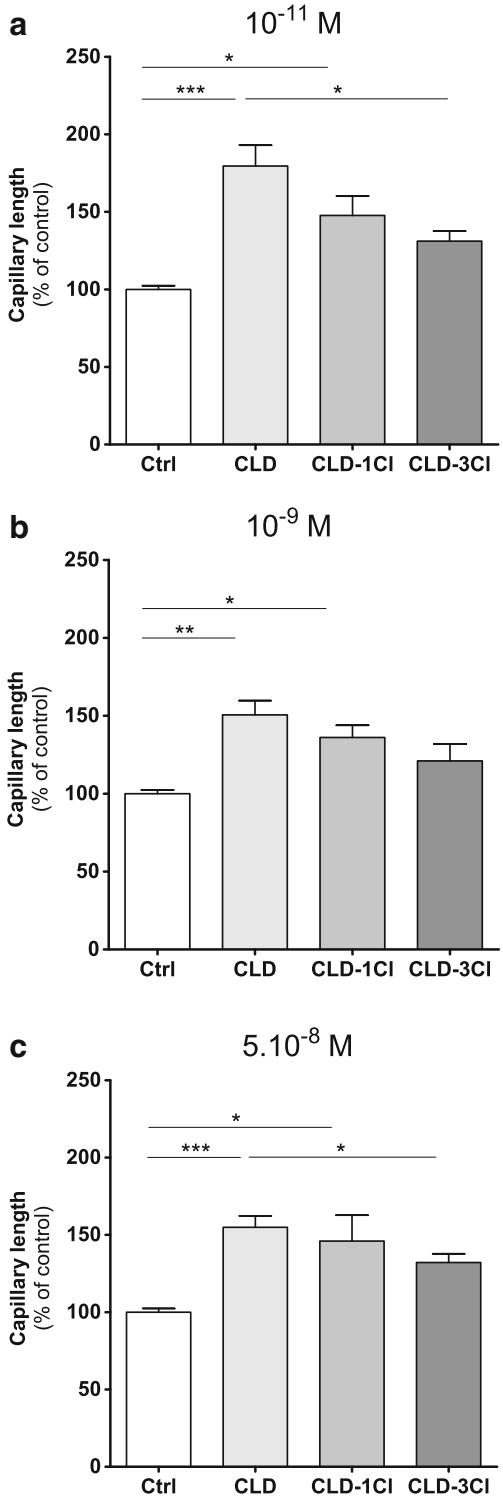

Fig. 1 Capillary length formed by HUVEC treated with CLD, CLD-1Cl, and CLD-3Cl for 24 h at 10^{-11} M (A), 10^{-9} M (B), and 3.10^{-8} M (C). $n = 6$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. No significant statistical difference was found between all the other conditions

have no genotoxic potential and thus no carcinogenic potential through a genotoxic mode of action is expected. The epigenetic mode of action was not specifically addressed.

The only referenced paper comparing the toxic effects of CLD and some of its alteration products relates to isolated rat liver mitochondria. It shows the following order of potency: CLD > CLD-1Cl > > CLD-2Cl (Soileau and Moreland 1983). The monodechlorinated-CLD (CLD-1Cl) considered, with the Cl in position 5b (according to CAS nomenclature) substituted by an H atom, is, however, different from the one studied here, the 5a- or 5-hydro-CLD. As for the didechlorinated-CLD (CLD-2Cl) studied by Soileau and Moreland, it does not correspond to a level of CLD dechlorination considered in our work.

In another paper aiming at the determination of CLD dechlorination products in finfish, oysters, and crustaceans, the authors (Carver and Griffith 1979) report that the acute toxicity in mysid shrimp of CLD-1Cl is approximately equal to that of CLD, while CLD-2Cl appears to be less acutely toxic by at least an order of magnitude. The source of these data is, however, not specified nor is the position of dechlorination of the compounds studied. With respect to the monodechlorinated-CLD, the position of dechlorination is likely to be the same as that of the work by Soileau and Moreland, since limited choice seemed to have been offered to the research laboratories at that time (Soileau and Moreland 1983).

Even though we addressed a monodechlorinated-CLD different from that of Soileau and Moreland and probably also from the one reported by Carver and Griffith, these three sets of observations point at a very consistent trend for CLD: Toxicity decreases as the level of dechlorination increases. The fact that the trend is observed whatever the position of dechlorination in the CLD-1Cl reinforces the trend itself.

When considering the overall benefit of ISCR, the CLD derivatives other than the two studied here must be taken into consideration. Based on the trend supported by three independent studies (the present work, Carver and Griffith 1979; Soileau and Moreland 1983), the tetra- and pentadechlorinated-CLD identified in soils (Mouvet et al. 2016b) and water (Belghit et al. 2015) treated by chemical reduction are therefore most likely to be less toxic than the tridechlorinated-CLD, much less than the monodechlorinated-CLD, and far less than the parent compound. Knowing also that the tetra- and pentadechlorinated CLD are formed by ISCR to a much lesser extent than the mono and tridechlorinated-CLD, it can be assumed that the overall suite of CLD dechlorination products formed by chemical reduction is less toxic than CLD. Nevertheless, experimental studies are needed to further explore and confirm or infirm such properties in a living model. The protein–ligand interactions leading to the binding of the main CLD derivatives to estrogen receptors deserve also to be studied. CLD has been shown to be significantly more efficacious for ERα than for Erβ, and significant differences appear between chemicals (Delfosse et al. 2014). Such study would notably enable structure-based computational methods that can be used in risk assessment (Delfosse et al. 2014).

Conclusion

CLD and its two derivatives studied here, a mono- and a tri-dechlorinated, induced neither chromosomal aberrations nor mutagenic activity meaning that dechlorination of CLD by ISCR process does not lead to degradation products of concern regarding these endpoints.

Furthermore, the present study demonstrated that CLD-dechlorinated derivatives are devoid of genotoxicity and mutagenicity, and have lower proangiogenic properties than CLD. Thus, these CLD derivatives are probably less toxic than CLD.

In view of the major environmental and health issue that soil contamination by CLD represents in the FWI, any possible way of lowering permanently the CLD soil concentrations and the exposure levels of inhabitants is of major interest. So far, ISCR is the only proven way to achieve such a goal. Sequestrating CLD in the soil by adding compost is efficient in lowering plant contamination and leaching to groundwaters. This positive effect has, however, not been proven to last more than 6 months (Woignier et al. 2012; Woignier et al. 2013). On another hand, public authorities are managing the contamination, e.g., restricting the marketing of food products contingent (Achard et al. 2007), issuing statutory limitations on human consumption of poultry and other animal food (Journal officiel de la République Française 2005) and banning consumption and commercialization of fish and seafood (Préfecture de la Région Guadeloupe 2010). These advices remain, however, to be strictly obeyed, and they do nothing to solve the issue of ecosystems contamination. A combination of the three strategies aforementioned, and possibly others that remain to be tested, will certainly be required. In the case of ISCR, the present results remove partly one of the obstacles to the application of the process over significant areas in situ.

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Compliance with ethical standards

Conflicts of interest The authors declare no competing financial interests.

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