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Linking Pesticide Exposure with Pediatric Leukemia: Potential Underlying Mechanisms

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Abstract

Leukemia is the most common cancer in children, representing 30% of all childhood cancers. The disease arises from recurrent genetic insults that block differentiation of hematopoietic stem and/or progenitor cells (HSPCs) and drives uncontrolled proliferation and survival of the differentiation-blocked clone. Pediatric leukemia is phenotypically and genetically heterogeneous with an obscure etiology. The interaction between genetic factors and environmental agents represents a potential etiological driver. Although information is limited, the principal toxic mechanisms of potential leukemogenic agents (e.g., etoposide, benzene metabolites, bioflavonoids and some pesticides) include topoisomerase II inhibition and/or excessive generation of free radicals, which may induce DNA single- and double-strand breaks (DNA-DSBs) in early HSPCs. Chromosomal rearrangements (duplications, deletions and translocations) may occur if these lesions are not properly repaired. The initiating hit usually occurs *in utero* and commonly leads to the expression of oncogenic fusion proteins. Subsequent cooperating hits define the disease latency and occur after birth and may be of a genetic, epigenetic or immune nature (*i.e.*, delayed infection-mediated immune deregulation). Here, we review the available experimental and epidemiological evidence linking pesticide exposure to infant and childhood leukemia and provide a mechanistic basis to support the association, focusing on early initiating molecular events.

Keywords: infant and childhood leukemia, hematopoietic stem/progenitor cells, chromosomal rearrangements, topoisomerase II, pesticides, DNA double-strand break, oxidative stress

1. Introduction

Leukemia is the most common childhood cancer, accounting for 30% of all cancers diagnosed in children under 15 years of age, with an annual incidence of up to 40 cases per million children in developed countries and an incidence peak between three and five years of age [1,2]. Pediatric acute leukemia is a phenotypically- and genetically-heterogeneous disease of immature hematopoietic stem and progenitor cells (HSPCs). Phenotypically, it can target B-cell progenitors (B-cell acute lymphoblastic leukemia (B-ALL)), T-cell progenitors (T-ALL) or myeloid progenitors (acute myeloid leukemia (AML)). Acute leukemia can be further stratified according to the differentiation stage at which HSPCs are blocked; for example, B-ALL can have a pro-B (proB-ALL) or pre-B phenotype (preB-ALL) [3]. Similarly, AML can affect both immature (subtype M0 of the French-American-British classification of AML) and mature lineage-committed types, such as erythroblastic or megakaryoblastic leukemia (subtypes M6 and M7, respectively). Seventy percent of pediatric acute leukemias are ALL and 30% are AML. Genetically, ALL and AML can be further stratified according to molecular cytogenetics [4,5], which represents a prognostic factor.

Fetal hematopoiesis begins in the aorta gonad-mesonephros region to subsequently colonize the fetal liver (FL) and ultimately, just before birth, the bone marrow [6]. FL hematopoiesis entails an active proliferation of progenitors, rendering fetal HSPCs susceptible to oncogenic transformation through DNA damage mediated by chemical exposure during pregnancy [7]. Although the etiology of ALL remains elusive, ionizing radiation, congenital genetic syndromes and *in utero* exposure to specific genotoxic chemicals, including household pesticides, represent prime etiological suspects [8]. Importantly, altered patterns of infection during early childhood might also contribute to acute leukemia in children [9,10,11].

We here review the available experimental and epidemiological evidence linking pesticide exposure with infant and childhood leukemia and provide a mechanistic basis to support the association, focusing on early molecular events. However, the paucity of mechanistic data is a major obstacle to fully understanding the toxicological pathways involved. Causation pathways are likely to be multifactorial, and it is possible that the risk of pediatric leukemia from environmental exposure is influenced by genetic susceptibility.

2. Evidence Linking Pesticide Exposure with Pediatric Leukemia

2.1. Epidemiological Studies Supporting the Association

There is a growing concern about whether chronic low-level pesticide exposure during pregnancy or childhood increases the risk of childhood leukemia. Epidemiological studies suggest that pesticide exposure may have a greater impact on children than adults [12,13]. Almost all of the available evidence has focused on pediatric leukemia without making a distinction between infant and childhood leukemia, which are etiologically and pathologically different entities. However, most epidemiological studies are limited because no specific pesticides have been directly associated with the risk of leukemia, but rather the broad term "pesticide exposure" [13,14]. Such associations are mainly based on subjects' recall of the pesticide exposure, which hampers the drawing of conclusions because of recall/information bias.

In contrast to childhood leukemia, very few studies have examined the risk of infant leukemia and pesticide exposure. An international collaborative study on transplacental chemical exposure and risk of infant leukemia found an increased risk after *in utero* exposure to household pesticides (propoxur and other methylcarbamate insecticides), the therapeutic analgesic dipyrone and hormonal intake (estrogens). In these cases, infant leukemia was associated with the mixed lineage leukemia (*MLL*) gene fusion, likely as a result of topoisomerase II inhibition [15,16]. Although the aforementioned study was based on a rather small sample size, an increased risk (Odds Ratio—OR: 2.18) of infant leukemia was shown in mothers exposed to domestic insecticides during pregnancy. Since estrogens can be metabolized to catechol estrogen-3,4-quinones [17], the association found for infant leukemia might be due to topoisomerase II inhibition caused by quinone metabolites generated during estrogen metabolism [7]. A further Brazilian study found that over use of pesticides during pregnancy was associated with ALL and AML (OR: 2.10 and 5.01, respectively) in children <1 year of age [18]. Moreover, maternal exposure to the insecticide permethrin (assessed by self-reporting)

was associated with a higher risk of leukemia in children <1 year of age, with an OR of 2.47 for ALL and 7.28 for AML. This finding was also supported by a case-control study in China where the use of pyrethroids (assessed by urine levels of major metabolites) was associated with a greater risk of ALL [19].

The presence of the herbicide chlorthal in household dust samples was also associated with an increased risk of ALL in children <8 years, with a significant dose-response trend [20]. The association was greater with the herbicide mixture chlorthal plus alachlor. Other studies, however, report no significant associations. For example, no significant risk of childhood leukemia was found with exposure to some agricultural and residential herbicides, such as metolachlor, bromoxynil, cyanazine and 2,4-dichlorophenoxyacetic acid [20,21]. Furthermore, a case-control study on leukemia in children <1 year old from the American Children's Oncology Group failed to find a significant association between household exposure to insecticides or rodenticides and ALL or AML [22].

Different meta-analyses have consistently shown an increased risk of childhood leukemia associated with pesticide exposure [13,23]. However, this review will focus on the latest quantitative synthesis of evidence from studies. A recent meta-analysis has shown that maternal occupational pesticide exposure during pregnancy and/or paternal occupational pesticide exposure near-to-conception increases the risk of leukemia in offspring [24]. The authors pooled data from 13 case-control studies participating in the Childhood Leukemia International Consortium (CLIC) and found an almost two-fold increased risk of AML in mothers exposed to pesticides during pregnancy, whereas no significant risk was found for paternal exposure around conception. In relation to ALL, the same study observed a 20% increased risk with paternal exposure around conception, which appeared to be more evident for pediatric T-cell ALL. By contrast, no significant association was found between maternal exposure during pregnancy and risk of B or T-cell ALL. In a separate study investigating residential pesticide exposure, Bailey *et al.* [25] pooled data from 12 case-control studies in the CLIC and found a significant increased risk of ALL associated with exposure to any pesticide shortly before conception, during pregnancy and after birth (OR: 1.39, 1.43 and 1.36, respectively). Little variation was observed with the type of pesticide. Regarding AML, an increased risk was found for exposure to any pesticide in the few months prior to conception and during pregnancy (OR: 1.49 and 1.55, respectively); however, exposure after birth failed to demonstrate an increased leukemogenic risk. A recent meta-analysis conducted by Chen *et al.* [12] pooled 16 case-control studies and found that childhood exposure to indoor, but not outdoor, residential insecticides was associated with an increased risk of pediatric leukemia (OR: 1.47). A slightly weaker association was found for herbicide exposure (OR: 1.26). Notwithstanding these positive associations, observational studies on pesticide exposure and pediatric leukemia have a number of weaknesses to claim causal relationships. The consistency of findings across meta-analyses may be due to the considerable overlap in the studies included in the different meta-analyses undertaken. Many epidemiological analyses have not been performed using methodologically-rigorous association studies. Limitations include the lack of an accurate exposure estimate (from both a qualitative and quantitative standpoint), lack of temporal concordance (most studies were case-control in design) and little information on the dose-response relationship. In addition, the available epidemiological evidence may be challenged by endogenous or exogenous factors, such as genetic susceptibility, lifestyle and co-exposure to other environmental agents.

2.2. In Vitro Studies

The few *in vitro* studies available so far have shown that captan and captafol (two related chloroalkylthiocarboximide fungicides) decrease the activity of topoisomerase II by 50% and 20%, respectively, at a concentration of 1 μM [26]. Similarly, thiram (a dithiocarbamate fungicide) inhibits topoisomerase II at 10 μM [27]. However, genotoxic potential (*i.e.*, genetic abnormalities, mutations) of these fungicides occurred only at very high doses (10–100 mM) *in vivo* using common fruit flies [26]. More

recently, the organophosphate (OP) insecticide chlorpyrifos has been reported to induce DNA double-strand breaks (DSBs) and *MLL* gene rearrangements in human fetal liver $CD34^+$ HSPCs as a consequence of topoisomerase II inhibition [14].

Other OP pesticides have been implicated in leukemogenesis, particularly isofenphos, diazinon and fenitrothion. An *in vitro* study using the human leukemic cell line K562 demonstrated metabolic changes consistent with a leukemogenic potential of isofenphos [28]. In addition, human peripheral blood lymphocytes exposed to isofenphos exhibited dose-dependent damage to chromosomal DNA, as well as disruption of the cholinergic nuclear signaling pathway, which collectively could lead to genomic instability and leukemogenesis [29]. In an *in vitro* study using diazinon, a concentration of 0.1 μM induced hypermethylation of several genes involved in cell cycle arrest, such as cyclin-dependent kinase inhibitor 1A (*CDKN1A*) and 1C (*CDKN1C*), and tumor suppressor genes, such as *p53* and *PTEN* [30]. Fenitrothion at low concentrations (1 μM) also induced chromosomal damage in the B-cell leukemia/lymphoma-2 cell line BCL-2 [31].

3. Gene-Environment Interactions

For most pediatric leukemias, multiple genetic polymorphisms of xenobiotic metabolizing enzymes may interact with environmental, dietary and maternal factors to modulate the development of the disease. For example, quinones, which are capable of inhibiting topoisomerase II and can cleave the *MLL* gene at topoisomerase II cleavage sites, may be poorly detoxified depending on the activity of NAD(P)H:quinone oxidoreductase 1 (NQO1), an enzyme that detoxifies chemicals with quinone rings, such as bioflavonoids and benzene metabolites. Thus, genetic polymorphisms of *NQO1* resulting in low-activity variants might be associated with an increased risk of infant leukemia. By contrast, in childhood ALL without *MLL* rearrangements, deficiency of the *NQO1* gene is not associated with the etiology of the disease [32].

Global DNA hypomethylation is associated with activation of oncogenes and neoplastic processes [33], whereas the hypermethylation of 5′ cytosine-phospho-guanine (CpG) islands in promoter regions of some tumor suppressor genes prevents their transcription and promotes the development of tumors [34]. The genetic regulation of folate metabolism may have an influence on the preleukemic clone origin via DNA hypomethylation of key regulatory genes, rendering the genome vulnerable to genomic instability [35]. The presence of some polymorphisms in genes involved in folate metabolism reduces enzyme activity, leading to inadequate folate levels and DNA hypomethylation, ultimately contributing to the neoplastic process [35,36]. The insufficient input of folate increases the plasma concentration of homocysteine and *S*-adenosylhomocysteine, with the latter being a general inhibitor of adenosylmethionine-dependent methyltransferases [37]. Inhibition of these enzymes may alter both DNA methylation and transcriptional regulation [36,38]. The 677C>T gene polymorphism in methylenetetrahydrofolate reductase (*MTHFR*) has been linked to a decreased risk of childhood ALL, likely as a result of higher production of 5,10-MTHF and thymidine, which improve the fidelity of DNA synthesis and repair [39]. On the other hand, inactivating polymorphisms of detoxifying enzymes involved in carcinogen metabolism, such as glutathione *S*-transferases (GST), in parents have been associated with the development of ALL in their children <1 year old. The deletion of both the *GSTT1* and *GSTM1* genes in either parent might affect the risk of infant leukemia [40]. Furthermore, genetic polymorphisms of xenobiotic transport and metabolism pathways are associated with the risk of childhood ALL. In particular, polymorphisms of the *ABCB1* gene, which encodes a membrane transporter of lipophilic compounds, may interact with household insecticide exposures to increase the risk of disease [41]. Genetic variability in DNA repair pathways and cell cycle checkpoints might also interact with environmental, dietary, maternal and other external factors affecting the development of ALL. In summary, the limited data available suggest that dietary and environmental exposure to substances targeting topoisomerases together with the reduced ability of fetuses or their mothers to detoxify such compounds because of polymorphic variants of given genes could contribute to the development of pediatric leukemia [8,42].

The International Childhood Acute Lymphoblastic Leukemia Genetics Consortium revealed limitations in current studies on genetic susceptibility and the risk of ALL because of difficulties in conducting statistically- and methodologically-rigorous investigations [43]. Genome-wide association studies of childhood ALL have provided robust evidence for four low-penetrance susceptibility variants, which confer only a modest increase in risk. Moreover, the well-recognized ethnic differences in the risk of ALL represent a weakness in assessing the interplay between inherited and non-genetic risk factors. Given the small frequency of many ALL subgroups, the identification of differential effects will realistically be possible only through multi-center pooled analyses [43].

4. Early Molecular Events Involved in Pesticide-Associated Pediatric Leukemogenesis

Despite the rather comprehensive epidemiologic evidence linking pesticide exposure during different reproductive stages (pre-conception, pregnancy and early postnatal life) and pediatric leukemia, robust underlying pathological mechanisms remain unknown. The initiating event at the molecular level might be the induction of chromosomal rearrangements as a result of pesticide exposure and subsequent topoisomerase II inhibition or generation of oxidative stress, leading directly or indirectly to DNA damage. A mechanistic explanation follows.

4.1. DNA Double-Strand Breaks (DSBs)

Under some circumstances, oxidative lesions can lead to DNA DSBs formation in HSPCs. Environmental exposures to numerous chemicals, including many pesticides, have been shown *in vivo* and *in vitro* to generate oxidative species that can ultimately induce DNA base or sugar oxidative damage, leading to single-strand breaks (SSBs) and DSBs formation in the DNA [44]. For example, OP insecticides (chlorpyrifos, methyl-parathion, malathion), methyl-carbamates (methomyl) and the herbicide paraquat all cause oxidative DNA damage followed by DNA SSBs and DSBs [45,46,47,48]. There is also evidence of pesticide-induced oxidative stress and DNA damage in agricultural workers [47]. Additionally, oxidative species may interact with biological molecules to disrupt normal DNA synthesis and repair, and so, inhibition/inactivation of antioxidant proteins or DNA repair enzymes may also be an underlying molecular mechanism [49]. Along this line, pesticides can disrupt a number of antioxidant enzymes, including superoxide dismutase and catalase [50], rendering oxidative stress [51].

DSBs can arise under different circumstances: (i) when two SSBs form close to each other on opposite strands; (ii) upon enzymatic DNA cleavage next to an SSB on the opposite strand; or (iii) when either DNA replication or transcription takes place at sites of misrepaired DNA. DSBs constitute the first molecular event in the generation of chromosomal aberrations [52]. For instance, chlorpyrifos is reported to cause DNA DSBs and further chromosomal rearrangements (*i.e.*, *MLL*) through oxidative stress in human FL HSPCs [53]. However, chlorpyrifos can also induce DNA DSBs as a result of topoisomerase II inhibition in FL HSPCs in a manner similar to that produced by etoposide [14]. Analogously, blood lymphocytes from pesticide sprayers have greater fragile site breakage than normal individuals following treatment with aphidicolin, an inhibitor of DNA polymerases [54]. Chromosomal fragile sites are regions of the genome prone to breakage following exposure to many chemicals, including environmental and chemotherapeutic agents. During DNA replication, fragile site-inducing conditions can uncouple the helicase complex from the DNA polymerase, resulting in long stretches of single-stranded DNA and further DNA breakage [55]. Aphidicolin can also induce fragile site breakage through a topoisomerase II-mediated mechanism [56].

Topoisomerase II has critical functions in both DNA replication and transcription processes, and the so-called "topoisomerase II poisons" disrupt the DNA-induced topoisomerase II cleavage-religation equilibrium through the stabilization of ternary (drug-DNA-enzyme) complexes, termed cleavage complexes [57]. Chemical-induced breakpoints are strongly associated with predicted topoisomerase II cleavage sites (*i.e.*, *MLL*), thus supporting a role for topoisomerase II-mediated breakage upon exposure to environmental agents. The high

frequency of topoisomerase II recognition sites in specific DNA regions and the high expression of this enzyme in human $CD34^+$ HSPCs represent favorable conditions for breakage following exposure to agents targeting topoisomerase II activity (*i.e.*, bioflavonoids and quinones). Because $CD34^+$ HSPCs appear to be more sensitive to DNA damage than committed progenitor cells, exposure to low levels of different chemicals may induce DNA breakage at certain sites in HSPCs, increasing the risk of chromosomal rearrangements. If affected cells survive, they continue growing and dividing, thus perpetuating DNA lesions and starting the chain of events that will eventually lead to leukemogenesis [55].

4.2. Chromosomal Translocations

Key molecular events leading to pediatric leukemia pathogenesis are chromosomal translocations. These generally result from the exchange of chromosomal arms between heterologous chromosomes, and DNA DSBs are prerequisites for their occurrence. Chromosomal translocations ultimately result in the deregulation of key cellular proteins, especially those encoded by proto-oncogenes and tumor suppressor genes, which are critical functional regulators of the cell [58]. Two functional classes of translocations are known. The first one relocates a proto-oncogene (or genes encoding for non-antigen receptors or transcription factors) into regulatory regions of actively-transcribed genes (such as those encoding for immunoglobulin chains or T-cell receptors), causing dysregulated expression of an intact protein. The second class of translocations juxtaposes two genes to encode a chimeric protein, which is functionally distinct from the wild-type proteins [1].

Although the mechanistic generation of chromosomal translocations is not well understood, they may arise from improper DNA repair or erroneous recombination of variable (V), diversity (D) and joining (J) gene segments (a process known as V(D)J recombination). As for improper DNA repair, reactive oxygen species (ROS)-induced DSBs in human FL $CD34^+$ HSPCs following maternal exposure to chemicals triggers recombination/repair pathways by non-homologous end-joining (NHEJ) [14]. The majority of damaged HSPCs may either successfully repair the DNA DSBs or fail to do so and undergo apoptotic cell death. In a fraction of cells, the repair of the DNA DSBs within particular breakpoint cluster regions (bcr) is not completed correctly, giving rise to chromosomal translocations or deletions [59]. For fusion genes to be leukemogenic, DSBs must occur simultaneously in two chromosomes and must also involve the coding region of the genes to generate an exon-exon in-frame functional chimeric gene product. Importantly, this has to occur in an HSPC that has managed to bypass cell death and displays a sustainable lifespan and clonal potential to propagate the chimeric gene product [60].

Erroneous V(D)J recombination usually occurs in developing lymphocytes during cell maturation, where V(D)J gene segments of immunoglobulin chains or T-cell receptors are rearranged to yield a wide range of immunoglobulins and T-cell receptors. The process entails the cleavage of the V(D)J gene at the flanking recombination signal sequences (RSS) by lymphocyte-specific recombination-activating gene (RAG) endonucleases and subsequent ligation of the segments via the classical NHEJ pathway [61]. In pediatric leukemia, chromosomal translocations and deletions often arise as a result of mistakes in V(D)J rearrangements because RAG enzymes can erroneously recognize and target RSS-like sequences. V(D)J-recombinase-mediated rearrangements may occur at both immune RSS and non-immune cryptic RSS (cRSS), which are widely distributed throughout the genome [62]. There is growing evidence that *in vivo* exposure to DNA-damaging agents, including pesticides, can increase the frequency and alter the recombination site distribution of V(D)J rearrangements at cRSS [63,64]. An increase in V(D)J-recombinase-mediated events at either immune or non-immune RSS following exposure to DNA-damaging agents could play an important role in environmentally-induced genetic alterations associated with leukemia development. Nonetheless, the mechanism by which exposure to DNA-damaging agents could increase the frequency of V(D)J-recombinase-mediated genomic rearrangements remains unclear [64].

5. Pathobiology of Pediatric Leukemias

Given the distinct natural history and pathogenesis of infant and childhood leukemia, both entities will be addressed separately, although a chromosomal translocation is frequently the common initiating oncogenic event in both entities.

5.1. Infant Leukemia

Infant acute leukemia shows unique clinical and biological features and is commonly associated with rearrangements in the *MLL* gene (*MLL*-r), a master gene located on chromosome 11q23 that regulates normal human hematopoietic development and differentiation [65]. The *MLL* gene encodes a methyltransferase with activity for lysine 4 of histone H3 (H3K4), which mediates changes in chromatin associated with epigenetic transcriptional activation that plays an essential role in regulating gene expression during early development and hematopoiesis [66]. Rearrangements involving the *MLL* gene have been reported to occur only in mice with defects in DNA damage response and not in wild-type animals [67]. *MLL*-r functions as the initiating, and perhaps the sole driving, oncogenic event by dysregulating epigenetic and/or transcriptional programs [33] (Figure 1). Epidemiological and genetic studies have suggested that *MLL*-r may result from transplacental exposure to DNA topoisomerase-II inhibitors during gestation, such as chemotherapeutic agents, benzene metabolites (*i.e.*, benzoquinone), quinolone antibiotics, bioflavonoids present in some fruits and vegetables and some pesticides [7,33,68]. However, exposure to topoisomerase-II inhibitors is not sufficient *per se* for rearrangement of *MLL*, and the genetic background, such as mutations in the DNA damage response pathway, may influence the likelihood of *MLL*-r [67].

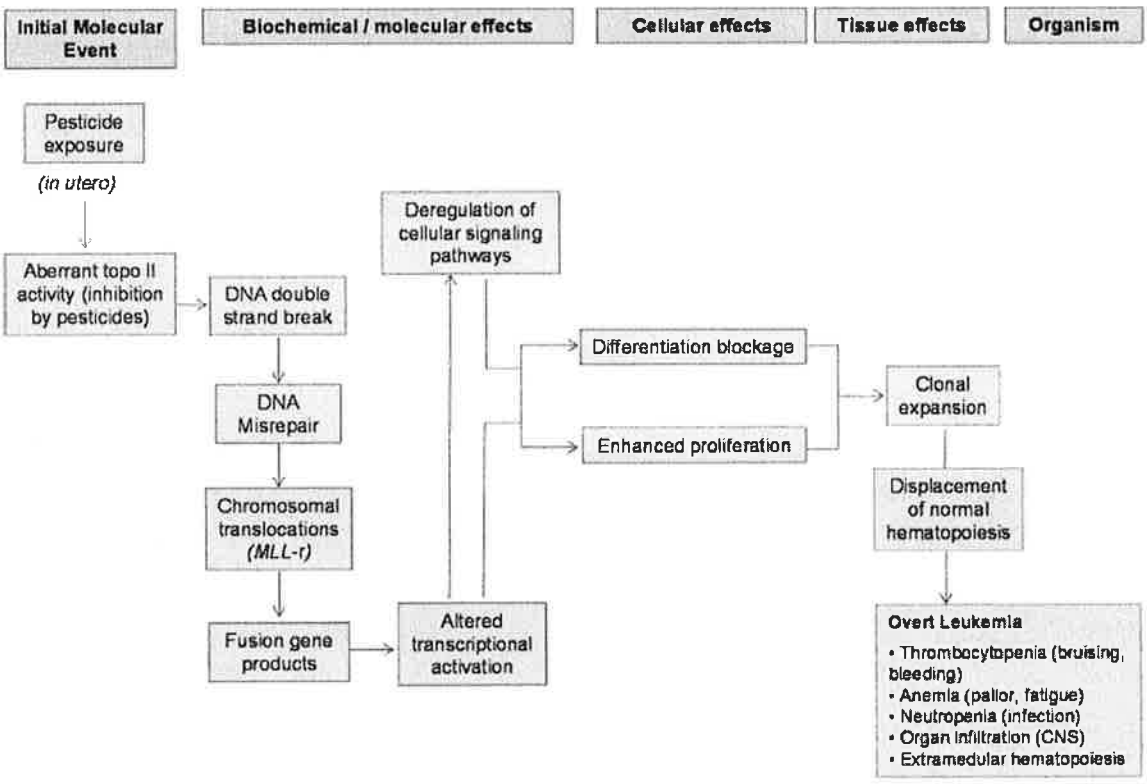

Figure 1

Chain of pathogenic events linking pesticide exposure to the development of infant *MLL*-rearranged acute leukemia.

The existence of recombination-prone sequences in the *MLL* bcr region supports the contention that *MLL*-r results from DNA breakage and recombination events. The genomic instability within *MLL* bcr may be the consequence of increased ROS generation [69]. The *MLL* fusion gene renders HSPCs more vulnerable to DNA repair and cell-cycle deregulation, facilitating the rapid acquisition of additional, secondary genetic changes, particularly upon continued exposure to genotoxic chemicals *in utero* [7,70]. These chemicals target early mesodermal precursors or HSPCs residing mainly in the FL where they inhibit topoisomerase-II activity and produce DNA DSBs within the *MLL* bcr, which are not properly repaired by homologous recombination or NHEJ. Because those mesodermal precursors or HSPCs are rapidly dividing and have high topoisomerase II content, they may be particularly sensitive to damage by topoisomerase II-targeting chemicals during a critical developmental window of vulnerability [33,71,72,73,74,75]. However, because of the very short latency of infant leukemia, it remains obscure whether the fusion gene generated from chromosomal translocations requires additional cooperating oncogenic hits for leukemogenesis. Although recurrent activating mutations of genes associated with cellular proliferation, such as components of the RAS signaling pathway, have been reported [76,77,78,79], functional studies revealed that these mutations are important for tumor maintenance rather than initiation in human HSPCs [80]. *MLL* breakage itself is not sufficient for the development of full-blown infant leukemia, even if the DNA damage response is defective. Activation of cellular proliferation by mutation of other genes might be necessary for overt leukemia [67]. The transformation mediated by the aberrant proteins encoded by fusion genes might depend on alternative (epi)-genetic cooperating lesions at a critical developmentally-earlier window of stem cell vulnerability to develop overt leukemia [33].

Intriguingly, and in contrast to the global dogma of cancer biology, *MLL*-r infant leukemia has been shown to have abnormal hypermethylation in non-enhancer, non-promoter regions, perhaps contributing to genomic stability and a silenced mutational landscape [76,81,82]. Extensive hypermethylation of tumor suppressor genes resulting in gene silencing has been observed in some cases of *MLL*-r infant leukemia [83].

5.2. Childhood Leukemia

Childhood leukemia has a prevalence peak at ~3–5 years of age, suggesting that environmental exposures *in utero* or during early childhood might be risk factors [25]. Under the current paradigm, the first initiating oncogenic mutation usually involves structural or numerical chromosomal alterations, impairing normal cell differentiation, while secondary hits more commonly comprise mutations affecting developmentally-regulated master transcription factors or membrane-proximal signaling pathways conferring proliferation and survival advantages to the differentiation-blocked clone [1,7,8,84,85]. The development of leukemia requires the activation of cell proliferation in addition to differentiation blockage [67]. Numerical aberrations (*i.e.*, hyperdiploidy) are also common hallmarks in childhood B-cell ALL.

The most common chromosomal aberrations are *E2A-PBX1*, *TEL-AML1* and *MLL*-r for B-ALL and *AML1-ETO* and *MLL*-r for AML. Similar to *MLL* rearrangements, the resulting aberrant chimeric proteins alter the normal transcriptional program and block normal B-cell and/or myeloid differentiation [8,86,87,88] (Figure 2). Although the *AML1* gene has been linked to anti-topoisomerase II agents, similar to the *MLL* gene, *TEL-AML1* is not sufficient to cause the disease by itself. As this fusion gene is observed in cord blood from about 1% of normal newborns, a significant proportion of the population carries self-limiting preleukemic clones, and the majority of them do not result in disease [3]. The longer latency observed in childhood leukemia unequivocally indicates that the initiating chromosomal translocation itself is unlikely to convert a preleukemic clone into an overt disease, thus suggesting the need for secondary cooperating (epi)-genetic events.

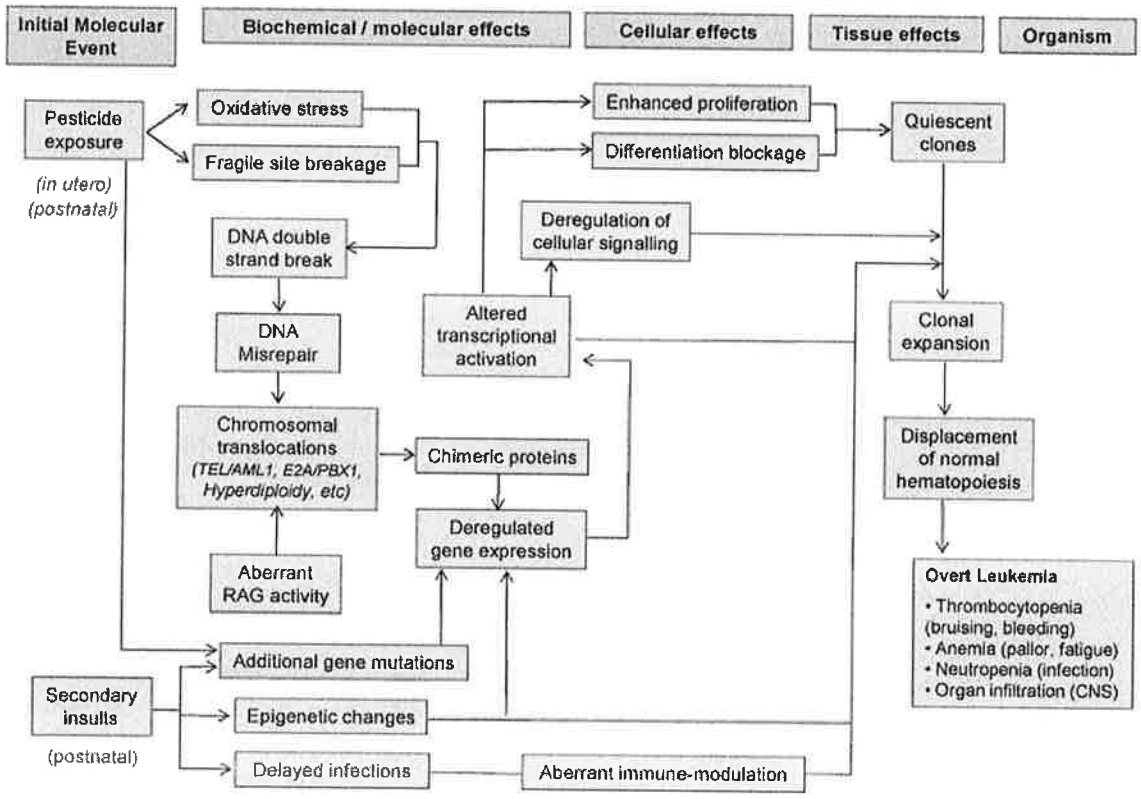

Figure 2

Chain of pathogenic events linking pesticide exposure to the development of childhood leukemia. Blue arrows indicate events related to the "first hit" and red arrows events related to the "second hit" (for more details, see Section 5.2).

Dysfunction of the immune system and delayed infections have been linked to childhood leukemia [9,89]. Two distinct underlying mechanisms might explain this association: (i) a lower repertoire of infections during early immune development; and (ii) an altered congenital responder status to infection resulting in functionally-aberrant clinical presentation of occasional infections. Thus, an untimely and excessive inflammatory response abolishes normal hematopoiesis, promoting selective expansion of a preleukemic clone (Figure 2) because of proliferative advantage and increased likelihood for a second mutation required for the development of the disease to occur [33]. In turn, early childhood infections or vaccination may reduce the likelihood of leukemia [90]. Importantly, the major histocompatibility genes might play a role in the linkage between patterns of infection and leukemia risk, as several HLA haplotypes have been associated with childhood leukemia [3]. However, other studies have suggested that major histocompatibility complex-defined variation in immune-mediated response is unlikely to be a major risk factor [91].

Aberrant RAG activity resulting in genomic rearrangements may be a crucial secondary mechanism leading to B-cell ALL. Aberrant RAG activities can result in various oligoclonal V(D)J recombination events and the inactivation of genes required for B-lineage differentiation [87]. A clear link between RAG and childhood leukemia through inflammatory mechanisms has been recently reported [89], further connecting immune system-RAG-childhood leukemia.

6. Role of Acetylcholinesterase in Leukemogenesis

Moderate acetylcholine (ACh) levels are crucial for controlling immune and inflammatory functions in peripheral tissues. An increase in ACh above a certain threshold can suppress the production of pro-inflammatory cytokines. Acetylcholinesterase (AChE) contributes to regulating ACh levels and, thus, modulates inflammation [92]. In particular, ACh produced by the vagus nerve and/or by peripheral leukocytes [93] can potently modulate several classical immune reactions by activating the α7-nicotinic ACh receptor on the leukocyte membrane, which in turn blocks the nuclear factor kappa B (NF-κB)-mediated production of pro-inflammatory cytokines, such as IL1β and tumor necrosis factor alpha [92]. Because mesenchymal stromal/stem cells carry both nicotinic and muscarinic ACh receptors [94], niche-derived cholinergic signals may play a role in hematopoiesis by regulating proliferation and apoptosis of HSPCs undergoing erythroid and myeloid differentiation [95].

The *ACHE* gene includes multiple putative binding sites for hematopoietic transcription factors. Alternative splicing gives rise to "synaptic" (AChE-S) multimers, which control ACh levels in the brain and muscles, "erythrocyte" (AChE-E) dimers and stress-induced "read-through" (AChE-R) monomers [96]. AChE-R is involved in cell proliferation, whereas AChE-S can be induced during apoptosis [97]. Under stress responses, blood AChE-R undergoes C-terminal cleavage rendering a C-terminal peptide (ARP) of 55 kDa, which promotes the myeloproliferation and thrombopoiesis characteristics of cellular stress [98]. Because ARP functions as a hemopoietic growth factor promoting proliferation of $CD34^+$ HSPCs, circulating AChE-R and/or ARP might be involved in directing $CD34^+$ HSPCs towards prolonged granulocytosis [96]. Furthermore, *ACHE* has been reported to play a role in hematopoiesis by regulating proliferation, differentiation and apoptosis of erythroid and myeloid progenitors. This might explain, at least in part, the association of perturbations in *ACHE* gene expression with myeloid leukemia [99], particularly after exposure to anticholinesterase insecticides, such as OPs.

ACHE is located on chromosome 7q22 within a critical region subject to non-random chromosomal abnormalities. The remarkable abundance of SINEs (short interspersed elements), in particular Alu repeats, in the *ACHE* locus implies exceptional susceptibility to retrotransposition events, which are assisted by the existence of chromosomal breakages. Alu repeats also facilitate unequal crossing-over, altogether contributing to the instability of this region. Chromosomal rearrangements could result in the loss of upstream transcription factor binding sites and, thus, may affect *ACHE* gene expression under stress or exposure to anti-AChE agents. This explains the reported chromosomal aberrations involving 7q22 in leukemic patients [100]. The proximal promoter of the *ACHE* gene contains consensus motifs for the leukemia-associated factor AML1/Runx1 and c-fos, a transcription factor known to regulate *ACHE* gene expression under stress [101]. Hence, the loss of DNA on chromosome 7 may play a significant role in AML [95,96,97,98,99,100,101,102]. Furthermore, a study of 1880 children with ALL reported that 4% of them had DNA losses involving chromosome 7 [103].

A pivotal role of AChE has been suggested in apoptosis. While the 55-kDa AChE protein is selectively induced during apoptosis, its suppression inhibits apoptosome formation and rescues cells from apoptosis [104]. The 55-kDa AChE protein is negatively regulated by the activation of the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) pathway [104,105]. This signaling cascade is crucial to cell cycle progression, transcription, translation, differentiation, apoptosis, motility and metabolism [106]. The decrease in AChE activity and the consequent increased level of ACh could cause cholinergic overstimulation and enhance cell proliferation in lung cancer [97]; however, whether a similar effect can occur in leukemogenesis is unknown. On the other hand, AChE can hydrolyze lipid peroxides, raising the possibility that a reduction in enzyme activity increases oxidative stress and cellular damage [97].

7. Conclusions

Overall, there is sustained epidemiological evidence to suggest a risk of pediatric leukemia upon exposure (*in utero* and/or after birth) to some classes of pesticides, but scientific/mechanistic studies to definitively support this association are lacking. Pesticides may induce topoisomerase II inhibition or generation of oxidative stress,

consistently leading to misrepaired DNA cleavage and further chromosomal aberrations in HSPCs. This early molecular event might be sufficient for triggering infant leukemia, but not childhood leukemia, which requires further postnatal events for overt disease. The combination of epidemiological and case-based genomic studies together with cell biology analyses would be useful to elucidate the etiology of pediatric leukemia. In particular, this approach would help to better understand the biological and genetic evidence that is pertinent to the mechanisms by which pesticides might impact on the risk of pediatric leukemia.

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Author Contributions

Antonio F. Hernández and Pablo Menéndez conceived of and wrote the review.

Conflicts of Interest

The authors declare no conflict of interest.

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Brain anomalies in children exposed prenatally to a common organophosphate pesticide.

Rauh VA[1], Perera FP, Horton MK, Whyatt RM, Bansal R, Hao X, Liu J, Barr DB, Slotkin TA, Peterson BS.

Author information

Abstract

Prenatal exposure to chlorpyrifos (CPF), an organophosphate insecticide, is associated with neurobehavioral deficits in humans and animal models. We investigated associations between CPF exposure and brain morphology using magnetic resonance imaging in 40 children, 5.9-11.2 y, selected from a nonclinical, representative community-based cohort. Twenty high-exposure children (upper tertile of CPF concentrations in umbilical cord blood) were compared with 20 low-exposure children on cortical surface features; all participants had minimal prenatal exposure to environmental tobacco smoke and polycyclic aromatic hydrocarbons. High CPF exposure was associated with enlargement of superior temporal, posterior middle temporal, and inferior postcentral gyri bilaterally, and enlarged superior frontal gyrus, gyrus rectus, cuneus, and precuneus along the mesial wall of the right hemisphere. Group differences were derived from exposure effects on underlying white matter. A significant exposure × IQ interaction was derived from CPF disruption of normal IQ associations with surface measures in low-exposure children. In preliminary analyses, high-exposure children did not show expected sex differences in the right inferior parietal lobule and superior marginal gyrus, and displayed reversal of sex differences in the right mesial superior frontal gyrus, consistent with disruption by CPF of normal behavioral sexual dimorphisms reported in animal models. High-exposure children also showed frontal and parietal cortical thinning, and an inverse dose-response relationship between CPF and cortical thickness. This study reports significant associations of prenatal exposure to a widely used environmental neurotoxicant, at standard use levels, with structural changes in the developing human brain.

Comment in

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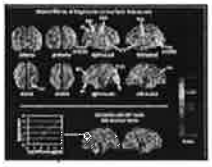

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Chlorpyrifos Induces *MLL* Translocations Through Caspase 3-Dependent Genomic Instability and Topoisomerase II Inhibition in Human Fetal Liver Hematopoietic Stem Cells

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ABSTRACT

Household pesticide exposure during pregnancy has been associated with a more than 2-fold increased risk in infant leukemia, and chlorpyrifos (CPF) is among the most frequently applied insecticides. During early fetal development, liver is a hematopoietic organ with majority of cells being $CD34^+$ hematopoietic stem cells ($CD34^+$HSC). The *in utero* injury to $CD34^+$HSC has been known to underlie the pathogenesis of several blood disorders, often involving rearrangements of the mixed-lineage leukemia (*MLL*) gene on 11q23. In this study, we evaluated the leukemogenic potential of CPF in human fetal liver-derived $CD34^+$HSC. Specifically, exposure to 10 μM CPF led to decrease in viability, inhibition in proliferation and induction of DNA double-strand breaks (DSBs) and occurrence of MLL^+ rearrangements. In particular, we observed CPF-mediated cell cycle disturbance as shown by G0/G1 arrest, in contrast to etoposide (VP-16), an anticancer drug used as a positive control and known to induce G2/M arrest. Further study on mechanisms underlying DNA DSBs and MLL^+ rearrangements revealed that CPF might act as topoisomerase II poison, a mechanism of action similar to VP-16. On the other hand, CPF was also shown to induce early apoptosis through active caspase-3 activation, a pathway known to underlie DNA DSBs and MLL^+ translocations. Our data indicate that *in utero* injury of $CD34^+$HSC by CPF may contribute to the increased risk of infant leukemia. Future work will elucidate the mechanism and the type of CPF-induced MLL^+ translocations in HSC.

Key words: chlorpyrifos; hematopoietic stem cells; *MLL* rearrangement; topoisomerase II; DNA double strand break; apoptosis

ABBREVIATIONS:

CPF,	chlorpyrifos;
VP-16,	etoposide;
QT,	quercetin;
MB,	merbarone;
HSC,	hematopoietic stem cells;
MLL gene,	mixed-lineage leukemia gene;
BCR,	breakpoint cluster region;
IAL,	infant acute leukemia;
DSB,	double-strand break;
Topo II,	topoisomerase II;
TARDIS,	trapped in agarose DNA immunostaining;
LDH,	Lactate dehydrogenase;
CAD,	caspase-activated DNase;
ROS,	reactive oxygen species;
DDR,	DNA damage response;
D-NHEJ,	DNA–PK dependent nonhomologous end joining;
HRR,	homologous recombination repair;
PBS,	phosphate-buffered saline;
DMSO,	dimethyl sulfoxide.

Chlorpyrifos (CPF, O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), as an important organophosphate (OP) insecticide, has been extensively used in residential and indoor pest control, especially for cockroaches and termites. Epidemiological studies have suggested that maternal exposure to certain household pesticides during pregnancy may increase the risk of childhood leukemia developed after birth (Daniels *et al.*, 1997; Infante-Rivard *et al.*, 1999; Ma *et al.*, 2002; Turner *et al.*, 2010; Zahm and Ward, 1998). Although the results in these studies are limited due to a broad spectrum of pesticides, with no specific pesticides being identified to be directly associated with the risk of leukemia, due to the fact that CPF is one of the most frequently used insecticides in the study areas, it may be one of main contributors to the increased risk of childhood leukemia after birth.

Of the childhood leukemia, some cases are diagnosed within one year of life, termed 'Infant acute leukemia' (IAL). IAL is characterized by a short latency, lack of a pre-leukogenic phase, and poor prognosis. Approximately 75% of infants with acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL) have cells that display a characteristic chromosome translocation involving the mixed lineage leukemia, or myeloid/lymphoid leukemia (*MLL*) gene located on 11q23 (Cimino *et al.*, 1993; Thirman *et al.*, 1993). The human *MLL* gene (also known as HRX, ALL-1, and HTRX1), which is a homolog of the Drosophila trithorax gene, may play an important role in normal hematopoietic development and differentiation (Ernst *et al.*, 2002).

MLL rearrangements usually occur within an 8.3-kb breakpoint cluster region (BCR) with many translocation partner genes (Meyer *et al.*, 2013). Retrospective analysis of *MLL* rearranged sequence in neonatal blood spots from Guthrie cards of children afflicted with leukemia indicates an *in utero* origin of the *MLL* rearrangements (Hjalgrim *et al.*, 2002; Yagi *et al.*, 2000). The high concordance rate in monozygotic twins to develop infant leukemia and short disease onset suggest that *MLL* abnormalities in the appropriate human fetal hematopoietic stem cells (HSC) may be the molecular basis for infant leukemia (Gale *et al.*, 1997). Accordingly, the initiation of molecular injuries in uncommitted hematopoietic progenitors during fetal development may predispose the offspring to the risk of IAL after birth.

It was agreed that the bone marrow-derived HSC are the target in benzene-mediated leukemia (Snyder, 2012); however, little is known regarding the *in utero* cell-specific origins of IAL. During early fetal development, the liver is the primary hematopoietic organ in which a large proportion of hepatic cell populations are comprised of HSC. HSC are capable of initiating long-term hematopoiesis, and therefore genetic injuries to these cells through maternal exposure to certain chemicals may be manifested with hematopoietic disorders after birth. Maternal exposure to certain pharmaceutical drugs, pesticides, and dietary agents has been associated with the increased risk for IAL in offspring (Barjesteh van Waalwijk van Doorn-Khosrovani *et al.*, 2007; Ma *et al.*, 2002; Shaw *et al.*, 2004; Spector *et al.*, 2005).

We have previously demonstrated that human fetal liver derived $CD34^+$HSC are the most relevant model in studies of diseases with origins during pregnancy, due to the distinct physiological nature of these cells coupled with their unique *in utero* residing microenvironment (Moneypenny and Gallagher, 2005; Moneypenny *et al.*, 2006; Shao *et al.*, 2006, 2007). $CD34^+$HSC are a cell population with functional heterogeneity and variability, involving stem cells and progenitor cells that retain both pluripotency and differentiation potential (a proportion of these cells are in cell cycle or in G0 phase/dormant cells) (Passegué *et al.*, 2005). Our studies have suggested that human fetal liver $CD34^+$HSC have a relatively low constitutive cytochrome P450 biotransformation capacity (Shao *et al.*, 2006), and are more sensitive to oxidative stress induced by certain environmental chemicals such as 4-HNE and PBDEs (Moneypenny and Gallagher, 2005; Shao *et al.*, 2007). In fact, injury to fetal liver $CD34^+$HSC during pregnancy has been proposed to underlie the development of certain blood disorders manifested after birth (Alexander *et al.*, 2001; Shu, 1997; Tavassoli, 1991; Woodruff, 2004).

The *MLL* gene rearrangements occur not only *in utero*, and also in adult de novo leukemia following treatment with DNA topoisomerase II (Topo II) inhibitors, such as etoposide (VP-16), suggesting a similar mechanism of DNA damage. Epidemiological studies have also shown that maternal dietary consumption of Topo II inhibitors may increase the risk of *MLL*-associated IAL (Spector *et al.*, 2005). In addition, it has been shown in our previous study that exposure to VP-16 can induce *MLL* mispairing in human fetal liver $CD34^+$HSC (Moneypenny *et al.*, 2006). Collectively, these data may raise the question whether exposure to certain pesticides during pregnancy can induce *MLL* rearrangements in hematopoietic precursors, which are consistent with the role for dietary Topo II inhibitors in infant leukemia etiology.

To provide direct evidence for the potential of CPF to cause leukemia-associated *MLL* translocations, a preliminary action was to test a series of widely used pesticides (from the NCCLS inventory) for the potential to induce *MLL* translocations (Daniels *et al.*, 1997). By an *in vitro* Topo II inhibition assay using a purified Topo II (TopoGen), CPF and chlorpyrifos oxon (CPO) expressed a much stronger inhibitory effect than VP-16 on Topo II activity (data not published). In the current project, we further examine the clastogenic effect of CPF in human fetal liver $CD34^+$HSC. The ability of CPF to induce *MLL* gene rearrangements is compared with that of a model Topo II poison and

known inducer of *MLL* recombinations in humans, VP-16 (Moneypenny *et al.*, 2006). Based on the evidence that most *MLL*$^+$-leukemia originate in CD34$^+$ precursors, fetal liver derived CD34$^+$HSC would be the most relevant *in vitro* model for studying the clastogenic and leukemogenic effects of CPF. For the first time, our data demonstrate that human fetal liver CD34$^+$HSC are susceptible to CPF-mediated chromosomal translocations similar to those associated with IAL.

MATERIALS AND METHODS

Reagents. Iscove's Modified Dulbecco's media (IMDM), penicillin, streptomycin, and heat-inactivated fetal bovine serum (FBS) were purchased from HyClone Thermo (Carlsbad, California). Recombinant human stem cell factor (SCF), recombinant human granulocyte colony stimulating factor (G-CSF), and recombinant human interleukin 3 (IL-3) were obtained from Prospec-Tany TechnoGene Ltd (Rehovot, Israel). Vented culture flasks were purchased from Nest (Jiangsu, China). CD34$^+$ isolation columns were purchased from Miltenyi Biotec (Auburn, California). Histopaque©-1977 was obtained from Sigma (St Louis, Missouri). Bovine serum albumin was purchased from Roche (Basel, Switzerland). Amphotericin B-fungizone and gentamicin Sulfate were purchased from Amresco (Solon, Ohio). Hoechst dye and 40,6-diamidino-2-phenylindole were purchased from Molecular Probes (Eugene, Oregon). The *MLL* locus-specific DNA probe was obtained from Vysis (Downers Grove, Illinois). The DNA topoisomerase II assay kit was purchased from TopoGEN (Columbus, Ohio). Topo IIα-specific (Cat: 20233-1-AP) and TOP IIβ-specific (Cat: 20549-1-AP) polyclonal antibodies (both raised in rabbits), and peroxidase-conjugated affinipure goat anti-rabbit IgG(H+L) secondary antibody (Cat: SA00001-2) were purchased from ProteinTech Group, China Branch (Wuhan, China). Cell cycle, LDH cytotoxicity assay kit, apoptosis analysis kit, DNA ladder (BeyoRed) marker, DNA ladder extraction kit, Caspase-3 activity assay kit, Caspase inhibitor Z-VAD-FMK (20 mM), Bradford protein assay kit were purchased from Beyotime Institute of Biotechnology (Nanjing, China). CPF (98.5%, C 11600000 and Lot 10222) was purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). VP-16, quercetin (QT) and merbarone (MB) were purchased from Sigma Chemical (St Louis, Missouri). Ampicillin trihydrate (≥96%, Cat: 1016024) and n-butyl chloride (≥99%, Cat: 1020970) of analytical grade were purchased from Xiya Chemical Co. Ltd. (Chengdu, China). AlamarBlue was purchased from Beijing CellChip Biotechnology CO., Ltd (Beijing, China). Trypan blue was purchased from Solarbio (Beijing, China). ASC grade dimethyl sulfoxide (DMSO) and Agarose L.M.P were purchased from Amresco LLC (Solon, Ohio). Agarose N.M.P of molecular biology grade was purchased from Invitrogen (Life Technologies Co., Grand Island, New York).

Human fetal livers and CD34$^+$HSC isolation. All use of human tissues was approved by the Dalian Medical University (DMU) Ethics Committee and the specimens were provided by the DMU Medical Center on the informed consent of the participants. Primary CD34$^+$HSC (>95% purity) were isolated from human fetal livers which were typically 16–21 weeks of gestational age, as described in our previous studies (Shao *et al.*, 2006, 2007). Briefly, the fetal liver tissue was dissociated under sterile conditions, and the total cell crude (including hepatocyte and non-hepatocyte fractions) were repeatedly washed in 1 × phosphate-buffered saline (PBS) supplemented with 0.3% bovine serum albumin, 2.5 μg/ml amphotericin B-fungizone and 50 μg/ml gentamicin sulfate. The cell mixture was centrifuged over 1.077 g/ml Histopaque©-1977 at 400 g for 30 min at room temperature and the mononuclear layer was collected. The CD34$^+$HSC were acquired by repeatedly enriching with magnetic bead separation (Miltenyi Biotec). Cells were seeded at approximately 6250 cells/ml of IMDM supplemented with 15% FBS, 20 ng/ml SCF, 2 ng/ml IL-3, 1 ng/ml G-CSF, 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37°C in 95% O_2/5% CO_2 for 14 days. The cell amplification and enumeration of live and dead HSC were monitored over the culture period using Trypan blue exclusion (Shao *et al.*, 2007). On day 7, cells were treated with different concentrations of CPF for analysis. Vehicle control consisted of DMSO (0.1% v/v, denoted as '0 μM CPF' in the Results section) and positive control consisted of VP-16 known to induce *MLL* gene rearrangements (Barjesteh van Waalwijk van Doorn-Khosrovani *et al.*, 2007).

Cell viability and proliferation. In a 6-well culture plate, HSC were treated with CPF for 24 h in culture at concentrations ranging from 0 to 100 μM, and the cell viability was assessed by Trypan blue exclusion. For the cell proliferation assay, HSC were exposed to a single acute dose of CPF, VP-16, or vehicle, for 24 h. The cells were then placed in fresh culture medium and the cell growth was monitored over 168 h post-exposure. At each time point, cells at 10 000 cells/200 μl were transferred to a 96-well plate and incubated with 20 μl AlamarBlue (10%) for 12 h, a predetermined optimal time for evaluating treatment-related effects on cell viability. The change in AlamarBlue reduction was measured at 565 nm (reference 600 nm) on a microplate reader (Multiscan Ascent, Thermo Fisher Scientific, Waltham, Massachusetts).

Lactate dehydrogenase cytotoxicity assay. The quantification of plasma membrane damage is typically used to determine cell death or cytotoxicity. When the integrity of plasma membrane is compromised, lactate dehydrogenase (LDH), a stable cytoplasmic enzyme present in all cells, is rapidly released into the cell culture supernatant. In this study, CPF-mediated cytotoxicity was examined based on the measurement of activity of LDH released from damaged cells using a commercial LDH-cytotoxicity assay kit (refer to 'Supplementary Materials' for more details).

Cell cycle analysis. The cell cycle effect of CPF on HSC was examined using PI stain (Krishan, 1975). Briefly, HSC at 1×10^6/ml were treated with CPF at 0, 1, 10, and 50 μM for 24 h in culture, harvested, and washed twice with ice-cold PBS. The cells were fixed/permeablized with 70% ethanol overnight at 4°C, washed twice with PBS at 1000 g for 5 min, and then stained with PI (50 μg/ml) and Rnase-A (100 mg/ml) for 2 h at room temperature in the dark per the manufacturer's instructions (Beyotime Institute of Biotechnology, Nanjing, China). The cell cycle evaluation was performed with a BD Biosciences FACSCalibur flow cytometer at 488 em/630 ex with 10 000 events recorded for each condition, and the data were analyzed using MOD FIT software (Verity Software House, Topsham, Maine).

Analysis of active caspase-3. Caspase-3 is a key protease that is brought into action from its inactive state during early apoptotic stages (Hars *et al.*, 2006). Like other members in the caspase family, it is synthesized to be a pro-enzyme and is activated in cells undergoing apoptosis by self-proteolysis and/or cleavage by the other protease. Active caspase-3 then takes a role in driving downstream apoptotic events by proteolytically cleaving and activating other caspases, as well as relevant targets in the cytoplasm (eg, Bcl-2 and D4-GDI) and in the nucleus (eg, CAD

and PARP), leading to downstream events. Caspase-3 activity can be monitored using a caspase-3 activity kit (C1115, Beyotime, China, refer to 'Supplementary Materials' for more details).

DNA fragmentation. DNA fragmentation occurs in two stages during apoptosis (Wyllie, 1980). The initial stage is the formation of high molecular weight (HMW) fragmentation by an endonucleolytic activity that cleaves DNA into 50–300 kb fragments. This degree of cleavage is sufficient to cause the chromatin to undergo condensation. The second stage is the formation of internucleosomal DNA fragmentation (DNA laddering) catalyzed by an endonucleolytic activity distinct from that causing HMW fragmentation, resulting in DNA fragments that are multiples of 180–185 bp in length (refer to 'Supplementary Materials' for more details).

Neutral Comet assay. The potential for CPF to induce double strand DNA damage was determined using neutral Comet assay specific for the detection of double strand break (DSB) formation (Olive *et al.*, 1991). HSC were treated with CPF at 0, 1, 10, and 50 μM, along with 10 μM VP-16 for 24 h in culture, and gently suspended in 0.5% of low-melting point agarose at 4×10^5/ml at 37°C and scattered on microscope slides coated with 1.5% normal melting point agarose (Invitrogen). The slides were allowed to solidify for 30 min at 4°C under cover slips and then incubated overnight at 4°C in lysis buffer containing 2.5 M NaCl, 100 mM ethylene diaminetetraacetic acid, 10 mM Tris, 250 mM NaOH, 1% sodium lauryl sarcosinate, 10% DMSO and 1% Triton X-100, pH = 10). The slides were subsequently subjected to electrophoresis (25 V, 300 mA,) at 4°C for 20 min (buffer: Tris 90 mM, boric acid 90 mM and ethylene diaminetetraacetic acid 2 mM, pH = 7.5), fixed with 100% ethanol and stained with 20 μg/ml EB. The cells were then scored with fluorescence microscopy (Olympus, U-RFLT50). The assay was performed three times and 50 cells were scored per slide. The percentage of DNA in the tail was measured for statistical analysis of DSB induction using the CASP software (Comet Assay Software Project Lab http://www.cometassay.com).

MLL-associated gene rearrangements. The presence of *MLL* translocations was detected using the fluorescence *in situ* hybridization (FISH) and a dual color (orange and green) DNA probe (Vysis, Abbott Molecular Inc, Illinois) as described previously (Moneypenny *et al.*, 2006). Cells with normal *MLL* structure yield two orange/green fusion signals, whereas those with *MLL* rearrangements exhibits one orange/green fusion signal and distinct green and orange signals (Moneypenny *et al.*, 2006; Yamamoto *et al.*, 2004). In the occurrence of a large deletion, distally from the *MLL* breakpoint, one of the two orange signals can be eliminated, generating one fusion signal and one isolated green signal, reflecting a concomitant translocation and deletion (refer to 'Supplementary Materials' for more details).

DNA Topo II activity assay. The ability of CPF to inhibit DNA Topo II isolated from human fetal liver HSC was determined with a Human Topoisomerase II Assay Kit (TopoGen), specific for assessing the activity of eukaryotic Topo II, based upon the decatenation of kinetoplast DNA (kDNA).

In this assay, 1 μl nuclear extract from human fetal liver HSC (containing Topo II, refer to 'Supplementary Materials' for 'Nuclear extract preparation') was incubated with reaction buffer (10 mM Tris [pH 7.9], 50 mM NaCl, 50 mM $MgCl_2$, 100 mM EDTA, 0.015 mg/ml BSA, and 1 mM ATP) and kDNA (TopoGen), in the presence of CPF (0, 1, 10, 50, and 100 μM) or VP-16 (50 and 100 μM) for 1 h at 30°C. The DNA was fractionated by electrophoresis in 1% agarose (containing 0.5 μg/ml ethidium bromide) to separate the decatenated products containing nicked open circular and covalently closed circular (relaxed) minicircle DNA. Linear DNA (reflecting 'κDNA degradation') migrates between the nicked and relaxed species. Linear and decatenated κDNA markers (TopoGen) were used to identify positions of different isomers. The gels were visualized by UV illumination and photographed. The densitometric scanning of different isomers in the gels, reflecting the degree of Topo II inhibition, was analyzed using a BioRad Fluor-S imaging system.

Stabilization of Topo II cleavage complex by trapped in agarose DNA immunostaining assay. The trapped in agarose DNA immunostaining (TARDIS) assay is a well accepted cell-based assay that is specific for visualization of inhibitor-DNA-Topo II ternary complexes in individual cells rather than a cell-free system (Padget *et al.*, 2000; Willmore *et al.*, 1998). In this study, CPF-mediated stabilization of Topo II cleavage complexes in human fetal liver HSC was further validated quantitatively using the TARDIS assay. Briefly, exponentially growing cells (approximately 4×10^5/ml) were treated with CPF (0, 10, and 50 μM) or VP-16 (10 and 50 μM) for 1 h and embedded in agarose on microscope slides before staining with antibodies against Topo IIα or Topo IIβ, and Hoechst 33258 (10 mM in PBS) for nuclei localization (refer to 'Supplementary Materials' for details on 'Agarose embedding and staining'). The blue (Hoechst-stained DNA) fluorescence and the red (Rhodamine-stained Topo IIα or Topo IIβ) immunofluorescence were visualized separately on an epifluorescence microscope (Olympus IX81; Olympus U-HGLGPS [130w]) and appropriate sets of optical filters (Omega Optical, Inc.).

Fluorescent TARDIS images were analyzed with ImagePro plus6.0 (Media Cybernetics, USA) according to Willmore *et al.* (1998) (refer to 'Supplementary Materials' for more details on 'Quantitative fluorescence microscopy and image analysis'). Briefly, 5 pairs of images of randomized fields of view per treatment were captured from replicate slides giving rise to approximately 100 cells for each dose (Olympus DP73). All images were subject to background correction and blue and red shade correction. The corrected images for Hoechst were used to define the areas occupied by the DNA for each cell. Valid objects were those defined by the computer software that did not touch the edge of the image. Objects consisting of more than one cell were excluded from this analysis.

Examination on the specificity of the test systems using non-genotoxic compounds. To test the specificity of the assay system in this study, the neutral Comet assay, the dual-color FISH analysis and the TARDIS assay were assessed using two classical non-genotoxic controls, ampicillin trihydrate and n-butyl chloride, along with VP-16 as a positive control. Ampicillin trihydrate was listed as negative in *in vivo* genotoxicity tests, non-genotoxic in *in vitro* mammalian cell tests for up to 5000 μg/ml (≈12.4 mM), and non-carcinogenic in rat and mouse models (Kirkland *et al.*, 2008). A typical testing range for ampicillin trihydrate is 156–5000 μg (0.3–12.4 mM), therefore, we have chosen 0.5, 12.4, and 25 mM for specificity experiments (Kirkland *et al.*, 2008; Mitchell *et al.*, 1997). N-butyl chloride was listed as no data in *in vivo* genotoxicity tests, non-genotoxic in *in vitro* mammalian cell tests for up to 5000 μg/ml (≈54 mM), and non-carcinogenic in rat and mouse models (Kirkland *et al.*, 2008). A typical testing range for n-butyl chloride is 31.25–5000 μg (0.3–54 mM), therefore, we have chosen 0.5 and 54 mM for specificity experiments (Kirkland *et al.*, 2008; Mitchell *et al.*, 1997).

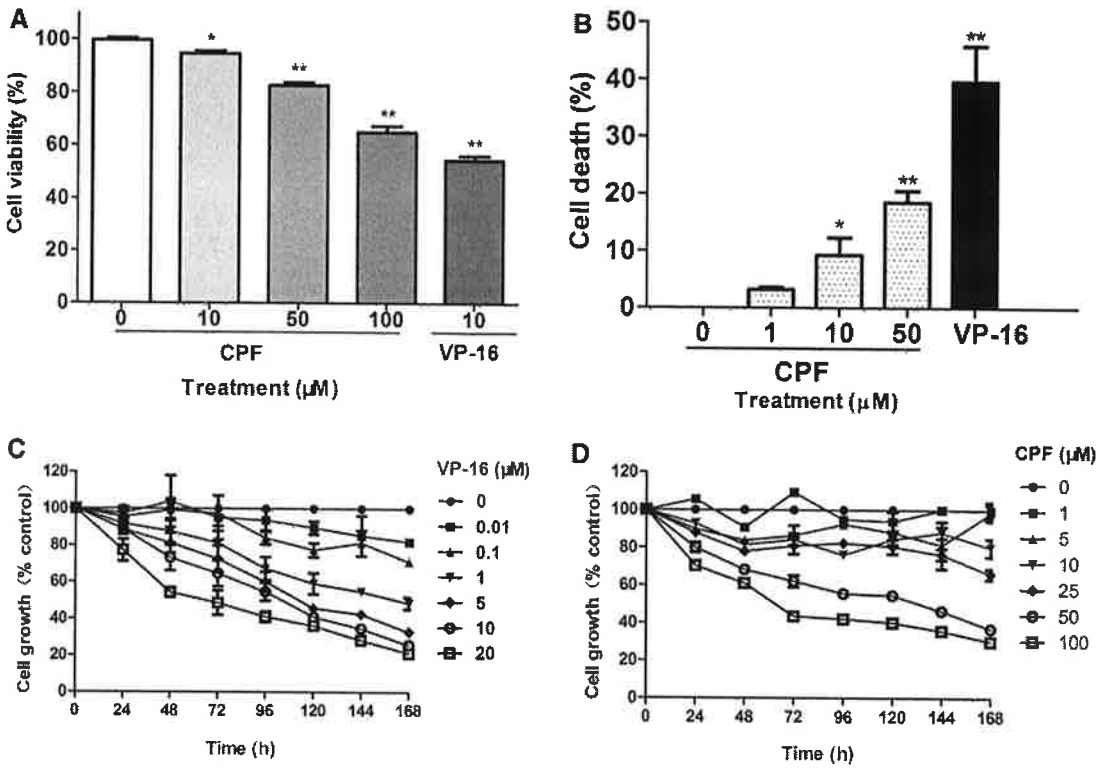

FIG. 1. CPF reduces viability and induces cytotoxicity in human fetal liver HSC. A, shows the cell viability as determined by Trypan blue exclusion. B, shows the cytotoxicity as measured by LDH release assay. C, VP-16 induces a dose- and time-dependent inhibition on cell growth at levels ≥0.1 μM as measured by AlamarBlue reduction assay. D, CPF induces a dose- and time-dependent inhibition on cell growth, especially at levels ≥ 25 μM. Data represent the mean ± SEM of 3 experiments. $^{*}p < .05$, $^{**}p < .01$.

Statistical analysis. All data on cell viability and proliferation represent the mean ± SEM of 3 experiments with each experiment performed in triplicate. Flow cytometry data represent the mean ± SEM of 3 replicates of 10 000 events. The significance of CPF on cell viability, cell proliferation, active caspase-3, cell cycle, and DSBs in human fetal live HSC were determined using one-way analysis of variance by SPSS (SPSS11.5, Chicago, Illinois). FISH data were analyzed using χ^2 analysis. Treatment related effects were considered significant at $p \leq .05$.

RESULTS

CPF Reduces Cell Viability and Inhibits Cell Proliferation in Human Fetal Liver HSC

To evaluate the cytotoxicity of CPF to the HSC cell model, viability of cells was assessed by Trypan blue exclusion after exposure to various CPF concentrations (0, 10, 50, and 100 μM) for 24 h (Fig. 1A). Although 10 μM VP-16 caused a quite amount of cell death (54.67%), the percentage of viable cells at 10, 50, and 100 μM CPF was 95.35, 84.10, and 64.88%, respectively. An additional analysis on CPF-induced cell death using LDH cytotoxicity assay revealed that CPF at 1, 10, 50 μM led to 3.18, 9.28, and 18.57 increase in LDH release, respectively, in a dose-dependent manner, while 10 μM VP-16 caused 39.69% cell death (Fig. 1B).

CPF was previously described as an anti-proliferative agent in other cell models (Slotkin *et al.*, 2007). The effect of CPF on cell growth was also examined in HSC. Because 100 μM CPF induced only moderate cell death, a broader range of CPF concentrations were employed in this experiment. HSC were exposure to a single dose of CPF for 24 h over a range of 1–100 μM, or VP-16 over a range of 0.001–20 μM and then allowed to recover in fresh culture medium. The rate of cell growth was monitored over the 7-day course by addition of 10% AlamarBlue followed by fluorescent measurement of AlamarBlue reduction. As observed, VP-16 ≥ 0.1 μM (Fig. 1C) and CPF ≥ 25 μM (Fig. 1D) elicited a dose-dependent decrease in HSC proliferation at all time points. Although the overall rate of cell growth was consistently reduced throughout the 7-day period, the dramatic decrease occurred within 4 days post-exposure and slowed down at 5 days post-exposure for both VP-16- and CPF-treated cells.

CPF Induces Cell Cycle Arrest in Human Fetal Liver HSC

The inhibitory effect of CPF on HSC proliferation may be due to its ability to modulate cell cycle. Therefore, the effect of CPF on the distribution of HSC in the cell cycle was characterized using flow cytometry (Fig. 2 and Table 1). When the typical cell cycle distribution in HSC is 34.28% in G0/G1 phase, 63.09% in S phase, and 2.64% in G2/M phase, exposure to 10 μM VP-16 induces massive cell cycle arrest at the G2/M phase (54.01%) and dramatic reduction in G0/G1 (10.42%) and S phase (35.57%), consistent with our previous findings (Moneypenny *et al.*, 2006). In contrast, CPF induces a G0/G1 cell cycle arrest with a concentration-dependency. For example, exposure to 50 μM CPF dramatically shuffled the cells in the cell cycle compartments as evidenced by a considerable accumulation of cells in G0/G1 phase (58.50%), and a profound reduction of cells in S phase (31.79%). In addition, CPF has also led to an increasing number of cells, though not statistically significant, arrested in G2/M compartment (9.71%). Further analysis on S-phase promoting factor (SPF) and proliferation index (PI) suggested the inhibitory effects of both CPF and VP-16 on DNA synthesis and cell growth, consistent with the studies on cell cycle arrest and cell proliferation (Table 1).

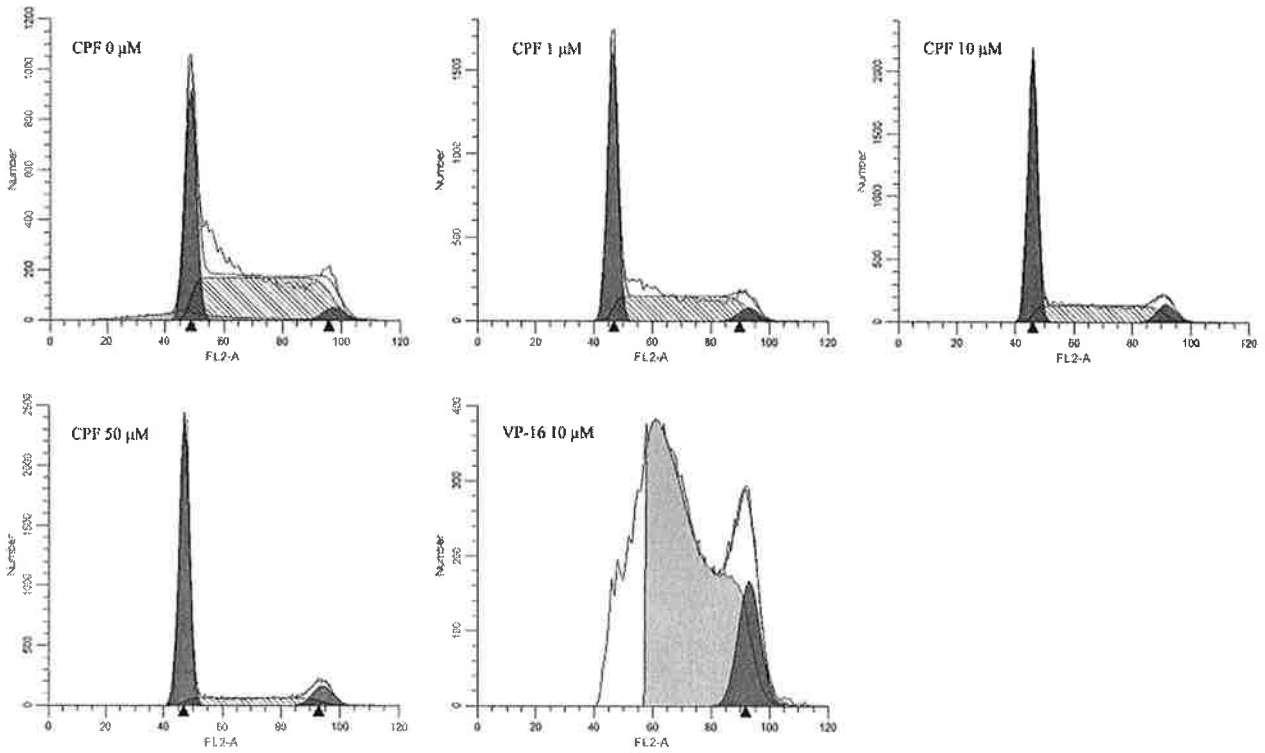

FIG. 2. CPF induces cell cycle arrest in human fetal liver HSC. HSC at 1×10^6/ml were treated with CPF at 0, 1, 10, and 50 μM for 24 h in culture, and the effect of CPF on the distribution of HSC in the cell cycle was characterized using flow cytometry by counting 10 000 events. The data interpretation of CPF on cell cycle distribution were conducted using a MOD FIT software. The statistical variations are summarized in Table 1.

TABLE 1. Disturbance of Cell Cycle by CPF in Human Fetal Liver HSC

	G0/G1	S	G2/M	SPF	PI
0	34.28 ± 4.01	63.09 ± 4.38	2.64 ± 0.99	0.63 ± 0.04	0.66 ± 0.04
1	44.50 ± 4.15	50.34 ± 3.75	5.15 ± 0.45	0.50 ± 0.04	0.56 ± 0.04
10	54.63 ± 1.69**	36.86 ± 4.48	8.51 ± 2.91	0.37 ± 0.04	0.45 ± 0.02**
50	58.50 ± 9.91**	31.79 ± 11.55*	9.71 ± 4.60	0.32 ± 0.12*	0.42 ± 0.10**
VP-16	10.42 ± 4.84**	35.57 ± 20.98*	54.01 ± 16.48**	0.36 ± 0.21*	0.90 ± 0.05**

Data represent the mean ± SEM of three replicates of 10 000 flow cytometric events.
*$p < .05$; **$p < .01$.

CPF Induces DNA Damage and MLL Gene Rearrangements in Human Fetal Liver HSC

The CPF-mediated DSB formation, as measured by neutral Comet assay, was compared with VP-16, known to induce DNA DSB formation in human fetal liver HSC (Moneypenny *et al.*, 2006). As shown in Figure 3A, 10 μM VP-16 induced a dramatic increase in DNA tails relative to DMSO control and exposure to CPF led to an increase in DNA tails with a dose-dependent manner. The quantification of DSB formation revealed that CPF at 1, 10, and 50 μM, or VP-16 at 10 μM, led to significant increase in the '% DNA in the tail' (Table 2). In particular, 1 μM CPF was sufficient to cause DSBs, indicating that CPF is a strong inducer of DSBs in human fetal liver HSC.

To test specificity of the assay, the data of CPF-mediated DSB formation were verified by including two known non-genotoxic chemicals in mammalian cells, ampicillin trihydrate (0.5, 12.4, and 25 mM) and n-butyl chloride (0.5 and 54 mM), following the same procedure as in Figure 3A. As demonstrated in Figures 3B and C, both non-genotoxic compounds within the testing range (ie, the cytotoxic level) were not able to induce DSBs, while VP-16 led to a dose-dependent increase in the Comet tails. However, ampicillin trihydrate at 25 mM (twice its upper range) can cause a moderate level of DSB formation.

Because CPF can cause DNA damage in human fetal liver HSC, the occurrence of *MLL* translocations due to error prone DSB processing was then investigated (Fig. 4 and Table 3). HSC were exposed to a single dose of DMSO (0.1% v/v), CPF of 1 and 10 μM or 1 μM VP-16 for 24 h and allowed recovery in culture. The presence of *MLL* rearrangements was determined in a subset of HSC using dual-color FISH analysis at 24 h, 72 h, and 7 days post-exposure. Throughout the 7-day period, control cells maintained normal *MLL* structure as reflected by 2 orange/green fusion signals. HSC exposed to 1 μM VP-16 showed *MLL* rearrangements as revealed by the presence of distinct green and orange signals, and the number of cells positive for *MLL* rearrangements persisted over 7-day culture period, with 4/100 (4%) at 24 h, 6/100 (6%) at 72 h, and 10/100 (10%) cells on day 7. Similarly, 1 μM CPF was found sufficient to elicit *MLL* rearrangements, as evidenced by the detection of a split signal in 1/100

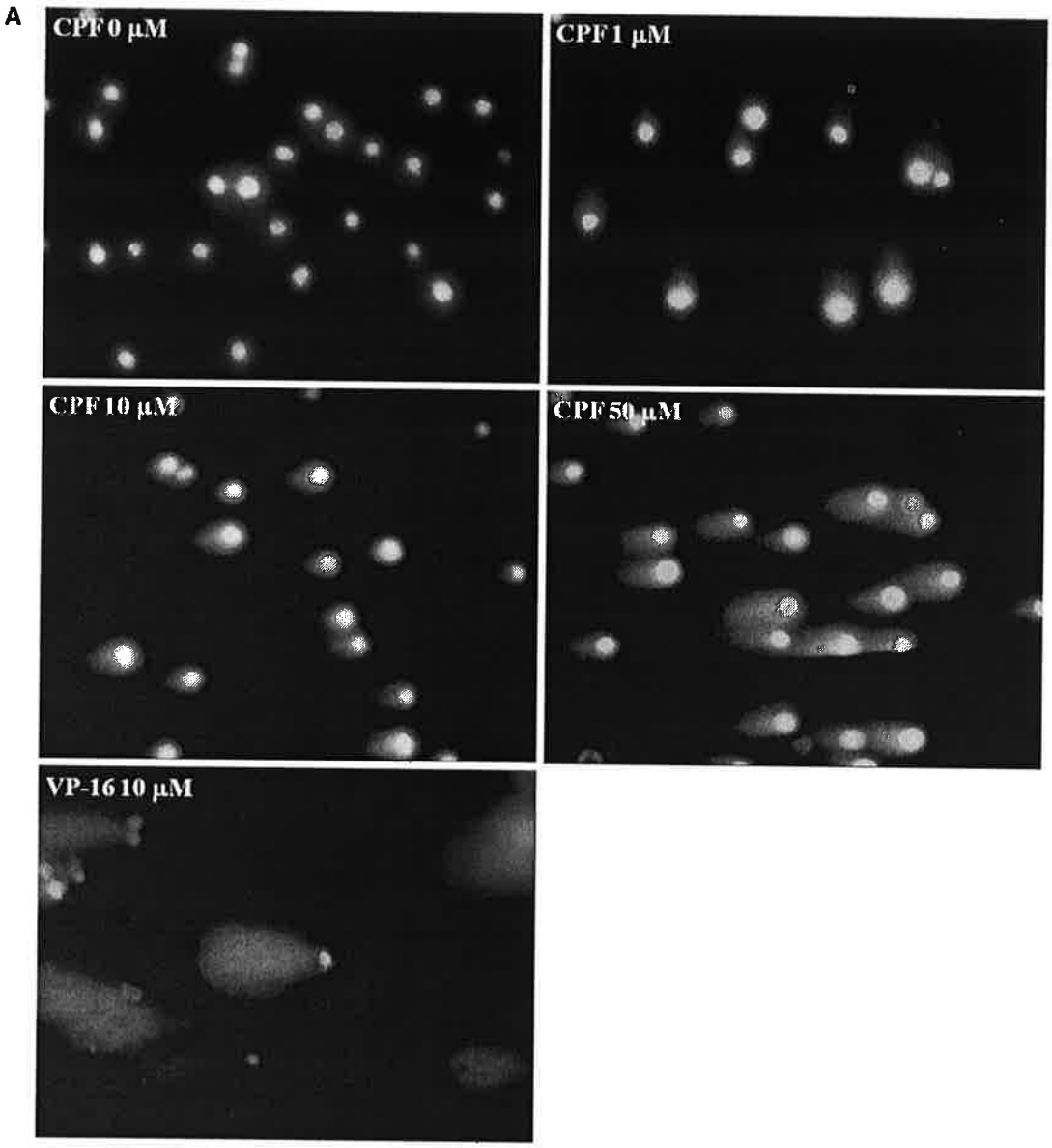

FIG. 3. CPF induces DNA DSBs in human fetal liver HSC. A, HSC were treated with CPF at 0, 1, 10, and 50 μM, or 10 μM VP-16 for 24 h in culture, and the DNA tailing was measured by neutral Comet assay. The statistical variations are summarized in Table 2. B, The specificity of the neutral Comet assay was verified by including non-genotoxic controls, ampicillin trihydrate at 0.5, 12.4, and 25 mM and n-butyl chloride at 0.5 and 54 mM, in comparison with the positive control, VP-16 at 10 and 50 μM. As shown, ampicillin trihydrate and n-butyl chloride, when within the testing range (Mitchell *et al.*, 1997), did not induce DSBs; however, ampicillin trihydrate of 25 mM (a dose twice its upper range) was able to cause a moderate level of DSB formation. C, The statistical analysis of the result in Figure 3B, by the % DNA in tail and tail moment. Data represent the mean ± SEM of three experiments. $^{***}p < .001$.

(1%) at 72 h and 2/100 (2%) cells on day 7. Exposure to 10 μM CPF resulted in an increased number of cells scored positive for *MLL* rearrangements and at earlier time point, ie, 3/100 (3%) at 24 h, 5/100 (5%) at 72 h, and 7/100 (7%) cells on 7 days post-exposure.

In this study, we also detected one fusion signal and one isolated green signal in some HSC, reflecting CPF-mediated concomitant occurrence of *MLL* rearrangements and deletions. Loss of an orange signal was detected in both VP-16 and CPF treated groups and among the cells positive for *MLL* rearrangements (Fig. 4 and Table 3). Due to the limited number of cells scored, no monosomy or trisomy of *MLL* gene was observed, as reflected by simultaneous gain or loss of an orange/green fusion signal (Barjesteh van Waalwijk van Doorn-Khosrovani *et al.*, 2007).

The specificity of the dual-color FISH analysis and the validity of CPF-mediated *MLL* translocations were testified by examining the response of non-genotoxic chemicals, ampicillin trihydrate, and n-butyl chloride to FISH detection. Following the same treatment protocol in Figure 4, the samples were analyzed through the service of The Diagnostic Laboratory, The First Hospital of Dalian Medical University, Dalian, Liaoning, China. As demonstrated in Table 4, all doses of ampicillin trihydrate (0.5, 12.4, and 25 mM) and n-butyl chloride (0.5 and 54 mM) responded negatively to FISH detection, while VP-16 (1 and 5 μM) led to a dose-dependent increase in *MLL* rearrangements.

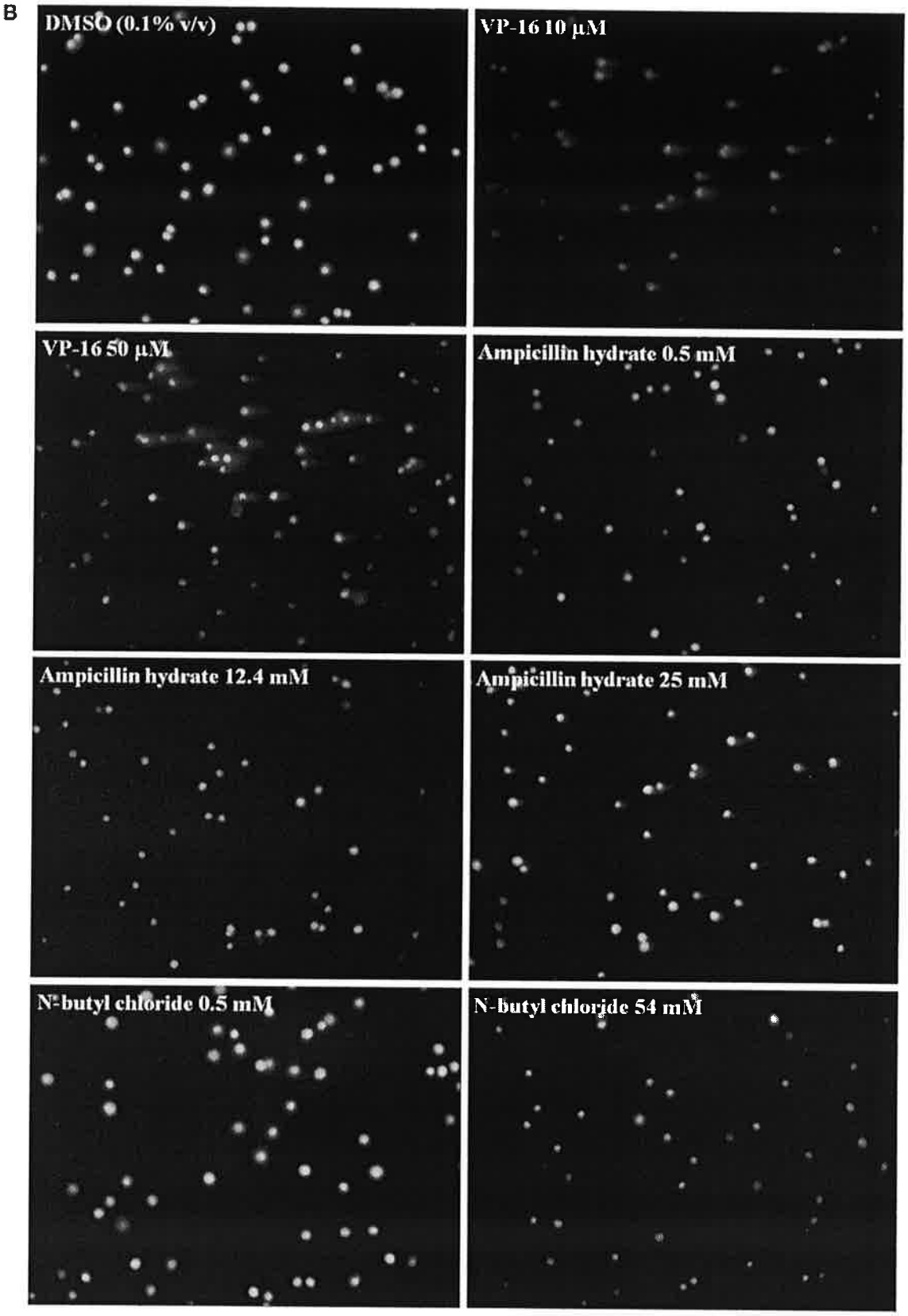

FIG. 3. Continued

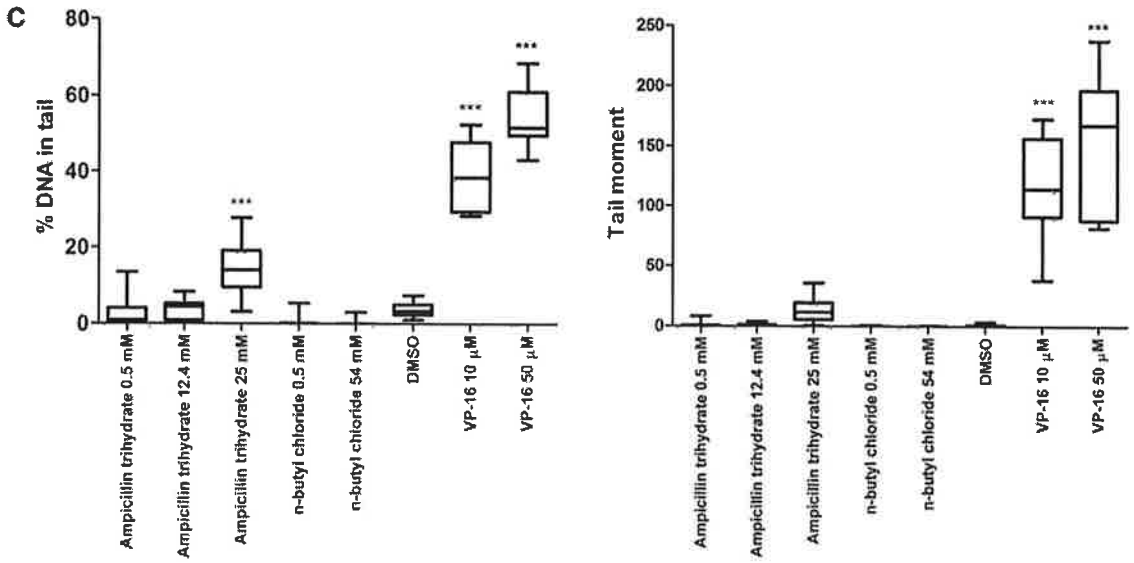

FIG. 3. Continued

TABLE 2. Quantitation of DSB Formation in CPF or VP-16-Treated Human Fetal Liver HSC as Measured by Neutral Comet Assay

Treatment	Dose	N	% DNA in Tail
CPF (μM)	0	50	1.36 ± 1.46
	1	50	6.57 ± 2.32*
	10	50	16.26 ± 4.12***
	50	50	34.15 ± 5.57***
VP-16, 10 (μM)		50	81.87 ± 7.97***

Data represent the mean ± SEM of 3 experiments.
*$p < .05$; ***$p < .001$.

CPF Induces Apoptosis and DNA Fragmentation Through Caspase-3 Mediated Pathway in Human Fetal Liver HSC

The intracellular active caspase-3, a key marker for early apoptosis, was evaluated in HSC following exposure to CPF (0, 1, 10, and 50 μM) or 10 μM VP-16 for 24 h in culture. As shown in Figure 5A, CPF induced a dose-dependent increase in active caspase-3. And the analysis on apoptosis by Annexin V-FITC/PI also revealed a dose-dependent pattern (Fig. 5B, upper panel) and the statistical significance showed at and above 10 μM (Fig. 5B, lower panel).

The ability of CPF to activate caspase-3 in HSC has led us to investigate a downstream event of DNA laddering, the resultant of CAD activation by active caspase-3. In this experiment, two positive controls were used to confirm the presence of DNA ladder, 10 μM VP-16 and 50 μM QT, which were known to induce *MLL* rearrangements through the same mechanism of action. As expected, a dose-dependent DNA laddering was observed, following exposure to CPF (0, 10, 50, and 100 μM) or 10 μM VP-16 and 50 μM QT for 24 h in culture (Fig. 5C).

The role of caspase-3 in apoptosis and DNA laddering was also confirmed in parallel using Z-VAD-FMK, a cell permeable pan caspase inhibitor. HSC at 10×10^6 were pre-incubated with 20 μM Z-VAD-FMK for 30 min before treating with CPF at 0, 1, 10, and 50 μM, or 10 μM VP-16 for 24 h, and the level of apoptosis and DNA laddering was evaluated. As expected, the retardation in caspase activity can greatly protect cells from CPF- or VP-16-induced downstream events, as evidenced by a decrease in apoptosis (Fig. 5B) and alleviation in DNA laddering (Fig. 5D). The alleviation of DNA ladder and apoptosis by caspase inhibitor suggests that CPF may induce apoptosis in HSC through death-receptor pathway (Betti *et al.*, 2003). It is noticed that the fragments caused by both VP-16 and CPF included a 1.5 Kb fragment, implicating that the fragment may be generated from a cleavage of *MLL* BCR when targeted by genotoxic chemicals (Sim and Liu, 2001).

CPF Stimulates Formation of Cleavable Complex—Is CPF a Topo II Poison in Human Fetal Liver HSC?

Inhibition of DNA Topo II has been directly related to DSB formation and believed to underlie the *MLL* translocations. In this study, the ability of CPF to stimulate DNA cleavage was initially tested using crude HSC nuclear extract (containing Topo II). Supercoiled plasmid DNA (κDNA, TopoGen) was treated with HSC Topo II in the presence of various amounts of CPF or VP-16. As shown in Figure 6A, with the increase of CPF concentrations (0–100 μM in lanes 2–6), the catenated form of κDNA (ie, the substrate retaining in the well) was progressively converted to the nicked circular, relaxed circular and linear forms, similar to the cleavable complexes seen in VP-16 (50 and 100 μM in lanes 7 and 8). Two markers, linearized κDNA marker and decatenated κDNA marker, were resolved in parallel on the side lanes to allow unambiguous detection of Topo II. The observed DNA cleavage was believed to be the result of the cleavable complex formation between CPF, Topo II and DNA rather than to nucleases or chemical degradation (Lown and Sim, 1977; Nelson *et al.*, 1984). As summarized in Table 5, both CPF and VP-16 induced a dose-dependent reduction in the formation of the nicked open circular and a dose-dependent increase in the percentage of the linear DNA, reflecting the ability of CPF to induce the formation of cleavable complex (also see Fig. 6B for better presentation). CPF also induced a reduction in the percentage of relaxed circular but with no concentration dependency. The fact that CPF induced more reduction of the closed circular and more formation of the linear DNA than VP-16 at the same concentrations suggests that CPF may be a stronger Topo II poison.

Because Topo II inhibition can also be achieved through catalytic inhibition of the enzyme, not involving the DNA cleavable complex, further analysis was conducted to clarify the

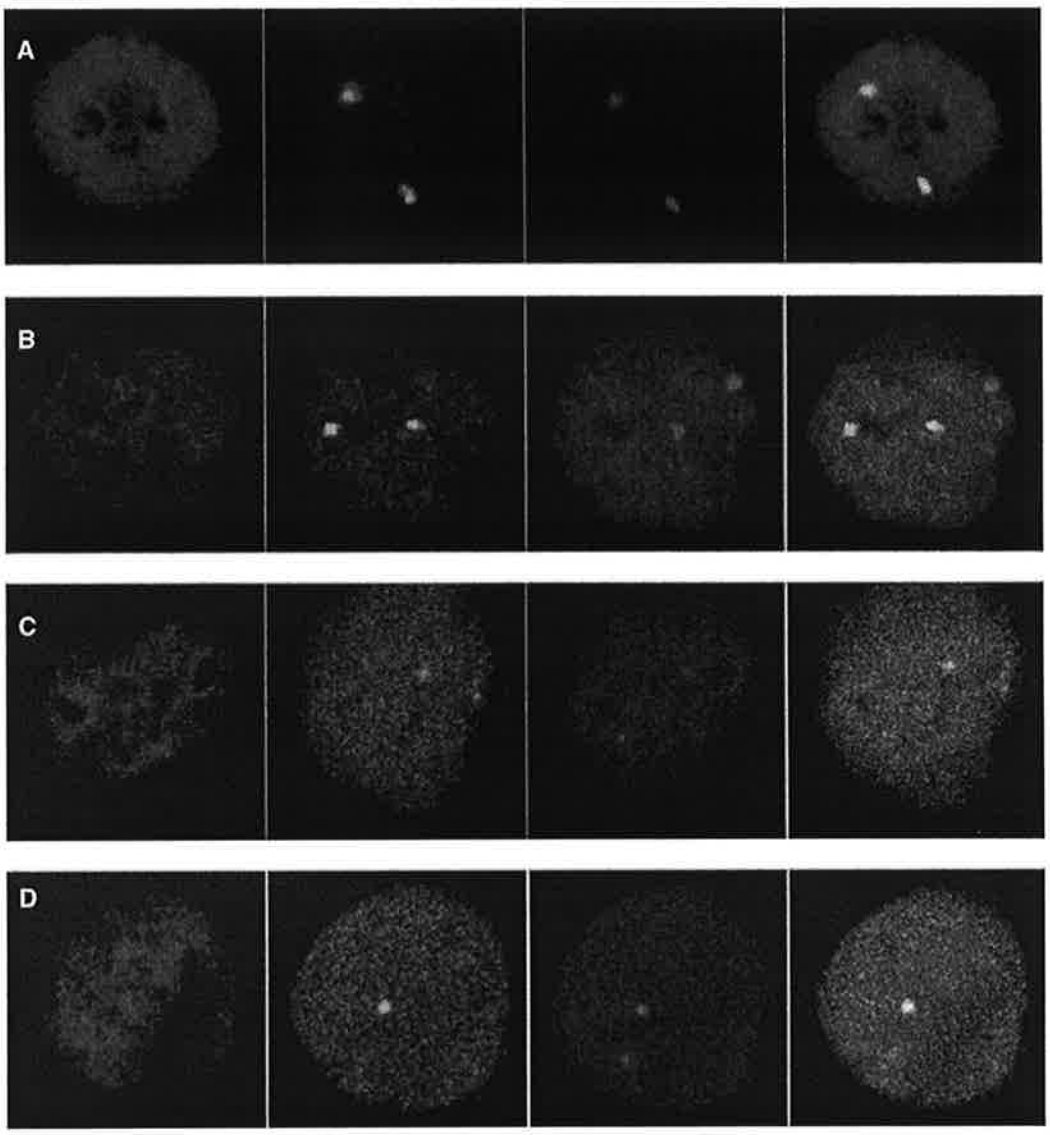

FIG. 4. CPF induces *MLL* gene rearrangements in human fetal liver HSC. HSC were exposed to CPF or DMSO (0.1% v/v) and *MLL* gene rearrangements was evaluated by FISH. **A**, Control cells show a normal chromosomal structure, as evidenced by green and orange signals either adjacent to each other or overlapping. In (**B**), 10 μM CPF induces *MLL* translocations as reflected by a split signal in which green and orange probes are separated. **C**, 1 μM VP-16, a model compound, induces *MLL* translocations as reflected by a split signal in which green and orange probes are separated. **D**, A pattern of *MLL* rearrangements with one fusion signal and one isolated green signal reflecting a concomitant translocation and deletion induced by 1 μM VP-16.

TABLE 3. Induction of *MLL* Abnormalities by CPF in Human Fetal Liver HSC as Examined by Dual-Color FISH Analysis

Time	Treatment	Split Signal	Loss of One Signal
24 h	DMSO (0.1% v/v)	0/100 (0%)	
	CPF, 1 μM	0/100 (0%)	
	CPF, 10 μM	3/100 (3%)	0/100 (0%)
	VP-16, 1 μM	4/100 (4%)	1/100 (1%)
72 h	DMSO (0.1% v/v)	0/100 (0%)	
	CPF, 1 μM	1/100 (1%)	0/100 (0%)
	CPF, 10 μM	5/100 (5%)	1/100 (1%)
	VP-16, 1 μM	6/100 (6%)	2/100 (2%)
7 days	DMSO (0.1% v/v)	0/100 (0%)	
	CPF, 1 μM	2/100 (2%)	0/100 (0%)
	CPF, 10 μM	7/100 (7%)	1/100 (1%)
	VP-16, 1 μM	10/100 (10%)	3/100 (3%)

TABLE 4. Specificity Testing on the Dual-Color FISH Analysis in Human Fetal Liver HSC

Treatment		Split Signal
DMSO (0.1% v/v)		0/200 (0%)
Ampicillin hydrate	0.5 mM	0/200 (0%)
	12.4 mM	0/200 (0%)
	25 mM	0/200 (0%)
N-butyl chloride	0.5 mM	0/200 (0%)
	54 mM	0/200 (0%)
VP-16	1 μM	6/200 (3%)
	5 μM	11/200 (5.5%)

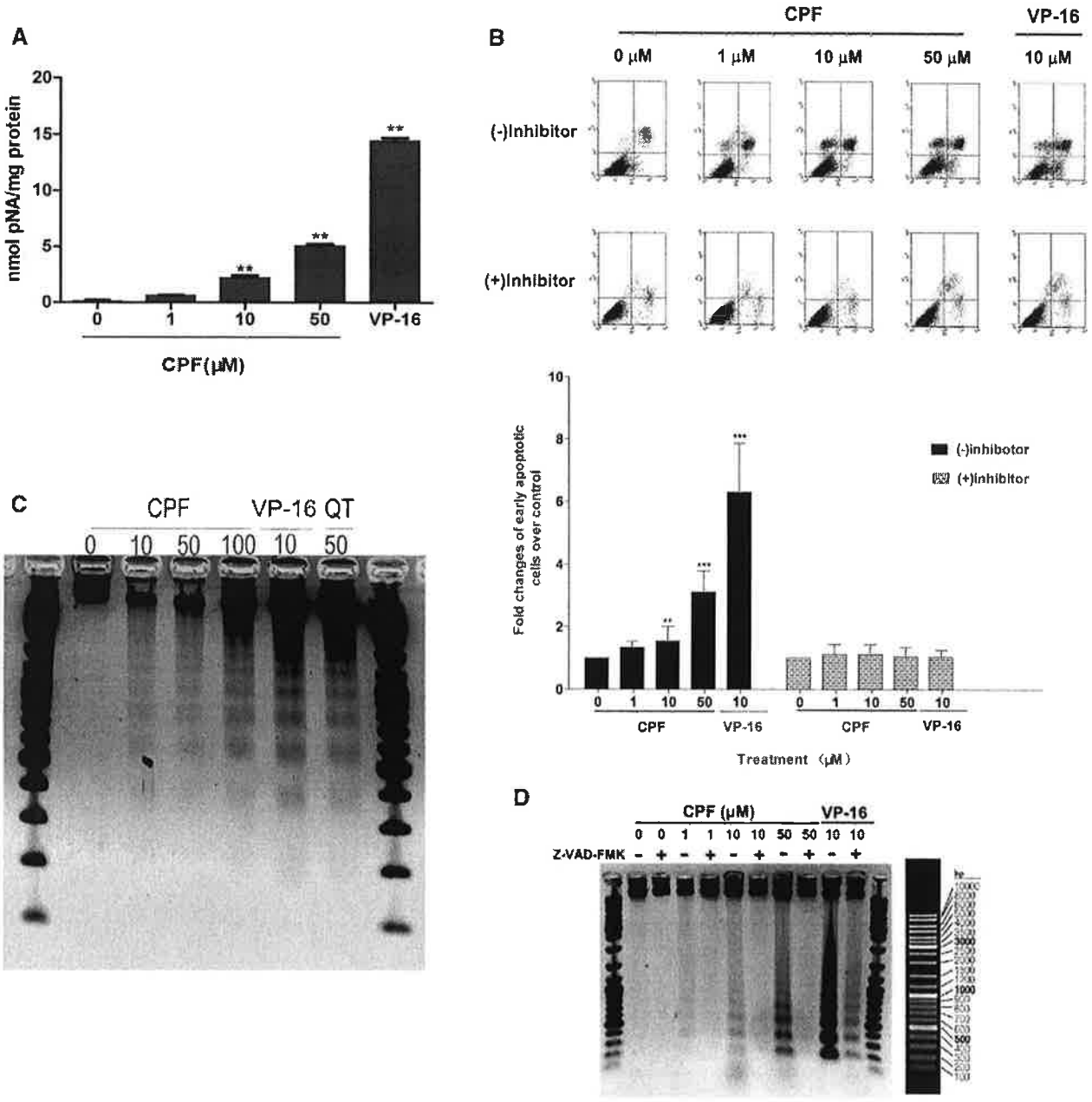

FIG. 5. CPF induces apoptosis and DNA fragmentation through caspase-3 mediated pathway in human fetal liver HSC. A, CPF activates intracellular caspase-3 as reflected by the level of a metabolic product of caspase-3 activity, pNA. Data represent the mean ± SEM of 3 experiments. $^{**}p < .01$. B, CPF induces apoptosis, as evaluated by a flow cytometry assay, with/without the pan caspase inhibitor (upper panel); the lower panel shows the data analysis of CPF-induced apoptosis. Data represent the mean ± SEM of 3 experiments. $^{**}p < .01$; $^{***}p < .001$. C, CPF induces DNA laddering as resolved by electrophoresis. In this experiment, two positive controls were used to confirm the presence of DNA ladder, 10 μM VP-16 and 50 μM QT, which were known to induce *MLL* rearrangements through the same mechanism of action. D, CPF induces DNA laddering through caspase-3-mediated pathway. In the presence of pan caspase inhibitor, Z-VAD-FMK, the induction of DNA laddering was greatly alleviated, confirming the spatial relationship between caspase-3 and DNA fragmentation.

mechanism of CPF-induced Topo II inhibition, by incubating MB, a catalytic inhibitor of Topo II, with the nuclear extract of HSC for 15 min prior to CPF exposure. As demonstrated in Figure 6C, compared with the background level in DMSO group (lane 4), 10 μM MB wiped off at least 50% of Topo II activity (lane 10), as shown by a low level of κDNA cleavage; 50 μM CPF (lane 5) or 50 μM VP-16 (lane 7) led to dramatic increase in linear DNA and nicked circular formations; and preincubation with MB prior to CPF (lane 6) or VP-16 (lane 8) exposure led to only a slight decrease in the decatenated product, confirming that the inhibition of Topo II by CPF is possibly through a mechanism similar to that of VP-16 by forming Topo II-DNA cleavable complex.

One observation worth of mentioning is that we also detected the formation of linear kDNA in both control- and MB-treated cells (Fig. 6A and C), possibly reflecting the degradation of κDNA ('κDNA degradation') by the nuclease activity in the crude cell extract (TopoGen Topoisomerase II Assay Kit User Manual). Nevertheless, the level of 'κDNA degradation' went up with increasing CPF concentrations, opposite to the pattern of

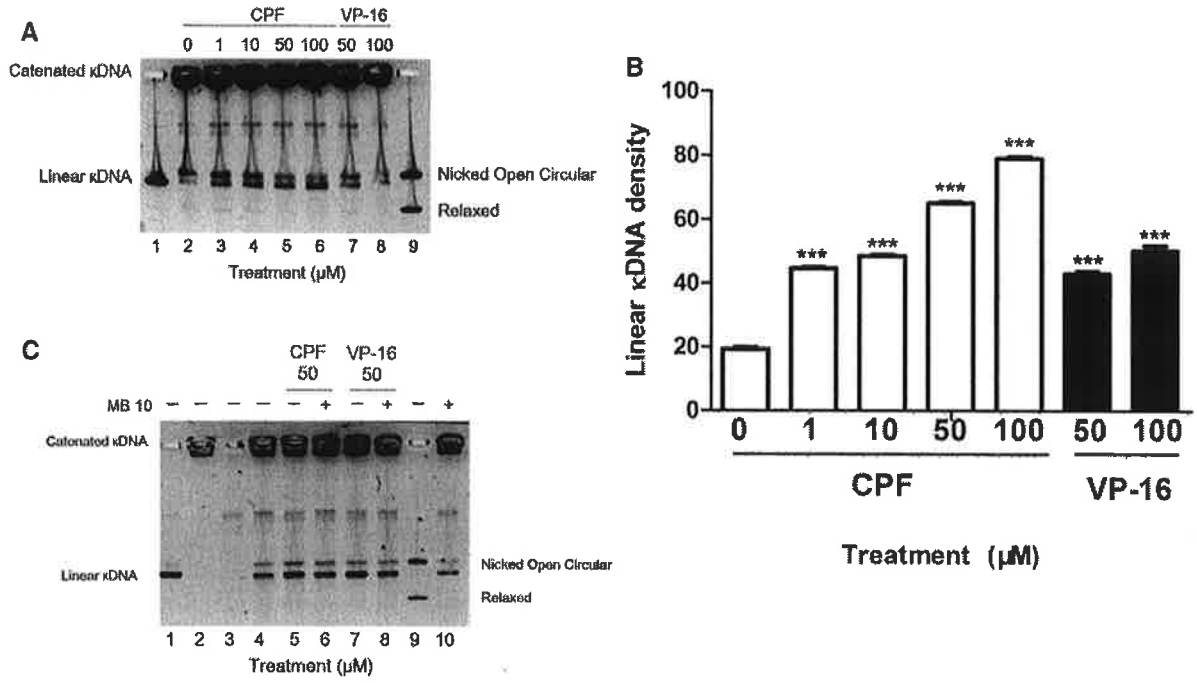

FIG. 6. CPF inhibits Topo II activity in human fetal liver HSC. The nuclear extract (containing DNA Topo II) isolated from HSC was treated with CPF or VP-16 for 1 h at 30°C. The inhibition of Topo II by CPF was determined using a Human Topoisomerase II Assay Kit (TopoGen) based upon the decatenation κDNA. A, A representative plot of Topo II activity assay. Lanes 2-6 for CPF at 0, 1, 10, 50, and 100 μM; lanes 7 and 8 for VP-16 at 50 and 100 μM. B, The statistical analysis of linear κDNA formation on Topo II activity assay. Data represent the mean ± SEM of 3 experiments. ***$p < .001$. C, CPF is not a catalytic Topo II inhibitor in human fetal liver HSC. As shown, pre-incubation of the nuclear extract (containing DNA Topo II) with MB, a catalytic inhibitor of Topo II, does not abolish CPF-mediated inhibition in Topo II activity. Lanes 1. linear κDNA marker; 2. catenated DNA; 3. the nuclear extract for background; 4. DMSO (0.1% v/v); 5. 50 μM CPF; 6. MB + 50 μM CPF; 7. 50 μM VP-16; 8. MB + 50 μM VP-16; 9. decatenated κDNA marker; 10. 10 μM MB.

TABLE 5. Inhibition of Topo II Activity by CPF in Human Fetal Liver HSC

Decatenation Product (%)	CPF (μM)					VP-16 (μM)	
	0	1	10	50	100	50	100
Nicked open circular	75.35 ± 0.93	52.44 ± 0.96**	49.62 ± 0.5**	32.99 ± 1.41**	19.78 ± 0.21**	54.63 ± 0.55**	48.95 ± 3.36**
Linear κDNA	19.30 ± 1.06	44.62 ± 0.68**	48.58 ± 0.65**	65.14 ± 0.69**	79.14 ± 0.97**	43.13 ± 1.09**	50.34 ± 2.76**
Relaxed circular	5.35 ± 1.98	2.94 ± 1.36	1.81 ± 0.12*	1.87 ± 0.78*	1.07 ± 0.94**	2.24 ± 1.21	0.71 ± 0.65*
Total	100	100	100	100	100	100	100

Data represent the mean ± SEM of 3 experiments.
*$p < .05$; **$p < .01$.

'nicked circular' and 'relaxed circular' (Fig. 6A and Table 5). The possible explanations may be that (1) the Topo II inhibitors can lead to the activation of nucleases, resulting in increased cleavage of κDNA, and (2) the inhibition of Topo II leaves out more κDNA substrates available for the nucleases, as evidenced by the fact that the sum of three products is the same for all treatment groups (Table 5).

To validate the findings from *in vitro* Topo II inhibition assay, TARDIS, a specific assay for visualization of stabilized cleavage complexes in CPF-treated cells rather than a cell-free system, was conducted using Topo II isoform-specific antibodies (Willmore *et al.*, 1998). Figure 7A shows formation of Topo IIα cleavable complexes *in vivo* by CPF at 0, 10, and 50 μM or VP-16 at 10 and 50 μM at a dose-dependent manner; and Figure 7B shows formation of Topo IIβ cleavage complexes *in vivo* by CPF at 0 and 10 μM or VP-16 at 10 and 50 μM at a dose-dependent manner. As in the neutral Comet, two non-genotoxic controls, ampicillin trihydrate, and n-butyl chloride, were included in the assay for specificity testing. Based on the result from the neutral Comet, only one dose was tested for ampicillin trihydrate (25 mM) and n-butyl chloride (54 mM), and no clear evidence for the formation of Topo IIα or Topo IIβ cleavable complexes was detected. As an additional control for the assay specificity, VP-16 at 50 μM tested with no Topo IIα or Topo IIβ antibody staining showed no formation of cleavage complexes (data not shown). Our data suggested that, as with VP-16, both isoforms of human DNA Topo II are potential targets for CPF *in vivo* (Willmore *et al.*, 1998).

DISCUSSION

CPF, as a widely used OP insecticide, was used to be believed neither a mutagen nor a teratogen or a carcinogen (Breslin *et al.*, 1996; Deacon *et al.*, 1980; Gollapudi *et al.*, 1995; Yano *et al.*, 2000). However, the opinion has been challenged by many recent studies, in which CFP was demonstrated with genotoxic

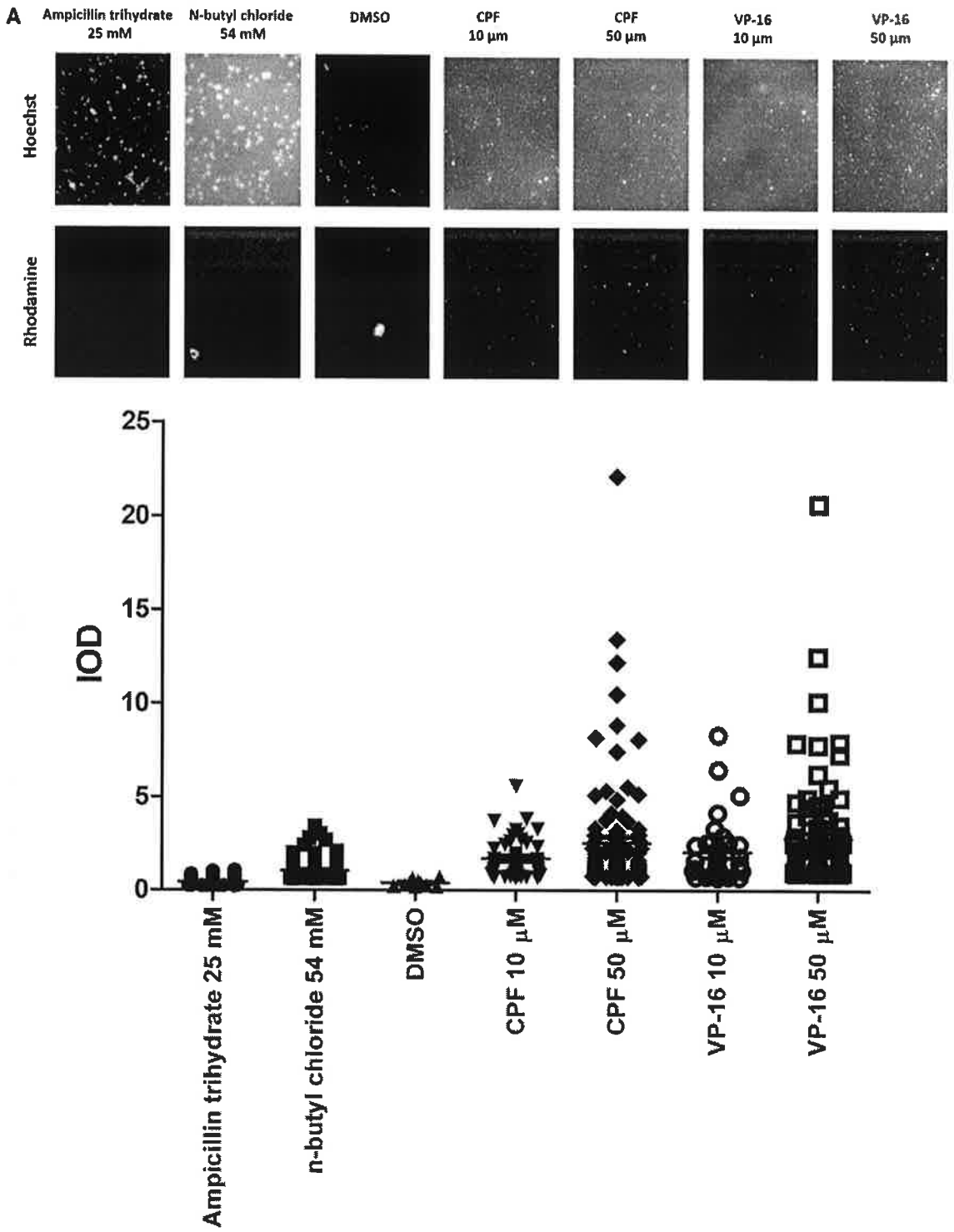

FIG. 7. CPF stabilizes Topo II cleavable complexes in human fetal liver HSC. HSC were treated with CPF (0, 10, and 50 µM) or VP-16 (10 and 50 µM) and the TARDIS assay was conducted using isoforms-specific antibodies. Blue fluorescence (Hoechst) shows the area occupied by DNA from an individual cell and red immunofluorescence shows the formation of Topo II cleavage complexes in the same field of view. **A**, Topo IIα TARDIS. CPF or VP-16 can induce stabilized Topo IIα cleavable complexes at a dose-dependent manner, while ampicillin trihydrate (25 mM) and n-butyl chloride (54 mM) were not responsive. **B**, Topo IIβ TARDIS. CPF or VP-16 can induce stabilized Topo IIβ cleavable complexes at a dose-dependent manner, while ampicillin trihydrate (25 mM) and n-butyl chloride (54 mM) were not responsive. Cells treated with VP-16 50 µM but without Topo IIα or Topo IIβ antibody staining did not show red immunofluoresence (data not shown).

and carcinogenic potential in a variety of *in vitro* and *in vivo* test models. For example, CPF can induce DNA breaks and micronuclei formation in rat lymphocytes, *in vivo* mouse bone marrow and Chinese toad using Comet assay and Micronuclei test (Ojha and Srivastava, 2014; Yaduvanshi *et al.*, 2012; Yin *et al.*, 2009), and induce structural aberrations of the polytene chromosomes in Anopheles mosquito larvae (Chaudhry and Anand, 2005). CPF has also been suggested as an environmental

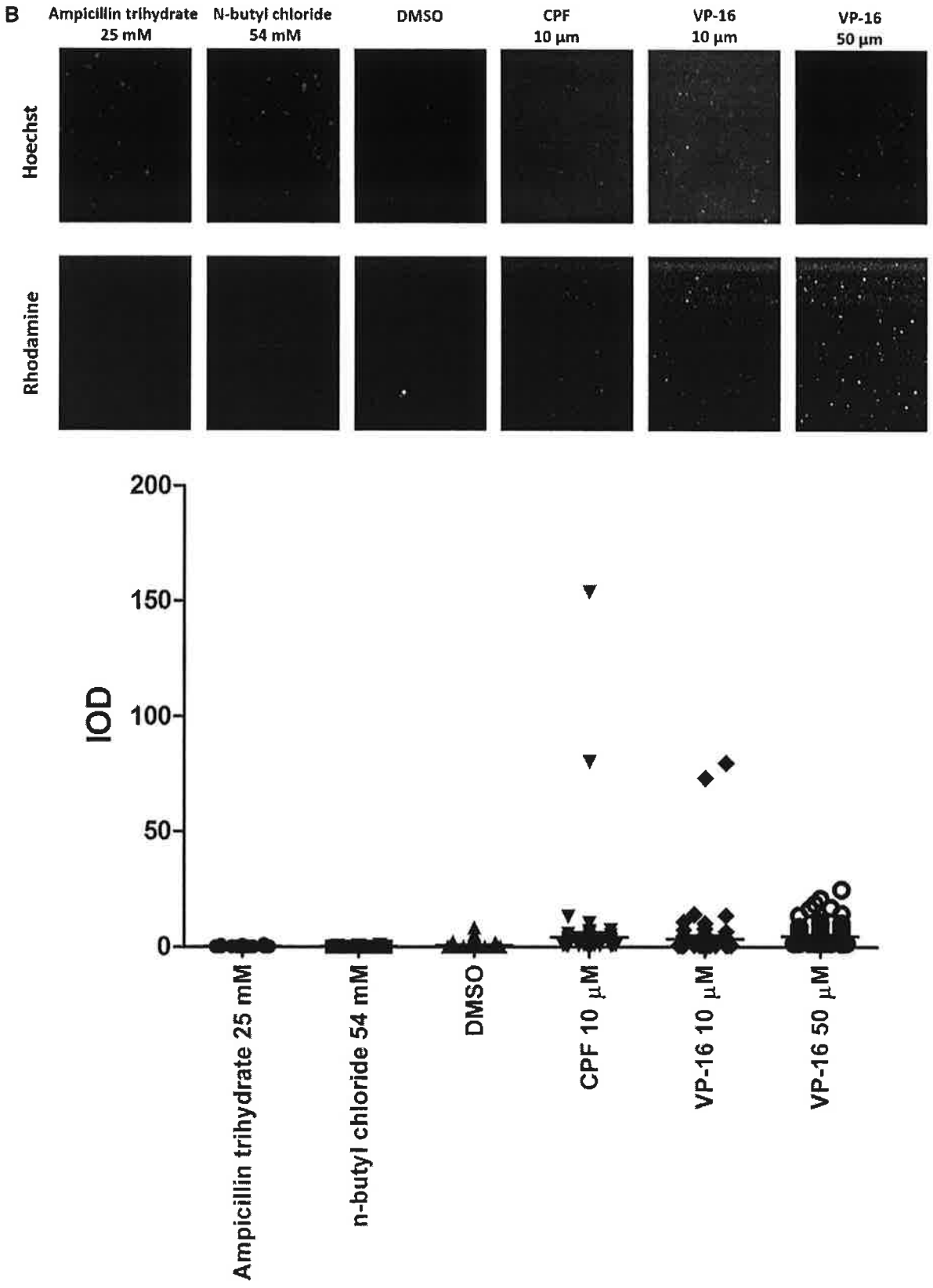

FIG. 7. Continued

risk factor for breast cancer (Ventura *et al.*, 2012), and a population-based, case-control study in Canada has shown that CFP use may be associated with an increased risk of developing Hodgkin lymphoma in men (Karunanayake *et al.*, 2012). The carcinogenicity of CPF is further confirmed by the ability of CPF to disrupt genomic DNA methylation (Wang *et al.*, 2014). In addition, exposure to residential pesticides during pregnancy has been shown to associate with an increased risk of early childhood leukemia (Ma *et al.*, 2002; Turner *et al.*, 2010). Although no specific pesticide was identified for this result, based on household inventories conducted in the study areas, CPF was one of the most commonly used home insecticides, possibly, at least in part, accounting for this increased risk. Therefore, CPF should be subject to extensive testing for its genotoxicity and carcinogenicity.

Though CFP can cause DNA breaks *in vitro* and *in vivo*, the consequences of DNA lesion have not been studied. In this study, for the first time, we demonstrated that CPF induced not only DNA DSBs, and also *MLL* gene rearrangements in human fetal liver-derived HSC. As previously stated, human fetal liver HSC, used as an *in vitro* model in our experiments, are physiologically relevant to metabolism and biotransformation of environmental chemicals and hematopoiesis during early fetal development. The genetic injuries induced by CPF exposure in human fetal liver HSC is similar to those observed in blood cells following VP-16 chemotherapy or in most of IAL cases. Therefore, further understanding of genotoxic mechanism of CPF in human fetal liver HSC may be important to define the relationship between maternal chemical exposure and risk of malignancy.

Some evidences support the contention that the *in utero* origins of *MLL* rearrangements in IAL may be the result of transplacental exposure to DNA Topo II inhibitors such as VP-16 (Alexander *et al.*, 2001; Greaves, 2002; Ross *et al.*, 1996). We also have shown in our previous study that VP-16 can induce DNA strand breaks and *MLL* rearrangements in human fetal liver HSC (Moneypenny *et al.*, 2006). It is well known that Topo II inhibitors may increase DNA DSBs by forming inhibitor-enzyme-DNA complexes that decrease DNA religation (Felix, 2001). A specific site within *MLL* BCR has been identified to be highly sensitive to double strand DNA cleavage induced by Topo II inhibitors (Broeker *et al.*, 1996). The model for the VP-16-induced *MLL* rearrangements may include initial stabilization of Topo II DNA DSBs, forcing cells to invoke non-homologous recombination (Felix, 2001). If such rearrangements containing leukogenic fusion genes occur sub-lethally, these translocations result in the production of fusion proteins that may accelerate the process of leukogenesis (Blanco *et al.*, 2001). Therefore, in this study, we selected VP-16, epipodophyllotoxin, as a major positive control to test cytotoxic and genotoxic effects and mechanisms of CPF.

CPF caused dose-dependent reductions in human fetal liver HSC viability at concentrations >10 μM as early as 24 h post-exposure, but the IC_{50} value at 24 h for CPF (>100 μM) to induce cell death was much higher than that of VP-16 (around 10 μM). The data indicate that human fetal liver HSC may be more susceptible to VP-16-induced cytotoxicity than that of CPF. However, the conclusion may be questioned. CPF is usually considered as a pro-poison that requires metabolic activation to become a more toxic agent. In human liver and intestine, CPF is metabolized to the corresponding oxon, CPO, the toxic metabolite responsible for acetylcholinesterase inhibition, and/or detoxified to 3,5,6-trichloro-2-pyridinol by different CYP450 isoforms (Eyer *et al.*, 2009; Leoni *et al.*, 2012). No studies on fetal metabolism of CPF have been reported. However, the parent CPF could be detected in maternal blood, urine, and the cord blood of infants (Arnold *et al.*, 2015; Mink *et al.*, 2012). Because we have previously shown that human fetal liver HSC may have a relative low CYP 450 biotransformation capacity (Shao *et al.*, 2006), unlike an *in vivo* condition, CPF may be limited to be activated into CPO in human fetal liver HSC. Therefore, it would be more relevant to study the genotoxic and clastogenic effects of CPF in our *in vitro* model. In addition, CPF also inhibited cell proliferation of human fetal liver HSC in a time-dependent manner, and the same tremendous reduction in proliferation was observed 4–5 days after exposure to both CPF and VP-16, suggesting that CPF may exert the same mechanism as VP-16 to induce cytotoxicity in HSC.

With regard to the relationship between concentrations used in the experiments and those likely encountered in exposed humans, several cohort studies were referenced during the designing phase of the present study. The studies in the urban minorities in New York City reported similar levels of maternal and umbilical cord blood CPF concentrations (3.9 ± 4.8 pg/g for maternal blood and 3.7 ± 5.7 pg/g for cord blood), indicating that CPF was readily transferred from mother to fetus during pregnancy (Whyatt *et al.*, 2005). The significant associations between prenatal exposure to CPF (>6.17 pg/g) in umbilical cord plasma and some adverse neurodevelopmental endpoints were reported (Rauh *et al.*, 2006, 2011). Taking both the human exposure scenario and the nature of our *in vitro* cell model into considerations, we used a broad range of CPF concentrations to examine the potential of CPF to induce DNA damage and *MLL* rearrangements in human fetal liver HSC. As previously stated, while human fetal liver HSC can be the most relevant model for studying the leukemogenic potential of some transplacental compounds, it only allows a narrow window for carrying out the *in vitro* experiments. The culture period for primary human fetal liver HSC is about 14 days post-isolation, and day 7 is the point when cells expand to large enough population for experiment while maintaining a moderate pluripotency (Moneypenny *et al.*, 2006). Although the lowest concentration of CPF of 1 μM (≈35 000 pg/ml) may not be environmentally relevant, it is justified for inducing genotoxic effect in our *in vitro* model, given the fact that we only exposed the cells to an acute and a single dose of CPF.

Rapidly dividing cells such as progenitor cells have a high Topo II content, and thus may be particularly sensitive to damage by Topo II-targeting chemicals (Potter *et al.*, 2002). In this study, we found that, similar to VP-16, CPF could exert as Topo II poison to induce genome cleavage by forming the ternary complexes with Topo II–DNA, as evidenced by both *in vitro* Topo II inhibition assay using the nuclear extract from human fetal liver HSC and the cell-based TARDIS assay (Padget *et al.*, 2000; Willmore *et al.*, 1998). As DNA replication requires chromatin to be in an open state, the accumulation of cleavable complexes by CPF may lead to generation of permanent DNA strand breaks (an early marker of DNA damage), which trigger recombination/repair pathways, mutagenesis, and chromosomal translocations in human fetal liver HSC. When such breaks overwhelm the cells, they initiate apoptosis or death.

Interestingly, the sensitivity of CPF-induced DNA injuries and *in vitro* Topo II poisoning using the nuclear extract from human fetal liver HSC appears to exceed its cytotoxic effects. A very low dose of CPF was sufficient to induce DNA DSBs and Topo II inhibition in human fetal liver HSC, and the results are similar to that of VP-16. The explanations could be that CPF-mediated cell viability was measured by Trypan blue exclusion,

which only stained the cells with a loss of membrane integrity. For those cells carrying DNA damage and/or at early apoptotic stage induced by low micromolar concentrations of CPF, the DNA repair mechanisms may be triggered and the membrane integrity may well be maintained. As described earlier, the experiment on proliferation by AlamarBlue reduction, which reflects the mitochondrial vitality of the cells, may better reflect the cytotoxic effect of CPF in human fetal liver HSC. Even 1 and 5 μM CPF can lead to fluctuation on cell proliferation, indirectly supporting our assumption that cells, when chemically challenged, may undergo some repair processes before completely die. The Topo II inhibition assay was conducted in a cell-free system, in which the nuclear extract (containing Topo II) was first extracted from human fetal liver HSC and then incubated with CPF *in vitro*. Therefore, even low micromolar concentrations of CPF could show effect of Topo II poisoning in a relatively simple system.

Mammalian cells have two distinct Topo II isoforms, Topo IIα and Topo IIβ, with two being differentially regulated and functioning differently in living cells. When Topo IIα is essential for cell growth and believed to be crucial in the cytotoxic effects of VP-16, Topo IIβ is responsible for transcription, a role that may underlie VP-16-mediated therapy-related AML and IAL involving *MLL* translocations (Azarova *et al.*, 2007; Cowell and Austin, 2012a,b). A favored working model for chromosomal translocations is that genes on the same and different chromosomes share transcriptional factories, ie, the areas concentrated with RNA polymerase complexes. When Topo IIβ normally introduces transient DSBs for genes undergoing transcription, the presence of Topo II poisons stabilize DSBs, leading to the opportunity for illegitimate end joining and translocations between two different transcribing genes engaged in the same transcriptional factory. In the case of *MLL* rearrangements, the breakpoints are localized to telomeric 1 kb of *MLL* BCR, an open chromatin structure with DNase I hypersensitivity, cryptic promoter activity and VP-16-mediated cleavage (Cowell *et al.*, 2012; Cowell and Austin, 2012a,b). In this study, CPF is found to be of similar action to VP-16 in Topo II inhibition, *MLL* rearrangements and genotoxicity. It is reasonable to assume the same mechanism of Topo IIβ and transcriptional factories in the *MLL* BCR may play a major role in CPF-mediated DSBs and subsequent *MLL* translocations due to a failure in nonhomologous end joining (NHEJ) repairs. Because *MLL* and its partner genes, eg, *AF9* and *AF4*, have been shown to share the same transcriptional factories in some nuclei (Cowell *et al.*, 2012), in the future study, we will examine the presence of *MLL/AF9*, *MLL/AF4*, along with other *MLL* fusion genes.

Alternatively, some studies indicate that VP-16- and non-VP-16 chemical-induced *MLL* translocations may be independent of Topo II inhibition, but caused by early chromatin fragmentation during apoptosis (Hars *et al.*, 2006). Apoptotic nucleases, such as caspase-activated DNase (CAD), have been suggested to be responsible for DNA cleavage within the *MLL* BCR. CAD is the major apoptotic nuclease to initiate HMW along which the translocations usually occur and followed by internucleosomal DNA cleavage (DNA laddering). The activation of CAD requires cleavage of its inhibitor (ICAD) mainly by caspase-3. In this study, we have shown that both VP-16 and CPF activate cell early apoptosis and generate 1.5 Kb fragment by activation of caspase-3. So far, the mechanism through which CFP induces apoptosis in human fetal liver HSC is not clear. CPF has been known to cause oxidative stress by generation of reactive oxygen species (ROS) in various tissues and cells of target organisms (Goel *et al.*, 2005; Jett and Navoa, 2000). The relationship between CPF-induced apoptosis and ROS generation has also been proved by other researchers (Gupta *et al.*, 2010; Lee *et al.*, 2012). Thus, CPF may cause DNA DSBs and *MLL* rearrangements through oxidative stress-induced early apoptosis in human fetal liver HSC.

DNA DSBs, through either Topo II inhibition or CAD-mediated apoptotic cleavage by CPF and VP-16, could activate DNA repair pathways for genomic instability (Felgentreff *et al.*, 2014; Soni *et al.*, 2014; Thompson, 2012). There are 2 major distinct pathways for DNA DSB repair in higher eukaryotes, DNA-PK dependent NHEJ (D-NHEJ) and homologous recombination repair (HRR) (Soni *et al.*, 2014). HRR is thought to be a high fidelity mechanism (error-free), and not to cause cytotoxicity and genotoxicity, while D-NHEJ is an error-prone repair pathway which may lead to precursor lesions for chromosome translocations. In addition, an alternative NHEJ pathway, called backup NHEJ (B-NHEJ), is considered to be a complementary repair mechanism when D-NHEJ and HRR fail (Schipler and Iliakis, 2013). Unlike D-NHEJ, which achieves repair within a few minutes through an optimized synapsis mechanism, B-NHEJ is an evolutionarily older pathway with a less optimized synapsis mechanism that rejoins DNA ends with kinetics of several hours (Perrault *et al.*, 2004). The slow kinetics and suboptimal synapsis mechanisms of B-NHEJ allow more time for exchanges through the joining of incorrect ends and therefore may cause more chromosome aberrations or cell death than D-NHEJ (Schipler and Iliakis, 2013).

The decision for cells to choose a repair pathway for DNA DSBs is influenced by stage within the cell cycle at the time of damage (Heyer *et al.*, 2010; Iliakis, 2009). HRR pathway is known to restrict to late S and G2 phases of the cell cycle, while D-NHEJ predominates in many stages of the cell cycle, particularly in G0 /G1 phase. On the other hand, B-NHEJ operates robustly throughout G2 phase of the cell cycle. In this study, we found that VP-16 induced HSC cycle arrest at G2/M, consistent with our previous study (Moneypenny *et al.*, 2006), whereas CPF induced HSC cycle arrest at G0/G1. The observation indicate that the DNA DSBs induced by CPF might process a different pathway than by VP-16 for *MLL* rearrangements, supporting our observation that VP-16 induced more *MLL* rearrangements than CPF at the same dose of exposure. Apparently, the NHEJ pathways (rather than HRR) may be the underlying mechanisms for *MLL* rearrangements induced by CPF and VP-6, which will be further investigated in our future study.

As previously stated, during culture period, some human fetal liver HSC engage in differentiation into myeloid/lymphoid lineage(s) while maintaining a certain level pluripotency (Moneypenny *et al.*, 2006). DNA replication and the higher-order chromatin rearrangements, the crucial activities of S phase, present an unique opportunity during the cell cycle for the genetic and epigenetic regulations that may be involved in stabilizing the pluripotent state (Medina *et al.*, 2012). Therefore, the potential difference on the timing of DNA damage and DNA replication blockage by CPF and VP-16 may implicate the differences in the percentage of HSC with pluripotency and the composition of hematopoietic lineages, which may render clinical ramifications in the scenario of human exposure. The CPF-induced alterations on HSC pluripotency and differentiation pathways will be further determined using lineage-specific cell surface markers.

Genetic alterations in cells with longevity tend to be more significant than those in differentiated cells, because long-lived cells are allowed more time to acquire multiple genetic hits. HSC persist for a long time during which cells maintain

homeostasis through proliferation and hierarchical amplification of progeny in multiple lineages and differentiation stages. Hence, accumulated genetic injuries in HSC may not only be replicated in the HSC compartment, and also be propagated to downstream lineages. The clonal expansion of those cells carrying the *MLL* rearrangements may potentially evolve into leukemic progression (Ford *et al.*, 1993).

Overall, the findings from this study suggest that CPF has potential to cause DNA DSBs and *MLL* translocations in human fetal liver HSC derived from early human lives. However, due to low level detections of CPF in the umbilical cord blood, it should be cautious to make clinical ramifications that CPF may contribute to the increased risk of childhood leukemia associated with exposure to indoor insecticides during pregnancy. The conceptual working model that relates transplacental CPF exposure to the etiology of IAL is as follows: CPF enters the fetal circulation through maternal exposure, and, as a Topo II inhibitor, targets the hematopoietic precursor cells residing in liver and induces double strand DNA cleavage within the *MLL* BCR, either directly or indirectly. Alternatively, the genomic instability within *MLL* BCR may be the consequence of increased ROS generation by CPF (Gupta *et al.*, 2010), or of the caspase-3-activated, CAD-mediated DNA fragmentation. Majority of these cells may either successfully repair the break, or fail and die through secondary activation of apoptotic pathway. In a fraction of cells, the attempt to repair the double strand DNA cleavage within the *MLL* BCR is not completed properly, and then translocations or deletions may occur. The cells containing *MLL* rearrangements have a proliferative advantage conferred by the *MLL* mispairing, manifested clinically as leukemia.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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Specimen Label

RESTRICTED USE PESTICIDE

For retail sale to and use only by Certified Applicators or persons under their direct supervision and only for those uses covered by the Certified Applicator's certification.

Dow AgroSciences

Lorsban® Advanced

INSECTICIDE

®Trademark of The Dow Chemical Company ("Dow") or an affiliated company of Dow

For control of listed insects infesting certain field, fruit, nut, and vegetable crops.

Group	1B	INSECTICIDE

Active Ingredient:
chlorpyrifos: O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate 40.2%
Other Ingredients 59.8%
Total 100.0%

Contains 3.755 lb of chlorpyrifos per gallon.
Contains petroleum distillates.

Precautionary Statements

Hazard to Humans and Domestic Animals

EPA Reg. No. 62719-591

WARNING

May Be Fatal If Swallowed • Causes Skin Irritation • Causes Moderate Eye Irritation • Harmful If Inhaled • Prolonged Or Frequently Repeated Skin Contact May Cause Allergic Reactions In Some Individuals

Do not get on skin or on clothing. Avoid contact with eyes and breathing vapor or spray mist. Wash thoroughly with soap and water after handling and before eating, drinking, chewing gum, using tobacco or using the toilet.

First Aid

Organophosphate

If swallowed: Immediately call a poison control center or doctor. Do not induce vomiting unless told to do so by a poison control center or doctor. Do not give **any** liquid to the person. Do not give anything by mouth to an unconscious person.

If on skin or clothing: Take off contaminated clothing. Rinse skin immediately with plenty of water for 15-20 minutes. Call a poison control center or doctor for treatment advice.

If in eyes: Hold eye open and rinse slowly and gently with water for 15-20 minutes. Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. Call a poison control center or doctor for treatment advice.

If inhaled: Move person to fresh air. If person is not breathing, call 911 or an ambulance, then give artificial respiration, preferably mouth-to-mouth if possible. Call a poison control center or doctor for further treatment advice.

Note to physician: Chlorpyrifos is a cholinesterase inhibitor. Treat symptomatically. If exposed, plasma and red blood cell cholinesterase tests may indicate significance of exposure (baseline data are useful). Atropine, only by injection, is the preferable antidote. Oximes, such as 2-PAM/protopam, may be therapeutic if used early; however, use only in conjunction with atropine. In case of severe acute poisoning, use antidote immediately after establishing an open airway and respiration.

First Aid (Cont.)

Note to physician: Contains petroleum distillate – vomiting may cause aspiration pneumonia.

Have the product container or label with you when calling a poison control center or doctor, or going for treatment. You may also contact 1-800-992-5994 for emergency medical treatment information.

Personal Protective Equipment (PPE)

Materials that are chemical resistant to this product are barrier laminate and viton ≥14 mils.

Mixers and loaders using a mechanical transfer loading system and applicators using aerial application equipment must wear:
- Long-sleeved shirt and long pants
- Shoes and socks

In addition to the above, **mixers and loaders** using a mechanical transfer loading system must wear:
- Chemical-resistant gloves
- Chemical-resistant apron
- A NIOSH-approved dust mist filtering respirator with MSHA/NIOSH approval number prefix TC-21C or a NIOSH-approved respirator with any R, P, or HE filter

See Engineering Controls for additional requirements.

All **other mixers, loaders, applicators and handlers** must wear:
- Coveralls over long-sleeved shirt and long pants
- Chemical-resistant gloves
- Chemical-resistant apron when mixing or loading or exposed to the concentrate
- Chemical-resistant footwear plus socks
- Chemical-resistant headgear for overhead exposure
- A NIOSH-approved dust mist filtering respirator with MSHA/NIOSH approval number prefix TC-21C or a NIOSH-approved respirator with any R, P, or HE filter.

Discard clothing and other absorbent materials that have been drenched or heavily contaminated with this product's concentrate. Do not reuse them. Follow manufacturer's instructions for cleaning/maintaining PPE. If no such instructions for washables exist, use detergent and hot water. Keep and wash PPE separately from other laundry.

Engineering Controls

Mixers and loaders supporting aerial applications must use a mechanical transfer system that meets the requirements listed in the Worker Protection Standard (WPS) for agricultural pesticides [40 CFR 170.240(d)(4)] for dermal protection, and must:
- Wear the personal protective equipment required above for mixers/loaders
- Wear protective eyewear if the system operates under pressure, and
- Be provided and have immediately available for use in an emergency, such as broken package, spill, or equipment breakdown: coveralls, chemical resistant footwear and chemical-resistant headgear if overhead exposure

Pilots must use an enclosed cockpit in a manner that meets the requirements listed in the WPS for agricultural pesticides [40 CFR 170.240(d)(6).

Use of human flaggers is prohibited. Mechanical flagging equipment must be used.

When handlers use closed cab motorized ground application equipment in a manner that meets the requirements listed in the WPS for agricultural pesticides [40 CFR 170.240(d)(4-6)], the handler PPE requirements may be reduced or modified as specified in the WPS.

User Safety Recommendations

Users should:
- Wash hands before eating, drinking, chewing gum, using tobacco, or using the toilet.
- Remove clothing/PPE immediately if pesticide gets inside. Then wash thoroughly and put on clean clothing.
- Remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash thoroughly and change into clean clothing.

Environmental Hazards

This pesticide is toxic to fish, aquatic invertebrates, small mammals and birds. Do not apply directly to water, or to areas where surface water is present, or to intertidal areas below the mean high water mark. Drift and runoff from treated areas may be hazardous to aquatic organisms in water adjacent to treated areas. Do not contaminate water when disposing of equipment washwaters or rinsate. This product is highly toxic to bees exposed to direct treatment or residues on blooming crops or weeds. Do not apply this product or allow it to drift to blooming crops or weeds if bees are visiting the treatment area.

Directions for Use

Restricted Use Pesticide

It is a violation of Federal law to use this product in a manner inconsistent with its labeling.
Read all Directions for Use carefully before applying.

This product cannot be reformulated or repackaged into other end-use products.

Do not apply this product in a way that will contact workers or other persons, either directly or through drift. Only protected handlers may be in the area during application. For any requirements specific to your state or tribe, consult the agency responsible for pesticide regulation.

Agricultural Use Requirements

Use this product only in accordance with its labeling and with the Worker Protection Standard, 40 CFR Part 170. This Standard contains requirements for the protection of agricultural workers on farms, forests, nurseries, and greenhouses, and handlers of agricultural pesticides. It contains requirements for training, decontamination, notification, and emergency assistance. It also contains specific instructions and exceptions pertaining to the statements on this label about personal protective equipment (PPE) and restricted-entry interval. The requirements in this box only apply to uses of this product that are covered by the Worker Protection Standard.

Do not enter or allow entry into treated areas during the restricted entry interval (REI). The REI for each crop is listed in the directions for use associated with each crop..

Exception: If the product is soil-injected or soil-incorporated, the Worker Protection Standard, under certain circumstances, allows workers to enter the treated area if there will be no contact with anything that has been treated.

Certified crop advisors or persons entering under their direct supervision under certain circumstances may be exempt from the early reentry requirements pursuant to 40 CFR Part 170.

PPE required for early entry into treated areas that is permitted under the Worker Protection Standard and involves contact with anything that has been treated, such as plants, soil, or water, is:

- Coveralls over short-sleeved shirt and short pants
- Chemical-resistant gloves made out of any waterproof material
- Chemical-resistant footware plus socks
- Chemical-resistant headgear for overhead exposure

Notify workers of the application by warning them orally and by posting warning signs at entrances to treated areas.

Storage and Disposal

Do not contaminate water, food, or feed by storage and disposal.
Pesticide Storage: Store in original container in secured dry storage area. Prevent cross-contamination with other pesticides and fertilizers.
Pesticide Disposal: Wastes resulting from the use of this product must be disposed of on site or at an approved waste disposal facility.

Nonrefillable containers 5 gallons or less:
Container Handling: Nonrefillable container. Do not reuse or refill this container.
Triple rinse or pressure rinse container (or equivalent) promptly after emptying. **Triple rinse** as follows: Empty the remaining contents into application equipment or a mix tank and drain for 10 seconds after the flow begins to drip. Fill the container 1/4 full with water and recap. Shake for 10 seconds. Pour rinsate into application equipment or a mix tank or store rinsate for later use or disposal. Drain for 10 seconds after the flow begins to drip. Repeat this procedure two more times. **Pressure rinse** as follows: Empty the remaining contents into application equipment or a mix tank and continue to drain for 10 seconds after the flow begins to drip. Hold container upside down over application equipment or mix tank or collect rinsate for later use or disposal. Insert pressure rinsing nozzle in the side of the container, and rinse at about 40 psi for at least 30 seconds. Drain for 10 seconds after the flow begins to drip. Then offer for recycling if available or puncture and dispose of in a sanitary landfill, or by incineration, or by other procedures allowed by state and local authorities.

Refillable containers 5 gallons or larger:
Container Handling: Refillable container. Refill this container with pesticide only. Do not reuse this container for any other purpose. Cleaning the container before final disposal is the responsibility of the person disposing of the container. Cleaning before refilling is the responsibility of the refiller. To clean the container before final disposal, empty the remaining contents from this container into application equipment or a mix tank. Fill the container about 10% full with water and, if possible, spray all sides while adding water. If practical, agitate vigorously or recirculate water with the pump for two minutes. Pour or pump rinsate into application equipment or rinsate collection system. Repeat this rinsing procedure two more times. Then offer for recycling if available, or puncture and dispose of in a sanitary landfill, or by incineration, or by other procedures allowed by state and local authorities.

Storage and Disposal (Cont.)

Nonrefillable containers 5 gallons or larger:
Container Handling: Nonrefillable container. Do not reuse or refill this container.
Triple rinse or pressure rinse container (or equivalent) promptly after emptying. **Triple rinse** as follows: Empty the remaining contents into application equipment or a mix tank. Fill the container 1/4 full with water. Replace and tighten closures. Tip container on its side and roll it back and forth, ensuring at least one complete revolution, for 30 seconds. Stand the container on its end and tip it back and forth several times. Turn the container over onto its other end and tip it back and forth several times. Empty the rinsate into application equipment or a mix tank or store rinsate for later use or disposal. Repeat this procedure two more times. **Pressure rinse** as follows: Empty the remaining contents into application equipment or a mix tank and continue to drain for 10 seconds after the flow begins to drip. Hold container upside down over application equipment or mix tank or collect rinsate for later use or disposal. Insert pressure rinsing nozzle in the side of the container, and rinse at about 40 psi for at least 30 seconds. Drain for 10 seconds after the flow begins to drip. Then offer for recycling if available, or puncture and dispose of in a sanitary landfill, or by incineration, or by other procedures allowed by state and local authorities.

Product Information

Lorsban® Advanced insecticide is an emulsion in water for use in listed crops. This product resists washoff once it is dry. Target pests and application rates are provided in the accompanying tables.

Use Precautions and Restrictions

Insect control may be reduced at low spray volumes under high temperature and wind conditions.

Some reduction in insect control may occur under unusually cool conditions.

Flood irrigation: To avoid contamination of irrigation tail waters, do not flood irrigate within 24 hours following a soil surface or foliar application of Lorsban Advanced.

Do not aerially apply this product in Mississippi.

Insecticide Resistance Management (IRM)
Lorsban Advanced contains a Group 1B insecticide. Insect/mite biotypes with acquired resistance to Group 1B may eventually dominate the insect/mite population if Group 1B insecticides are used repeatedly in the same field or in successive years as the primary method of control for targeted species. This may result in partial or total loss of control of those species by Lorsban Advanced or other Group 1B insecticides.

To delay development of insecticide resistance:

- Avoid consecutive use of insecticides with the same mode of action (same insecticide group) on the same insect species.
- Use tank mixtures or premix products containing insecticides with different modes of action (different insecticide groups) provided the products are registered for the intended use.
- Base insecticide use upon comprehensive Integrated Pest Management (IPM) programs.
- Monitor treated insect populations in the field for loss of effectiveness.
- Contact your local extension specialist, certified crop advisor, and or manufacturer for insecticide resistance management and/or IPM recommendations for the specific site and resistant pest problems.
- For further information or to report suspected resistance, you may contact Dow AgroSciences by calling 800-258-3033.

Spray Drift Management
Do not allow spray to drift from the application site and contact people, structures people occupy at any time and the associated property, parks and recreation areas, non-target crops, aquatic and wetland sites, woodlands, pastures, rangelands, or animals.

Avoiding spray drift at the application site is the responsibility of the applicator. The interaction of many equipment and weather-related factors determine the potential for spray drift. The applicator is responsible for considering all of these factors when making the decision to apply this product.

Observe the following precautions when spraying Lorsban Advanced adjacent to permanent bodies of water such as rivers, natural ponds, lakes, streams, reservoirs, marshes, estuaries, and commercial fish ponds.

The following treatment setbacks or buffer zones must be utilized for applications around the above-listed aquatic areas with the following application equipment:

Application Method	Required Setback (Buffer Zone) (feet)
ground boom	25
chemigation	25
orchard airblast	50
aerial (fixed wing or helicopter)	150

Making applications when wind is blowing away from sensitive areas is the most effective way to reduce the potential for adverse effects.

The buffer distances specified in the below table are the distances in feet that must exist to separate sensitive sites from the targeted application site. Buffers are measured from the edge of the sensitive site to the edge of the application site.

Sensitive sites are areas frequented by non-occupational bystanders (especially children). These include residential lawns, pedestrian sidewalks, outdoor recreational areas such as school grounds, athletic fields, parks and all property associated with buildings occupied by humans for residential or commercial purposes. Sensitive sites include homes, farmworker housing, or other residential buildings, schools, daycare centers, nursing homes, and hospitals. Non-residential agricultural buildings, including barns, livestock facilities, sheds, and outhouses are not included in this prohibition.

Application Rate (lb ai/A)	Nozzle Droplet Type	Required Setback (Buffer Zones) (feet)		
		Aerial	Airblast	Ground
>0.5 – 1	coarse or very coarse	10	10	10
>0.5 – 1	medium	25	10	10
>1 – 2	coarse or very coarse	50	10	10
>1 – 2	medium	80	10	10
>2 – 3	coarse or very coarse	80[1]	10	10
>2 – 3	medium	100[1]	10	10
>3 – 4	medium or coarse	NA[2]	25	10
>4	medium or coarse	NA	50	10

[1]Aerial application of greater than 2 lb ai/A is only permitted for Asian Citrus Psylla control, up to 2.3 lb ai/A.
[2]NA is not allowed.

Only pesticide handlers are permitted in the setback area during application of this product. Do not apply this product if anyone other than a mixer, loader, or applicator, is in the setback area. Exception: Vehicles and persons riding bicycles that are passing through the setback area on public or private roadways are permitted.

Follow these spray drift **best management practices** to avoid off-target drift movement from applications.

Aerial Application

- The boom width must not exceed 75% of the wingspan or 90% of the rotor blade.
- Nozzles must always point backward, parallel with the air stream, and never be pointed downward more than 45 degrees.
- Nozzles must produce a medium or coarser droplet size (255 to 340 microns volume median diameter) per ASABE Standard 572.1 under application conditions. Airspeed, pressure, and nozzle angle can all effect droplet size. See manufacturer's catalog or USDA/NAAA Applicator's Guide for spray size quality ratings.
- Do not make applications at a height greater than 10 feet above the top of the target plants unless a greater height is required for aircraft safety. Making applications at the lowest height that is safe reduces exposure of droplets to evaporation and wind.
- Use upwind swath displacement and apply only when wind speed is 3 to 10 mph as measured by an anemometer. Do not apply product when wind speed exceeds 10 mph.
- If application includes a no-spray zone, do not release spray at a height greater than 10 feet above the ground or crop canopy.

Where states have more stringent regulations, they must be observed.

The applicator should be familiar with and take into account the information covered in the Aerial Drift Reduction Advisory.

Aerial Drift Reduction Advisory
This section is advisory in nature and does not supercede the mandatory label requirements.

Information on Droplet Size: The most effective way to reduce drift potential is to apply large droplets. The best drift management strategy is to apply the largest droplets that provide sufficient coverage and control. Applying larger droplets reduces drift potential, but will not prevent adverse effects from drift if applications are made improperly, or under unfavorable environmental conditions (see Wind, Temperature and Humidity, and Temperature Inversions).

Controlling Droplet Size:

- Volume - Use high flow rate nozzles to apply the highest practical spray volume. Nozzles with higher rated flows produce larger droplets.
- Pressure - Do not exceed the nozzle manufacturer's specified pressures. For many nozzle types, lower pressure produces larger droplets. When higher flow rates are needed, use higher flow rate nozzles instead of increasing pressure.
- Number of nozzles - Use the minimum number of nozzles that provide uniform coverage.
- Nozzle orientation - Orienting nozzles so that the spray is released parallel to the airstream produces larger droplets than other orientations and is the best practice. Significant deflection from horizontal will reduce droplet size and increase drift potential.
- Nozzle type - Use a nozzle type that is designed for the intended application. With most nozzle types, narrower spray angles produce larger droplets. Consider using low-drift nozzles. Solid stream nozzles oriented straight back produce the largest droplets and the lowest drift.

Boom Length: For some use patterns, reducing the effective boom length to less than 3/4 of the wingspan or rotor length may further reduce drift without reducing swath width.

Application Height: Do not make applications at a height greater than 10 feet above the top of the target plants unless a greater height is required for aircraft safety. Making applications at the lowest height that is safe reduces exposure of droplets to evaporation and wind.

Swath Adjustment: When applications are made with a crosswind, the swath will be displaced downwind. Therefore, on the up and downwind edges of the field, the applicator should compensate for this displacement by adjusting the path of the aircraft upwind. Increase swath adjustment distance with increasing drift potential (higher wind, smaller drops, etc.).

Wind: Drift potential is lowest between wind speeds of 2 to 10 mph. However, many factors, including droplet size and equipment type, determine drift potential at any given speed. Do not apply below 1.5 mph due to variable wind direction and high inversion potential. **Note:** Local terrain can influence wind patterns. Every applicator should be familiar with local wind patterns and how they affect spray drift.

Temperature and Humidity: When making applications in low relative humidity, set up equipment to produce larger droplets to compensate for evaporation. Droplet evaporation is most severe when conditions are both hot and dry.

Temperature Inversions: Do not make applications during a temperature inversion because drift potential is high. Temperature inversions restrict vertical air mixing, which causes small suspended droplets to remain in a concentrated cloud. This cloud can move in unpredictable directions due to the light variable winds common during inversions. Temperature inversions are characterized by increasing temperatures with altitude and are common on nights with limited cloud cover and light to no wind. They begin to form as the sun sets and often continue into the morning. Their presence can be indicated by ground fog; however, if fog is not present, inversions can also be identified by the movement of smoke from a ground source or an aircraft smoke generator. Smoke that layers and moves laterally in a concentrated cloud (under low wind conditions) indicates an inversion, while smoke that moves upward and rapidly dissipates indicates good vertical air mixing.

Sensitive Areas: Apply the pesticide only when the potential for drift to adjacent sensitive areas (e.g., residential areas, bodies of water, known habitat for threatened or endangered species, non-target crops) is minimal (e.g., when wind is blowing away from the sensitive areas).

Ground Boom Application
The following mandatory spray drift **best management practices** are required to reduce the likelihood of off-target drift movement from ground applications.

- Choose only nozzles and pressures that produce a medium or coarse droplet size (255 to 400 microns volume median diameter) per ASABE Standard 572.1. See manufacturer's catalog or USDA/NAAA Applicator's Guide for spray size quality ratings.
- Apply with nozzle height no more than 4 feet above the ground or crop canopy.
- Do not apply product when wind speed exceeds 10 mph as measured by an anemometer.

Orchard Airblast Application
The following mandatory spray drift **best management practices** are required to reduce the likelihood of off-target drift movement from airblast applications.

- Direct nozzles so spray is not projected above the canopies.
- Apply only when wind speed is 3 to 10 mph at the application site as measured by an anemometer outside of the orchard/vineyard on the upwind side.
- Outward pointing nozzles must be shut off when turning corners at row ends.

The applicator should take into account the following **best management practices** to reduce off-site spray drift. This section is advisory and does not supercede mandatory label requirements.

- Number of nozzles, nozzle orientation and spray volume, air speed and wind direction are key factors in adjusting airblast spray delivery to match the height and density of the crop canopy. Adjust airblast equipment to provide uniform coverage while minimizing the amount of spray movement over the top or completely through the crop canopy.
  - High air volumes deliver spray more efficiently than air at high speed. Reducing forward travel speed decreases the air speed necessary to deliver the spray to the top of the crop canopy.
  - Use air guides along with the number and orientation of spray nozzles to achieve the desired spray coverage and directional control.
- Take the following steps to minimize drift and the amount of non-target spray:
  - Orient nozzles and adjust air speed/volume/direction to force the spray through the crop canopy but not allow drift past the canopy.
  - Shut off spray delivery when passing gaps in crop canopy within rows.
  - Spray the outside rows of orchards from outside in, directing the spray into the orchard and shutting off nozzles on the side of the sprayer away from the orchard.
  - When treating smaller trees, vines or bushes, shut off top nozzles to minimize over the top spray movement.

Application Directions

Broadcast Foliar Application
Apply with conventional power-operated spray equipment using nozzles and spray pressures specified for insecticides. Apply Lorsban Advanced in a spray volume of not less than 2 gallons per acre (gpa) for aerial application equipment (fixed wing or helicopter) or not less than 10 gpa for ground equipment, unless otherwise specified. Increase spray volume to ensure adequate coverage with increased density and height of crop canopy.

Ground Application: Orient the boom and nozzles so that uniform coverage is obtained. The swath width should not be wider than the boom. Follow nozzle manufacturer's specifications for insecticide nozzles with respect to nozzle type, pressure, and spacing.

Broadcast Soil Application
Apply with conventional power-operated spray equipment that will apply the product uniformly to the soil surface. Use nozzles that produce medium or coarse droplets (255 to 400 microns). Unless otherwise indicated, a spray volume of 10 gpa or more is needed. For band application, use proportionally less spray volume.

Aerial Application
Use a minimum spray volume of 2 gpa. Mark swaths by mechanical flagging, permanent markers or GPS equipment.

Chemigation Application
Apply Lorsban Advanced through properly equipped chemigation systems for insect control in alfalfa, almond (orchard floors only), citrus (orchard floors only), corn (field and sweet), cotton, cranberry, peppermint, sorghum, soybeans, spearmint, sugarbeet, orchard floors (pecan and walnut), and wheat, or other crops as specified in Dow AgroSciences supplemental labeling. Do not apply this product by chemigation unless specified in crop-specific directions in this label or Dow AgroSciences supplemental labeling. Do not apply to labeled crops through any other type of irrigation system.

Note: Unless otherwise indicated in specific use directions, the application rates for chemigation are the same as those specified for broadcast application.

Directions for Sprinkler Chemigation: Apply this product only through the following sprinkler irrigation systems: center pivot, lateral move, end tow, side (wheel) roll, traveler, big gun, solid set, micro sprinkler, or hand move. Do not apply this product through any other type of irrigation system. Do not apply through sprinkler systems that deliver a low coefficient of uniformity such as certain water drive units.

Chemigation Equipment Preparation: The following use directions must be followed when Lorsban Advanced is applied through sprinkler irrigation systems. Thoroughly clean the chemigation system and tank of any fertilizer or chemical residues, and dispose of the residues according to state and federal laws. Flush the injection system with soap or a cleaning agent and water. Determine the amount of Lorsban Advanced needed to cover the desired acreage. Mix according to instructions in the Mixing Directions section and bring mixture to desired volume. Maintain continuous agitation during mixing and throughout the application period.

Chemigation Equipment Calibration: In order to calibrate the irrigation system and injector to apply the mixture containing Lorsban Advanced, determine the following: 1) Calculate the number of acres irrigated by the system; 2) Calculate the amount of product required and premix; 3) Determine the irrigation rate and determine the number of minutes for the system to cover the intended treatment area; 4) Calculate the total gallons of insecticide mixture needed to cover the desired acreage. Divide the total gallons of insecticide mixture needed by the number of minutes (minus time to flush out) to cover the treatment area. This value equals the gallons per minute output that the injector or eductor must deliver. Convert the gallons per minute to milliliters or ounces per minute if needed. 5) Calibrate the injector pump with the system in operation at the desired irrigation rate. It is suggested that the timed output of the injector pump be checked at least twice before operation, and the system monitored during operation.

Chemigation Equipment Requirements:

- The system must contain an air gap, an approved backflow prevention device, a functional check valve, vacuum relief valve (including inspection port), and low-pressure drain appropriately located on the irrigation pipeline to prevent water source contamination from back flow. Refer to the American Society of Agricultural Engineer's Engineering Practice 409 for more information or state specific regulations.
- The pesticide injection pipeline must contain a functional, automatic, quick-closing check valve to prevent the flow of fluid back toward the injection pump.
- The pesticide injection pipeline must also contain a functional, normally closed, solenoid-operated valve located on the intake side of the injection pump and connected to the system interlock to prevent fluid from being withdrawn from the supply tank when the irrigation system is either automatically or manually shut down.
- The system must contain functional interlocking controls to automatically shut off the pesticide injection pump when the water pump motor stops.
- The irrigation line or water pump must include a functional pressure switch that will stop the water pump motor when the water pressure decreases to the point where pesticide distribution is adversely affected.
- Systems must use a metering pump, such as a positive displacement injection pump (e.g., diaphragm pump) effectively designed and constructed of materials that are compatible with pesticides and capable of being fitted with a system interlock.
- To ensure uniform mixing of the insecticide into the water line, inject the mixture through a nozzle placed in the fertilizer injection port or just ahead of an elbow or tee in the irrigation line so that the turbulence will assist in mixing. The injection point must be located after all back-flow prevention devices on the water line.
- The tank holding the insecticide mixture must be free of rust, fertilizer, sediment, and foreign material, and equipped with an in-line strainer situated between the tank and the injector point.

Chemigation Operation: Start the water pump and irrigation system, and let the system achieve the desired pressure and speed before starting the injector. Check for leaks and uniformity and make repairs before any chemigation takes place. Start the injector system and calibrate according to manufacturer's specifications. This procedure is necessary to deliver the desired rate per acre in a uniform manner. When the application is finished, flush and clean the entire irrigation and injector system prior to shutting down the system.

Chemigation Precautions:

- Crop injury, lack of effectiveness, or illegal pesticide residues in the crop can result from non-uniform distribution of treated water.
- If you have questions about calibration, contact state extension service specialists, equipment manufacturers, or other experts.
- A person knowledgeable of the chemigation system and responsible for its operation, or under the supervision of the responsible person, shall operate the system and make necessary adjustments should the need arise and continuously monitor the injection.

Chemigation Restrictions:

- Do not add crop oil when Lorsban Advanced is applied by chemigation.
- Do not connect an irrigation system (including greenhouse systems) used for pesticide application to a public water system.
- The pesticide injection pipeline must contain a functional, automatic, quick-closing check valve to prevent the flow of fluid back toward the injection.

- The pesticide injection pipeline must contain a functional, normally closed, solenoid-operated valve located on the intake side of the injection pump and connected to the system interlock to prevent fluid from being withdrawn from the supply tank when the irrigation system is either automatically or manually shut down.
- The system must contain functional interlocking controls to automatically shut off the pesticide injection pump when the water pump motor stops, or in cases where there is no water pump, when the water pressure decreases to the point where pesticide distribution is adversely affected.
- Systems must use a metering pump, such as a positive displacement injection pump (e.g., diaphragm pump) effectively designed and constructed of materials that are compatible with pesticides and capable of being fitted with a system interlock.
- Do not apply when wind speed favors drift beyond the area intended for treatment. End guns must be turned off during the application if they irrigate non-target areas.
- Do not allow irrigation water to collect or runoff and pose a hazard to livestock, wells, or adjoining crops.
- Do not enter treated area during the reentry interval specified in the Agricultural Use Requirements section of this label unless required PPE is worn.
- Do not apply through sprinkler systems that deliver a low coefficient of uniformity such as certain water drive units.

Mixing Directions

Lorsban Advanced – Alone
To prepare the spray, add a portion of the required amount of water to the spray tank and, with the spray tank agitator operating, add Lorsban Advanced. Complete filling the tank with the balance of water needed. Maintain sufficient agitation during both mixing and application to ensure uniformity of the spray mixture.

Lorsban Advanced – Tank Mix
Lorsban Advanced is compatible with insecticides, miticides, and fungicides and non-pressure fertilizer solutions except for alkaline materials, such as bordeaux mixture and lime. Conduct a small jar compatibility test prior to tank mixing. Prepare tank mixtures in the same manner as directed above for use of Lorsban Advanced alone. When tank mixing Lorsban Advanced with herbicides, add wettable powders first, flowables second, and emulsifiable concentrates last. For best results when a fertilizer solution is involved, use a fertilizer pesticide compatibility agent, such as Unite or Complex. Maintain constant agitation during both mixing and application to ensure uniformity of the spray mixture. Do not allow spray mixtures to stand overnight.

Tank Mix Compatibility Test: Test compatibility of the intended tank mixture before adding Lorsban Advanced to the spray or mix tank. Add proportional amounts of each tank mix ingredient to a clear glass pint or quart jar with a lid, cap it, invert the jar several times. Observe the mixture for approximately 1/2 hour. If the mixture balls-up, forms flakes, sludges, jels, oily films or layers, or other precipitates that do not readily redisperse, it is an incompatible mixture that must not be used.

Uses

Alfalfa
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Apply as a broadcast foliar spray using aircraft or ground spray equipment. Use a higher rate in the rate range for increased pest pressure. Use a minimum spray volume of 2 gpa for aerial application (fixed wing or helicopter) or 10 gpa for ground equipment. Use a spray volume of 5 gpa or more by air or up to 20 gpa by ground when foliage is dense and/or pest population is high and/or under high temperature and wind conditions. Some reduction in insect control may occur under unusually cool conditions.

Chemigation: Lorsban Advanced may be applied through sprinkler irrigation systems to control listed foliar pests. Use specified broadcast application rates. See Chemigation Application section.

Target Pests	Lorsban Advanced (pint/acre)
corn rootworm adults (spotted cucumber beetle) grasshoppers leafhoppers	0.5 - 1
alfalfa blotch leaf miner alfalfa caterpillar alfalfa weevil larvae and adults armyworms blue alfalfa aphid chinch bug cowpea aphid crickets cutworms Egyptian alfalfa weevil larvae and adults (1) greenbugs green June beetle grubs mites (Bermuda grass stunt) (clover) (two-spotted) (winter grain) pea aphid plant bugs sod webworm sowbugs spittlebugs spotted alfalfa aphid (suppression) (not for use in California)	1 - 2
alfalfa webworm	1.5

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:
1. **In California:** For Egyptian alfalfa weevil control, apply the specified dosage in a minimum of 5 gpa of water when larvae are actively feeding.

Specific Use Precautions:
- Do not tank mix Lorsban Advanced with other pesticides, surfactants, or fertilizer formulations unless prior use has shown the combination to be non-injurious to alfalfa under current conditions of use. Some phytotoxic symptoms may be observed on young, tender, rapidly growing alfalfa treated with Lorsban Advanced. Alfalfa will outgrow these symptoms and no yield loss should be expected.
- This product is highly toxic to bees exposed to direct treatment on alfalfa. Do not apply if nearby bees are clustered outside of hives and bees are foraging in the treated area. Protective information may be obtained from your Agricultural Extension Service.
- To avoid contamination of irrigation tail waters, do not flood irrigate within 24 hours following an application of Lorsban Advanced.

Specific Use Restrictions:
- **Preharvest Interval:** Do not cut or graze treated alfalfa within 7 days after application of 1/2 pint of Lorsban Advanced per acre, within 14 days after application of 1 pint per acre, or within 21 days after application of rates above 1 pint per acre.
- Do not make more than four applications of Lorsban Advanced or other product containing chlorpyrifos per season or apply any product containing chlorpyrifos more than once per alfalfa cutting.
- Maximum single application rate is 0.94 lb ai chlorpyrifos (2 pints of Lorsban Advanced) per acre.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.

Apple Tree Trunk
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 4 days unless PPE required for early entry is worn.

Apply as a post-bloom application to the lower 4 feet of the apple tree trunk for borer control in states east of the Rockies only (except Mississippi). Mix with water and apply directly to trunk from a distance of no more than 4 feet using low volume handgun or shielded spray equipment. Do not allow spray to contact foliage or fruit.

Target Pests	Lorsban Advanced (quart/100 gal)
American plum borer apple bark borer broad necked root borer dogwood borer flatheaded appletree borer roundheaded apple tree borer tilehorned prionus	1.5

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 28 days before harvest.
- Do not make more than one application of Lorsban Advanced to the apple tree trunk per year as either a prebloom or post-bloom application.
- This product may not be used if a prebloom application of any other product containing chlorpyrifos has been made during the year.
- Do not allow meat or dairy animals to graze in treated orchards.
- Treat only the lower 4 feet of the apple tree trunk.
- Do not apply when wind speed is greater than 10 mph.

Asparagus

(For use only in Arizona, California, Idaho, Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, Oregon, South Dakota, Washington, and Wisconsin)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Apply as a ground broadcast foliar spray. Use sufficient volume of finished spray to ensure thorough coverage of crop foliage. **Note:** Lorsban Advanced may be applied aerially or with ground equipment for control of armyworms and grasshoppers.

Pests	Lorsban Advanced (pint/acre)
armyworms (1) asparagus aphids (1) asparagus beetles (1) cutworms (2) grasshoppers (1) symphylans (3)	2

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:
1. **Armyworms, asparagus beetles, asparagus aphids**, and **grasshoppers:** Apply during the fern stage when field counts or crop injury indicates that damaging pest populations are developing or present.
2. **Cutworms:** For best results, apply when the soil is moist and worms are active on or near the soil surface.
3. **Symphylans:** Apply it at least two weeks before harvest for optimum control.

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 1 day before harvest.
- Do not make more than one preharvest application per season.
- Do not make more than two postharvest applications during the fern stage.
- Maximum single application rate preharvest or postharvest is 0.94 lb ai chlorpyrifos (2 pints of Lorsban Advanced) per acre.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.

Brassica (Cole) Leafy Vegetables[1], Radish, Rutabaga, and Turnip

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours (3 days for cauliflower) unless PPE required for early entry is worn.

[1]*Brassica* (cole) leafy vegetables including broccoli, broccoli raab, Brussels sprout, cabbage, cauliflower, cavalo broccolo, Chinese broccoli, Chinese cabbage, collards, kale, kohlrabi, mizuna, mustard greens, mustard spinach, rape greens

Specific Use Restriction: If a preplant incorporation application for direct seeded or transplanted crops is made, **do not** apply this product as an at-plant or post plant soil application. If an at-plant or post plant soil application is made, **do not** apply this product as a preplant incorporation application for direct seeded or transplanted crops.

Preplant Incorporation Application for Direct Seeded or Transplanted Crops

Apply Lorsban Advanced as a broadcast spray to the soil surface using power-operated ground spray equipment. Use a total spray volume of 10 gpa or more. On the day of treatment, incorporate Lorsban Advanced into the top 2 to 4 inches of soil using a disc, field cultivator, or equivalent equipment.

Crop	Target Pests	Lorsban Advanced (pints/acre)
cauliflower	billbugs cutworms grubs root maggot symphylans wireworms	4
broccoli broccoli raab Brussels sprout cabbage cavalo broccolo Chinese broccoli Chinese cabbage collards kale kohlrabi mizuna mustard greens mustard spinach rape greens turnip		4.5
radish		5.5
rutabaga		4.5

Specific Use Precautions:

Insecticides, including Lorsban Advanced, may contribute to the stress of plants under certain environmental conditions. This stress may reduce plant stand or interfere with normal plant development. Herbicides used preplant incorporated may interact with insecticides and enhance this stress.

At-Plant or Post Plant Soil Application

Apply as indicated in Pest-Specific Use Directions. Use a higher rate in the rate range when there is increased pest pressure.

Crop	Target Pests	Lorsban Advanced (fl oz/1000 ft of row)
cauliflower	root maggot (1)	1.6 – 2.4
broccoli broccoli raab Brussels sprout cabbage cavalo broccolo Chinese broccoli Chinese cabbage collards kale kohlrabi mizuna mustard greens mustard spinach rape greens turnip		1.6 – 2.75
broccoli cabbage	root aphid (2)	1.2 (2.4 for double row plantings)
Radish	root maggot (3)	1
Rutabaga	root maggot (1)	1.6 – 3.3

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:
1. **Root maggot:**
   - **Direct seeded crops [broccoli, broccoli raab, Brussels sprout, cabbage, cauliflower, cavalo broccolo, Chinese broccoli, Chinese cabbage, collards, kale, kohlrabi, mizuna, mustard greens, mustard spinach, rape greens, rutabaga, turnip]:** Apply the specified dosage in a water-based spray as a 4-inch wide band over the row at planting time. Place band behind the planter shoe and in front of the press wheel to achieve shallow incorporation. Use a minimum of 40 gpa total spray volume.
   - **Transplanted crops [broccoli, broccoli raab, Brussels sprout, cabbage, cauliflower, cavalo broccolo, Chinese broccoli, Chinese cabbage, collards, kale, kohlrabi, mizuna, mustard greens, mustard spinach, rape greens, turnip]:** Apply

Lorsban Advanced as a water-based spray directed to the base of the plants immediately after setting. Use a minimum of 40 gpa total spray. Do not add any additional adjuvants, surfactants or spreader stickers. Do not apply as a foliage application.

2. **Root aphid (broccoli, cabbage):** Apply Lorsban Advanced in water or with liquid fertilizer injected as a sidedress on each side of the row after plants are established. See Mixing Directions section for Mixing Instructions for Liquid Fertilizer. Avoid mechanical damage to crop roots. Use a minimum of 15 gpa of total spray volume.
3. **Root maggot (radish):** Apply the specified dosage as a water-based drench in the seed furrows with the seed at planting time. Use a minimum of 40 gpa of total drench.

Specific Use Restrictions for Preplant Incorporation and At-Plant or Post Plant Soil Applications:

- **Soil applications (all labeled crops):**
  - ◊ **Preharvest Interval:** Do not apply within 30 days before harvest.
  - ◊ Do not foliarly apply any chlorpyrifos product labeled for foliar application (e.g., Lorsban 50W) within 10 days of a soil application of Lorsban Advanced.
  - ◊ Do not aerially apply this product in Mississippi.
- **Cauliflower:** Do not apply more than 2 pints of Lorsban Advanced to cauliflower planted in 40-inch rows. Use proportional amounts for other row spacings, but do not exceed 4 pints of Lorsban Advanced per acre. The maximum single application rate for cauliflower is 1.2 oz ai chlorpyrifos (2.4 fl oz of Lorsban Advanced) per 1000 ft of row.
- **Broccoli, broccoli raab, Brussels sprout, cabbage, cavalo broccolo, Chinese broccoli, Chinese cabbage, collards, kale, kohlrabi, mizuna, mustard greens, mustard spinach, rape greens, turnip:** Do not apply more than 2.6 pints of Lorsban Advanced per acre when planted in 40-inch rows. Do not apply more than 4.5 pints of Lorsban Advanced per acre to these crops when in 20-inch rows (or two rows per bed). Use proportional amounts for other row spacings, but do not exceed 4.5 pints of Lorsban Advanced per acre.
- **Radish:** Do not apply more than 5.5 pints of Lorsban Advanced per acre. The maximum single application rate for radish is 0.5 oz ai chlorpyrifos (1 fl oz of Lorsban Advanced) per 1000 ft of row.
- **Rutabaga:** Do not apply more than 4.5 pints of Lorsban Advanced per acre. The maximum single application rate for rutabaga is 1.6 oz ai chlorpyrifos (3.2 fl oz of Lorsban Advanced) per 1000 ft of row. Do not use rutabaga tops for food or feed purposes.

Foliar Application [*Brassica* (Cole) Leafy Vegetables Only]
Apply with conventional power-operated spray equipment in 20 to 150 gpa of water. For aerial applications, apply in a minimum of 5 gpa of water. Use a higher rate in the rate range when there is increased pest pressure. Consult your state agricultural experiment station, extension service specialist, or integrated pest control advisor for proper time to treat in your area.

To avoid phytotoxicity, do not treat plants under stress from extreme heat and/or lack of moisture. For best results, tank mix only if previous experience indiates that the combination will not result in phytotoxicity under the current conditions of use and the other pesticides and spray adjuvants are registered for this use. Read and carefully follow all applicable directions, restrictions, and precautions on other product labels used in combination with Lorsban Advanced. Tank mixing Thiodan 3EC, Thiodan 50WP, or cottonseed oil is not recommended.

Target Pests	Lorsban Adavnced (pint/acre)
armyworms cabbage aphid cutworms imported cabbage worm striped flea beetle (adult)	1 – 2

Specific Use Restrictions:

- **Preharvest Interval:** Do not apply within 21 days before harvest.
- Do not make more than three applications of any product containing chlorpyrifos per crop.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.
- Do not aerially apply this product in Mississippi.

Christmas Trees (Plantations Only)
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Unless otherwise indicated, apply as a foliar spray using power-operated ground equipment. Thorough coverage of foliage is essential. Use a minimum 10 gpa of finished spray with ground equipment. Use higher volume of finished spray, 20 gpa or more, when foliage is dense and/or pest density is high and/or under high temperature and wind conditions.

Target Pests		Lorsban Advanced
ants (4) aphids adelgids cooley eastern spruce gall Douglas fir needle midge European pine sawfly European pine shoot moth grasshoppers gypsy moth mites (1) European red spider two spotted spider	pales weevil (adult) pine needle midge pine spittlebug plant bugs scale (2) black pine pine needle pine tortoise spruce bud striped pine spittlebugs spruce budworm spruce needleminer	1 quart/acre
pales weevil (3)		3 quarts/100 gal

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:

1 **Spider mites:** When large numbers of eggs are present at the first application, a second application after 7 to 10 days may be required to control newly hatched nymphs and maintain effective control. **Not for control of mites in Washington and Oregon.**
2. **Scale:** For control, apply when scale crawlers are active.
3. Apply as a cut stump drench.
4. Excludes fire, harvester, carpenter, and pharaoh ants.

Specific Use Precautions:
Phytotoxicity: Do not apply under conditions of extreme heat or drought stress. Environmental factors and varietal differences significantly influence potential phytotoxic expression. **Testing has shown that Lorsban Advanced may be used at specified rates on the following conifer species without serious phytotoxicity: balsam fir, concolor fir, Douglas fir, eastern white pine, Fraser fir, grand fir, noble fir, Scotch pine, white spruce.** Before treating large numbers of other conifer species, treat a small block of plants and observe them 7 to 10 days for symptoms of phytotoxicity. **Note:** The user assumes responsibility for determining if it is safe to treat other conifer species with Lorsban Advanced under commercial growing conditions.

Specific Use Restrictions:

- Do not make more than three applications of Lorsban Advanced or other product containing chlorpyrifos per season.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 7 days of the first application.
- Do not allow meat or dairy animals to graze in treated areas.

Citrus Fruits[1]
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 5 days unless PPE required for early entry is worn.

[1]Citrus fruits including calamondin, chironja, citrus citron, citrus hybrids, grapefruit, kumquat, lemon, lime, mandarin (tangerine), pummelo, satsuma mandarin, sour orange, sweet orange, tangelo, tangor

Apply as a concentrate or dilute spray using conventional, power-operated spray equipment. Use a higher rate in the rate range when there is increased pest pressure. Use sufficient water to ensure thorough and complete coverage of the foliage and fruit. For dilute sprays (greater than 200 gpa), use a spray concentration of at least 0.5 pints of Lorsban Advanced per 100 gallons of finished spray. Complete coverage is not necessary for outside canopy sprays targeting certain pests such as lepidoptera insects and katydids. Treat when pests become a problem or in accordance with the local spray schedule as specified by your State Agricultural Experiment Station, certified Pest Control Advisor, or Extension Service Specialist. To avoid excessive ridging, do not apply Lorsban Advanced to citrus from December 1 up to the initiation of bloom (5% visible bloom).

Low Volume Application (for use in Florida only): Apply Lorsban Advanced at the rate of 3 to 5 pints per acre as a low volume application (e.g., 2 to 5 gallons of water) to control Asian citrus psyllid. Do not make low volume applications when wind speed is more than 5 mph. Regardless of the application method used (air, low volume, airblast), treat only a few acres when using the lower rate or a new application method to determine the effectiveness in the citrus grove.

Use of Spray Oils: To improve control of aphids, mealybugs, scale insects, and thrips, a petroleum spray oil specified for use on citrus trees may be added to spray mixtures at up to 1 gallon per 100 gallons of spray.

Target Pests	Lorsban Advanced (pint/acre)
aphids (including brown citrus aphid) brown marmorated stink bug glassywinged sharpshooter grasshoppers (1) katydids lepidopterous larvae avocado leafroller cutworms fruit tree leafroller orange dogs orange tortrix western tussock moth mealybugs (see below for California and Arizona) scale insects black scale brown soft scale (California red scale (see below for California and Arizona) chaff scale Florida red scale long scale purple scale snow scale thrips (see below for California and Arizona)	2 - 7
citrus rust mites (2) (3)	4 - 7
citrus psylla (4)	5
thrips suppression and mealybugs (California and Arizona, see restrictions)	6 - 12
California red scale (California and Arizona, see restrictions)	8 -12

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:

1. **Lubber grasshoppers:** Effective control requires direct contact with spray when grasshoppers are small (less than 1 inch in length).
2. **Citrus rust mites:** For control, use a spray concentration of at least 1 pint of Lorsban Advanced per 100 gallons.
3. In Los Angeles, Monterey, Orange, San Diego, San Luis Obispo, Santa Barbara, and Ventura Counties in California, Lorsban Advanced may be tank mixed with petroleum spray oils registered for control of mites in citrus. Follow all label directions and precautions for Lorsban Advanced and tank mix partners. Do not exceed 1.8% oil v/v or 1.8 gallons of oil per 100 gallons of spray. Use only on citrus species and varieties for which Lorsban Advanced is registered.
4. **Citrus psylla:** For control, add citrus oil at 2% v/v in a tank mix with Lorsban Advanced.

Specific Use Precautions:

- Observe local recommendations for tank mix combinations especially with regard to use of Lorsban Advanced with spray oil. Do not use penetrating surfactants in tank mixes with Lorsban Advanced. Consult with a county farm advisor, county agency, extension service personnel, agricultural commissioner, pest control advisor, or local Dow AgroSciences representative for local recommendations.
- Do not apply when trees are stressed by drought or high temperatures.
- Lorsban Advanced is highly toxic to bees exposed to direct treatment and must not be applied when bees are actively visiting the area. During the citrus bloom period in California, apply from 1 hour after sunset until 2 hours before sunrise.
- Do not use Lorsban Advanced in combination with spray oil when temperatures are expected to exceed 95°F on the day of application or for several consecutive days thereafter.

Specific Use Restrictions:

- **Preharvest Interval:** Do not apply within 21 days before harvest for applications of up to 7 pints of Lorsban Advanced per acre or within 35 days for application of rates above 7 pints per acre.
- Do not make more than two applications of Lorsban Advanced or other product containing chlorpyrifos per year (does not include citrus orchard floors).
- Do not apply more than a total of 7.04 lb ai chlorpyrifos (16 pints of Lorsban Advanced) per acre per year.
- Do not make a second foliar application of Lorsban Advanced or other product containing chlorpyrifos within 30 days of the first application.
- The use of application rates greater than 4 lb ai chlorpyrifos (8.5 pints of Lorsban Advanced) per acre are allowed only in the following counties in California: Fresno, Tulare, Kern, Kings, and Madera.
- Do not allow meat or dairy animals to graze in treated areas.

Citrus[1] Orchard Floors
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 5 days unless PPE required for early entry is worn.

[1]Citrus fruits including calamondin, chironja, citrus citron, citrus hybrids, grapefruit, kumquat, lemon, lime, mandarin (tangerine), pummelo, satsuma mandarin, sour orange, sweet orange, tangelo, tangor

Apply as a ground broadcast spray directed to the orchard floor to control foraging ants (excluding fire, harvester, carpenter and pharaoh ants). Do not apply spray to contact foliage or fruit. Apply in a total spray volume of 25 gpa or more using equipment that will apply the spray uniformly to the soil surface. Use a higher rate in the rate range for increased pest pressure. For best results, remove weed growth or other obstructions that might prevent the spray from reaching the soil surface. Foliar applications of Lorsban Advanced or other products containing chlorpyrifos may be made in addition to the orchard floor treatments but must comply with the 10 day re-treatment interval (see Specific Use Restrictions).

Chemigation: Lorsban Advanced may be applied to citrus orchard floors through sprinkler irrigation systems only if the system uniformly covers the soil surface at the base of the tree. Apply at specified broadcast application rates to control listed pests. See Chemigation Application section.

Note: Do not apply in tank mixture with Evik herbicide.

Target Pests	LorsbanAdvanced (pint/acre)
ants (1)	1.5 - 2

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:

1. Excludes fire, harvester, carpenter, and pharaoh ants.

Application with Dry Bulk Fertilizer: Most dry fertilizers can be used for impregnation with Lorsban Advanced. Apply Lorsban Advanced at the equivalent broadcast rate using a minimum of 200 lb per acre of dry bulk fertilizer.

Impregnation of Dry Bulk Fertilizer: Use a closed rotary drum mixer suitable for blending of dry bulk fertilizer equipped with an internal spray nozzle. Add the dry fertilizer to the mixer followed by the appropriate amount of Lorsban Advanced. After mixing the dry ingredients to ensure uniformity, add water through the spray nozzle in an amount sufficient to just dampen the mixture (4 to 8 pints of water per ton of fertilizer). Position the spray nozzle within the mixer to provide uniform coverage of the tumbling mixture of fertilizer and Lorsban Advanced. Addition of water will cause Lorsban Advanced to uniformly adhere to the dry bulk fertilizer. Apply bulk fertilizers impregnated with Lorsban Advanced immediately, **do not store it**. Foliar applications of Lorsban Advanced may be made in addition to the orchard floor treatments.

Compliance with any and all federal and state laws and regulations relating to the Lorsban Advanced and fertilizer mixture is the responsibility of the person offering such mixture for sale or distribution.

Specific Use Restrictions:

- **Preharvest Interval:** Do not apply within 28 days before harvest.
- Do not make more than three applications of Lorsban Advanced or other product containing chlorpyrifos per year (does not include foliar applications to citrus trees).
- Maximum single application rate is 1 lb ai chlorpyrifos (2 pints of Lorsban Advanced) per acre.
- Do not apply more than a total of 2.82 lb ai chlorpyrifos (3 quarts of Lorsban Advanced) per acre per year.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.
- Do not allow meat or dairy animals to graze in treated areas.

Corn (Field, Sweet, Seed)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Conservation Tillage: Preplant, At-Plant, or Preemergence Applications

Apply as a broadcast spray to surface trash and exposed soil using power-operated ground spray equipment. Use a total spray volume of 20 gpa or more. Use a higher rate in the rate range to extend residual control.

Tank Mixing: Lorsban Advanced may also be applied in tank mixtures with paraquat or glyphosate and/or liquid fertilizer solutions. See Mixing Directions section for tank mixing instructions. Read and carefully follow all applicable directions, restrictions, and precautions on labeling for each product used in combination with Lorsban Advanced.

Target Pests	Lorsban Advanced (pint/acre)
armyworms cutworms	1 - 2

Postemergence Application
Apply as a postemergence broadcast spray using sufficient spray volume to ensure thorough coverage of treated plants, but no less than 15 gpa for ground spray equipment or 2 to 5 gpa for aircraft equipment. Control may be reduced at low spray volumes under high temperature and wind conditions. Lorsban Advanced may be tank mixed with glyphosate products, such as Duramax® herbicide or Durango® DMA® herbicide, when application is to be made to glyphosate-tolerant corn.

Chemigation: Lorsban Advanced may be broadcast applied postemergence through sprinkler irrigation systems at specified application rates to control listed foliar pests. For best results, tank mix Lorsban Advanced with 2 pints of non-emulsifiable oil. See Chemigation Application section.

Target Pests	Lorsban Advanced (pint/acre)
grasshoppers	0.5 – 1
aphids armyworms chinch bugs (1) corn rootworm adults (2) cutworms (3) European corn borer (5) flea beetle adults (1) southern corn leaf beetle webworms (4) western bean cutworm	1 - 2
brown marmorated stink bug corn earworm southwestern corn borer (6)	1.5 - 2
billbugs (1) common stalk borer (9) corn rootworm larvae (7), (8) lesser cornstalk borer	2

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:
1. **Billbug, chinch bug,** or **flea beetle:** For best control, ground apply in a minimum spray volume of 20 to 40 gpa at 40 psi. If corn is less than 6 inches tall, apply in a 9- to 12-inch wide band over the row. For corn more than 6 inches tall, apply using drop nozzles directed to the base of the plant. Do not reduce the application rate for banded or directed applications. Concentrate the full labeled dosage rate in the treated zone. When chinch bugs continue to immigrate to corn over a prolonged period or under extreme pest pressure, a second application may be needed.
2. **Corn rootworm adults:** The specified dosage will control silk clipping.
3. **Cutworms:** It is preferable to apply Lorsban Advanced when soil is moist and worms are active on or near the soil surface. If ground is dry, cloddy, or crusted at time of treatment, worms may be protected from the spray and effectiveness will be reduced. Shallow incorporation using a rotary hoe or other suitable equipment immediately before or soon after treatment may improve control. A second application may be required if damage or density levels exceed economic thresholds established for your area.
4. **Webworm:** For control, shallow incorporation using a rotary hoe or other suitable equipment immediately before or soon after treatment is necessary.
5. **European corn borer:** For control, use 1.5 to 2 pints per acre when application is made with power-operated ground or aerial equipment, or 1 to 2 pints per acre when application is made through a sprinkler irrigation system. University research indicates that achieving greater than 50% control of first-generation European borer with a single liquid insecticide treatment is highly dependent upon timing, insecticide placement, and weather conditions.
6. **Southwestern corn borer:** A second application may be applied 21 days later if needed due to reinfestation.
7. **Corn rootworm larvae:** For postemergence control, apply at cultivation. Direct the spray to both sides of the row at the base of the plants just ahead of the cultivator shovels. Cover the insecticide with soil around the brace roots. A cultivation application of Lorsban Advanced may be made in addition to an at-planting application of Lorsban 15G.
8. Lorsban Advanced may also be applied through sprinkler irrigation systems at the rate of 2 pints per acre to control **corn rootworm larvae.** Time application to coincide with the appearance of the second instar larvae. Apply with enough water to wet the root zone to the depth control needed. If soils are wet, allow enough soil drying to occur such that an application using a minimum amount of water will not produce surface runoff. See Chemigation Application section for application instructions.
9. Do not use Lorsban Advanced in combination with a burndown herbicide for control of common stalk borer. For **common stalk borer** control, treat approximately 11 days after application of glyphosate or after burndown with paraquat herbicide is complete (3 to 5 days).

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 21 days before harvest of grain, ears, forage or fodder.
- Do not make more than three applications of Lorsban Advanced or any product containing chlorpyrifos per season, including the maximum allowed of two granular applications, at the 1 lb ai chlorpyrifos rate.
- Maximum single application rate is 1 lb ai chlorpyrifos (2.13 pints of Lorsban Advanced) per acre.
- Do not apply more than 3 lb ai chlorpyrifos (6.38 pints of Lorsban Advanced) per acre per season.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.
- If more than 1 lb ai granular chlorpyrifos per acre is applied at-plant (for a maximum of 1.3 lb ai per acre per season), only one additional application of a liquid product containing chlorpyrifos at 1 lb ai per acre is allowed per season, for a total of 2.3 lb ai chlorpyrifos per acre per season.
- Do not apply in tank mixes with Steadfast or Lightning herbicides.
- Do not aerially apply this product in Mississippi.

Cotton
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Apply as a broadcast foliar spray using aircraft or ground spray equipment (see separate rate table for Arizona and California). Use a higher rate in the rate range when there is increased pest pressure. Use sufficient spray volume to ensure thorough coverage of treated plants, but no less than 10 gpa for ground spray equipment or 2 gpa for aircraft equipment. Increase spray volume when foliage is dense and/or pest population is high and/or under high temperature and wind conditions. Treat when field counts indicate damaging insect populations are developing or present.

Chemigation: Lorsban Advanced may be applied through sprinkler irrigation systems at specified broadcast application rates to control listed foliar pests. See Chemigation Application section.

Proper application methods are necessary to ensure thorough spray coverage and correct rate, and minimize off-target drift. Follow Application Directions for ground and aerial application and Spray Drift Management recommendations in Product Information section of this label.

All States Except Arizona and California

Target Pests	Lorsban Advanced (pint/acre)
cotton fleahopper (1) plant bugs (1) (*Lygus, Mirids*)	0.37 - 1
grasshoppers thrips	0.5 - 1
cotton aphid fall armyworm yellowstriped armyworm	0.5 - 2
spider mites (2)	1
beet armyworm cotton bollworm (3) cutworms pink bollworm salt marsh caterpillar tobacco budworm (3)	1.5 - 2

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:

1. The 0.37 pint per acre rate will not provide a high degree of control but, compared to the 1 pint per acre rate, will minimize the damage from **plant bugs** and **cotton fleahoppers** and allow increased survival and build-up of beneficial insects to aid in the control of **bollworms** infesting cotton.
2. **Spider mites:** When large numbers of eggs are present, scout the treated area in 3 to 5 days. If newly hatched nymphs are present, make a follow-up application of a non-chlorpyrifos product that is effective against mites.
3. **Bollworms** and **budworms:** For best results, scout fields twice per week and apply when worms are 1/4 inch or less in length.

Arizona and California

Target Pests	Lorsban Advanced (pint/acre)
armyworms cotton aphid cotton fleahopper *Lygus* salt marsh caterpillar silverleaf whitefly (1) thrips	1 - 2
boll weevil cotton bollworm (2) cotton leaf perforator (suppression) cutworms pink bollworm spider mites (suppression) tobacco budworm (2)	2

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:

1. **Silverleaf whitefly:** Apply in tank mix combination with the specified rate of a pyrethroid insecticide labeled for control or suppression.
2. **Bollworms** and **budworms:** For best results, scout fields twice per week and apply when worms are 1/4 inch or less in length.

Specific Use Restrictions:

- **Preharvest Interval:** Do not apply within 14 days before harvest.
- Do not make more than three applications of Lorsban Advanced or other product containing chlorpyrifos per crop season.
- Maximum single application rate is 0.94 lb ai chlorpyrifos (2 pints of Lorsban Advanced) per acre.
- Do not apply more than 2.82 lb ai chlorpyrifos (6 pints of Lorsban Advanced) per acre per season.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.
- Do not allow meat or dairy animals to graze in treated areas.
- Do not feed gin trash or treated forage to meat or dairy animals.

Cranberry

(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Apply as a broadcast foliar spray. Use sufficient spray volume to ensure thorough coverage, but no less than 15 gpa. Except for control of cranberry weevil, treat when field counts indicate damaging insect populations are developing or present.

Chemigation: Lorsban Advanced may be applied through sprinkler irrigation systems to control listed pests. Apply at specified broadcast application rates. See Chemigation Application section.

Target Pests	Lorsban Advanced (pint/acre)
brown spanworm cranberry fruitworm cranberry weevil (1) cutworms fireworms sparganothis fruitworms	3

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:

1. **Cranberry weevil:** For control, apply once at flower bud development (late May, early June) and, if cranberry weevils are present, once after 100% bloom (early to mid-July).

Specific Use Precautions:

Apply only after the winter flood water has been removed. To avoid pesticide contamination of flood waters, do not apply when bogs are flooded.

Specific Use Restrictions:

- **Preharvest Interval:** Do not apply within 60 days before harvest.
- Do not make more than two applications of Lorsban Advanced or other product containing chlorpyrifos per season.
- Maximum single application rate is 1.41 lb ai chlorpyrifos (3 pints of Lorsban Advanced) per acre.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.

Fig

(For use only in California)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 4 days unless PPE required for early entry is worn.

Apply Lorsban Advanced as a dormant application in late winter prior to beetle emergence and prior to leaf formation. Use a spray volume of 10 gpa or more and apply as a broadcast spray to the soil surface using power-operated ground spray equipment. On the day of treatment, incorporate Lorsban Advanced into the top 3 inches of soil using suitable equipment.

Target Pest	Lorsban Advanced (quart/acre)
brown marmorated stink bug dried fruit beetle	2

Specific Use Restrictions:

- **Preharvest Interval:** Do not apply within 217 days (7 months) before harvest.
- Make only one application per year of Lorsban Advanced or other product containing chlorpyrifos.
- Maximum single application rate is 1.88 lb ai chlorpyrifos (2 quarts of Lorsban Advanced) per acre.

Grape

(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Soil Surface Application
(For use in areas east of the Continental Divide only)

Apply Lorsban Advanced just before the pest emerges from the soil. Apply 2 quarts of the diluted spray mixture to the soil surface on a 15-square foot area (4.4 foot circle) around the base of each vine.

Target Pest	Lorsban Advanced (pint/100 gal)
grape borer	4.5

Specific Use Restrictions for Soil Surface Application:

- Do not allow spray to contact fruit or foliage.
- Maximum single application rate for soil surface application is 2.12 lb ai chlorpyrifos (4.5 pints of Lorsban Advanced) per acre.

Prebloom Application
(For use in areas east of the Continental Divide only)

Apply as a spray drench ground application using a minimum spray volume of 25 gpa.

Target Pest	Lorsban Advanced (quart/acre)
brown marmorated stink bug climbing cutworm[1] grape mealybugs[2] grape scale	1

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:

1. **Cutworm:** For control, apply 1 quart of Lorsban Advanced per acre as a broadcast spray in a minimum spray volume of at least 50 gallons of water using power-operated ground spray equipment. Treat when cutworms first become active and when field counts indicate damaging insect populations are developing or present. Do not apply after bloom stage of growth. Consult your state agricultural experiment station or

extension service specialist concerning cutworm control practices in your area.

2. **Grape mealybug:** For control, apply 1 quart of Lorsban Advanced per acre in a minimum spray volume of at least 50 gallons of water per acre using power-operated ground spray equipment only prior to late budbreak. Applications after budbreak may result in transient leaf yellowing (Concords).

Specific Use Restrictions for Prebloom Application:
- Do not use in conjunction with soil surface application for grape borer control.
- Maximum single application rate for prebloom application to minimize phytotoxicity is 0.94 lb ai chlorpyrifos (1 quart of Lorsban Advanced) per acre.

Specific Use Restrictions for Soil Surface Application and Prebloom Application:
- **Preharvest Interval:** Do not apply within 35 days before harvest.
- Do not make more than one application of Lorsban Advanced or other product containing chlorpyrifos per season.
- Based upon available residue data, the use of Lorsban Advanced in grapes is restricted to areas east of the Continental Divide only. Do not use in the state of Mississippi.

Legume Vegetables (Succulent or Dried) (Except Soybean)[1]
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

[1]Legume vegetables including adzuki bean, asparagus bean, bean, blackeyed pea, broad bean (dry and succulent), catjang, chickpea, Chinese longbean, cowpea, crowder pea, dwarf pea, edible pod pea, English pea, fava bean, field bean, field pea, garbanzo bean, garden pea, grain lupin, green pea, guar, hyacinth bean, jackbean, kidney bean, lablab bean, lentil, lima bean, moth bean, mung bean, navy bean, pea, pigeon pea, pinto bean, rice bean, runner bean, snap bean, snow pea, southern pea, sugar snap pea, sweet lupin, sword bean, tepary bean, urd bean, wax bean, white lupin, white sweet lupin, yardlong bean

Preplant Broadcast Application
Apply Lorsban Advanced at a rate of 2 pints per acre to control seed maggots. Make a preplant broadcast application in a minimum of 10 gpa of spray to the soil surface using suitable ground equipment. To improve the activity against seed maggots, incorporate Lorsban Advanced into the top 1 to 3 inches of soil using suitable tillage equipment.

At-Plant T-Band Application
Apply 1.8 fl oz of Lorsban Advanced per 1000 feet of row at 30-inch row spacing. Apply the spray in a 3- to 5-inch wide band over the row behind the planter shoe and in front of the press wheel to achieve shallow incorporation. Mix the specified dosage in a minimum of 10 gpa of spray and apply to the soil surface using suitable ground spray equipment. Equivalent rates of insecticide spray required per 100 feet of row for listed row spacings are given in the accompanying table. To improve the activity of Lorsban Advanced against seed maggots, incorporate Lorsban Advanced into the top 1/2 to 1-inch of soil using tines or chains or other suitable equipment.

Spray Volume Per Acre (Gallons)	fl oz of Spray Volume per 100 Feet of Row			
	30-inch	28-inch	24-inch	22-inch
10	7.3	6.9	5.9	5.4
15	11	10.3	8.8	8.1
20	14.7	13.7	11.8	10.8

Specific Use Precautions: Insecticides, including Lorsban Advanced, may contribute to the stress of plants under certain environmental conditions. This stress may reduce plant stand or interfere with normal plant development. Herbicides used preplant incorporated may interact with insecticides and enhance this stress.

Specific Use Restrictions:
- Do not make more than one application of Lorsban Advanced per year.
- Do not apply more than 0.94 lb ai chlorpyrifos (2 pints of Lorsban Advanced) per acre.
- Do not apply Lorsban Advanced at-plant if the field was treated with a preplant incorporated treatment of Lorsban Advanced.

Onion (Dry Bulb)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

At-Plant Soil Drench Application
For direct seeded onions to control onion maggot, apply 32 fl oz of Lorsban Advanced per acre in a water-based spray as a 2- to 4-inch wide band over the row at planting time in a minimum of 40 gpa. Equivalent rates of insecticide spray required per 1000 feet of row for listed row spacings are given in the table below. Shallow incorporation is necessary. Place behind the planter shoe and in front of the presswheel. Phytotoxicity may occur if Lorsban Advanced is sprayed directly onto onion seeds. Do not mix Lorsban Advanced with other pesticide products. **Note**: The user should exercise reasonable judgment and caution with this product. Until familiar with results under user planting and growing conditions, limit application of this product to a small area to determine plant tolerance and extent of injury if such occurs prior to initiating large scale applications.

Lorsban Advanced (32 fl oz/acre)	Row Spacing			
	6-inch	10-inch	12-inch	18-inch
fl oz/1000 ft of row	0.37	0.61	0.74	1.1

Specific Use Restrictions:
- Do not make more than one application per year.
- Maximum single application rate is 0.032 lb ai chlorpyrifos per 1000 feet of row.
- Do not aerially apply this product in Mississipppi.

Postplant Soil Drench Application
Apply as an early season directed spray to the base of onion seedlings or transplants during peak egg laying. Use a minimum of 100 gpa for thorough wetting.

Target Pest	Lorsban Advanced (quart/acre)
onion maggot seedcorn maggot	1

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 60 days before harvest.
- Do not make more than two applications (at-plant plus postplant) per year.
- Maximum single application rate is 0.94 lb ai chlorpyrifos (1 quart of Lorsban Advanced) per acre.
- Do not aerially apply this product in Mississippi.

Peanut

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Apply to the soil surface as a preplant broadcast spray followed by immediate soil incorporation to a depth of 3 to 4 inches using a disc, field cultivator, or equivalent equipment. Use a minimum of 10 gpa total spray.

Target Pests	Lorsban Advanced (pint/acre)
wireworms (suppression)	4

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 21 days before harvest.
- Do not make more than one preplant application of Lorsban Advanced per season.
- Maximum single application rate is 1.88 lb ai chlorpyrifos (4 pints of Lorsban Advanced) per acre.
- The combined total of preplant and postplant applications of Lorsban Advanced, Lorsban 15G, or other product containing chlorpyrifos, must not exceed 4 lb ai chlorpyrifos per acre per season.
- Do not feed treated peanut forage or hay to meat or dairy animals.
- Do not aerially apply this product in Mississippi.

Pear
(For use only in California, Oregon and Washington)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Postharvest Application
Mix the specified dosage in 100 to 400 gpa of spray and apply using an airblast speed sprayer or other suitable ground equipment.

Target Pest	Lorsban Advanced (pint/acre)
brown marmorated stink bug codling moth	4

Specific Use Restrictions:
- Do not make more than one postharvest application (prior to dormancy) per year.
- Maximum single application rate is 1.88 lb ai chlorpyrifos (4 pints of Lorsban Advanced) per acre.
- Do not harvest or use treated fruit for food or feed.
- Do not allow meat or dairy animals to graze in treated orchards.
- If unauthorized entry into a treated orchard cannot be prevented, then the orchard must be posted with appropriate signs according to the Worker Protection Standard while treated, unharvested fruit remains on the tree.

Peppermint and Spearmint
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Apply as a broadcast spray using a total spray volume of 10 gpa or more using ground equipment.

Chemigation: Lorsban Advanced may be applied through sprinkler irrigation systems at specified broadcast application rates to control listed foliar pests. See Chemigation Application section.

Target Pests	Lorsban Advanced (pint/acre)
cutworm (1)	2 – 4
garden symphylans(2) mint root borer (3)	4

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:
1. **Cutworms:** Apply during May and June when field counts indicate damaging insect populations are developing or present. When larvae are less than 3/4 inch in length, use the 2 pint rate; otherwise, use a higher rate in the rate range.
2. **Garden symphylans:** Apply preplant to the soil surface. On the same day of treatment, incorporate the insecticide into the top 2 to 4 inches of soil using a disc, field cultivator, or equivalent equipment.
3. **Mint borer:** Apply postharvest when field counts indicate damaging insect populations are developing or present. If ground applied, follow with approximately 1 acre inch of sprinkler irrigation immediately after application to incorporate the insecticide into the soil or apply by chemigation.

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 90 days before harvest.
- Make only one application of Lorsban Advanced or other product containing chlorpyrifos during the growing season.
- Do not make more than one preplant incorporated application in the spring.
- Make only one postharvest application of Lorsban Advanced or other product containing chlorpyrifos per season.
- Maximum single application rate is 1.88 lb ai chlorpyrifos (4 pints of Lorsban Advanced) per acre.
- Do not use in conjunction with a broadcast foliar application of Lorsban Advanced for cutworm control.

Sorghum - Grain Sorghum (Milo)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Apply as a postemergence broadcast spray using sufficient spray volume to ensure thorough coverage of treated plants, but no less than 15 gpa for ground spray equipment or 2 to 5 gpa for aircraft equipment. Control may be reduced at low spray volumes under high temperature and wind conditions.

Chemigation: Lorsban Advanced may be applied through sprinkler irrigation systems at specified broadcast application rates to control listed foliar pests. See Chemigation Application section.

Target Pests	Lorsban Advanced (pint/acre)
sorghum midge (1)	0.5
grasshoppers yellow sugar cane aphid and other aphids	0.5 – 1
greenbug (2)	0.5 – 2
armyworms chinch bugs (3) cutworms lesser cornstalk borer (3)	1 – 2
webworms	1
European and southwestern corn borer	1.5 – 2
corn earworm	2

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:
1. **Sorghum midge:** Apply when 30% to 50% of the seed heads are in bloom.
2. **Greenbug:** Use a higher rate in the rate range when pest populations are high.
3. **Chinch bugs** and **lesser cornstalk borer:** Apply as a directed spray toward the base of the plant using power-operated ground spray equipment with sufficient water to ensure coverage of an 8- to 12-inch band centered in the row. For plants less than 6 inches high, apply an 8- to 12-inch band centered over the row. Do not reduce the dosage for banded or directed applications. Concentrate the full labeled dosage rate in the treated zone.

Specific Use Precautions:
- To minimize the potential for chemical injury, do not apply Lorsban Advanced to drought stressed grain sorghum within three days following irrigation or rain except where the product is applied in irrigation water.
- Be aware that sorghum lines used in seed production fields may be more susceptible to chemical injury. Susceptible inbred lines or hybrids are likely to be at greater risk of yield-reducing chemical injury when treated at the higher application rates. Users should not apply more than 1 pint of Lorsban Advanced per acre to seed sorghum if the additional risk of crop injury is unacceptable.

Specific Use Restrictions:
- **Preharvest Interval:** Do not harvest for grain, forage, fodder, hay, or silage within 30 days after application of 1 pint of Lorsban Advanced per acre or within 60 days after application of rates above 1 pint per acre.
- Do not make more than three applications of Lorsban Advanced or other product containing chlorpyrifos per use season.
- Maximum single application rate is 0.94 lb ai chlorpyrifos (2 pints of Lorsban Advanced) per acre.
- Do not apply more than 1.41 lb ai chlorpyrifos (3 pints of Lorsban Advanced) per acre per season.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.
- Do not treat sweet varieties of sorghum.
- Do not aerially apply this product in Mississippi.

Soybean
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Soil Application
Apply as a broadcast treatment to soil surface in a minimum spray volume of 10 gpa using suitable ground spray equipment or as a band application. Use a higher rate in the rate range when there is increased pest pressure. For band application, equivalent rates of insecticide spray required per 100 feet of row for listed row spacing are given in the table below. For at-plant treatments, apply in a 4- to 6-inch band centered over the row. Position the spray nozzle in front of the planter shoe or press wheel or after the press wheel followed by a drag chain for light incorporation. **Do not apply as an in-furrow treatment.** For a postemergence rescue treatment, apply as a directed spray in a 9- to 12-inch band at the base of the plant. For plants less than 6 inches tall, apply over-the-top in a 6- to 12-inch band.

Target Pests	At-Plant Treatment (Broadcast, T-band or Band) (pint/acre)	Postemergence Rescue Treatment (Band Only) (pint/acre)
cutworms lesser cornstalk borer	1 – 2	1 – 2

Fluid Ounces of Spray Required Per 100 Feet of Row for Listed Row Spacings and Spray Volumes				
Volume of Spray Per Acre (gal)	36"	32"	28"	24"
10	8.8	7.9	6.9	5.9
15	13.2	11.8	10.3	8.8
20	17.6	15.7	13.7	11.8

Foliar Application
Apply as a postemergence broadcast spray using sufficient spray volume to ensure thorough coverage of treated plants, but no less than 15 gpa for ground spray equipment or 2 to 5 gpa for aircraft equipment. Apply when field counts indicate damaging pest populations are developing or present. Lorsban Advanced may be tank mixed with glyphosate products, such as Duramax or Durango DMA, when application is to be made to glyphosate-tolerant soybeans. Use a higher rate in the rate range when there is increased pest pressure.

Chemigation: Lorsban Advanced may be applied through sprinkler irrigation systems at specified broadcast application rates to control listed foliar pests. See Chemigation Application section.

Target Pests	Lorsban Advanced (pint/acre)
grasshoppers green cloverworm spider mites (1) velvetbean caterpillar	0.5 – 1
armyworms bean leaf beetle corn earworm cutworms Mexican bean beetle potato leafhopper saltmarsh caterpillar and other woolly bears soybean aphid thistle caterpillar (painted lady butterfly)	1 - 2
brown marmorated stink bug European corn borer southern green stink bug	2

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:
1. **Spider mites:** When large numbers of eggs are present, scout the treated area in 3 to 5 days. If newly hatched nymphs are present, make a follow-up application of a non-chlorpyrifos product that is effective against mites.

Specific Use Precaution:
- On determinate soybeans, do not make more than one application after pod set.

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 28 days before harvest.
- Do not make more than three applications of Lorsban Advanced or other product containing chlorpyrifos per year.
- Maximum single application rate is 0.94 lb ai chlorpyrifos (2 pints of Lorsban Advanced) per acre.
- Do not apply more than a total of 2.82 lb ai chlorpyrifos (6 pints of Lorsban Advanced) per acre per season.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 14 days of the first application.
- Do not allow meat or dairy animals to graze in treated areas or otherwise feed treated soybean forage, hay, and straw to meat or dairy animals.

Strawberry
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Preplant Incorporation Application
Apply Lorsban Advanced in sufficient water to ensure uniform soil coverage and incorporate into the soil in the spring for protection of strawberries during the following year.

Target Pest	Lorsban Advanced (quart/acre)
garden symphylans grub	2

Foliar Application
Apply as a broadcast foliar spray when buds first appear and repeat application 10 to 14 days later. Use a minimum spray volume of 40 gpa.

Target Pest	Lorsban Advanced (quart/acre)
strawberry bud weevil	1

Postharvest Application
Apply as a directed spray to crown of strawberry plants immediately after harvest and after plants are topped. Repeat application, if required, 14 to 18 days later. Use a minimum spray volume of 100 gpa.

Target Pest	Lorsban Advanced (quart/acre)
strawberry crown moth	1

Specific Use Precautions:
- Do not tank mix Lorsban Advanced with pesticides, surfactants, or fertilizer formulations unless prior use has shown the combination non-injurious under your current conditions of use.
- Phytotoxicity may occur when Lorsban Advanced is applied to strawberries under conditions of high temperature and drought stress.

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 21 days before harvest.
- **Preplant Application:** Do not make more than one application of Lorsban Advanced or other product containing chlorpyrifos per year.
- **Foliar and Postharvest Applications:** Do not make more than two applications of Lorsban Advanced or other product containing chlorpyrifos per year.
- **Postharvest Application:** Do not sprinkle irrigate for one week following application.
- Maximum single application rate is 1.88 lb ai chlorpyrifos (2 quarts of Lorsban Advanced) per acre for preplant incorporation and 0.94 lb ai chlorpyrifos (1 quart of Lorsban Advanced) per acre for foliar and postharvest application.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first foliar application and within 14 days of postharvest application.
- **For prebloom use only.** Do not apply after berries start to form or when berries are present.

Sugarbeet
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Soil Application (At-Planting or Preplant Incorporated)
To reduce feeding damage from early season insects such as cutworms, apply at-planting or as a preplant treatment and incorporate to a depth of 1 to 2 inches. Do not apply as an in-furrow treatment. Apply 1 pint of Lorsban Advanced per planted acre to a 10-inch wide band centered over the row for furrows 30 inches apart. (For rows 30 inches apart, this is equivalent to 9.2 fl oz of Lorsban Advanced per 10,000 feet of row). For other row widths, adjust the spray volume per planted acre in proportion to the length of row actually treated.

Postemergence Application
Apply specified rate as a broadcast or banded foliar spray. Treat when field counts indicate that damaging insect populations are developing or present.

Broadcast Application: Apply the specified dosage in water using 2 to 5 gpa of finished spray when using aerial spray equipment or 10 to 30 gpa when using ground spray equipment. **Chemigation:** Lorsban Advanced may be applied through sprinkler irrigation systems at specified broadcast application rates to control listed foliar pests. See Chemigation Application section for application instructions.

Banded Foliar Spray: Apply the specified rate within the band using a minimum of 7 gallons of spray volume in a 5- to 7-inch wide band centered over the row. Do not reduce the rate for band applications. Concentrate the full labeled dosage rate (see band rates in table below) in the treated zone. For best results, lightly incorporate band-applied treatments, either mechanically or with irrigation.

Target Pests	Lorsban Advanced	
	Broadcast (pint/acre)	Band (pint/acre)
grasshoppers (1)	0.5 - 1	-
leafminers spider mites	1	0.67
tarnished plant bug (*Lygus*)	1	-
aphids fall armyworm yellowstriped armyworm webworms	1 - 2	0.67 – 1.33
beet armyworm	1.5 - 2	1 – 1.33
cutworms flea beetle adults	2	1.33
sugarbeet root maggot adults (2), (5)	0.5 - 1	-
sugarbeet root maggot larvae (3), (5)	-	1.33 - 2
sugarbeet root maggot larvae (4), (5)	2	1.33 - 2

Numbers in parentheses (-) refer to "Pest-Specific Use Directions".

Pest-Specific Use Directions:

1. **Grasshoppers:** The low rate will control small nymphs (1st through 3rd instar).
2. **Sugarbeet root maggot adults:** Apply anytime from 7 days before until 3 days after peak adult emergence in order to target adults present at time of application based upon local field trap monitoring.
3. **Sugarbeet root maggot larvae:** Use as primary treatment to control root maggot larvae. Base application timing on local field trap monitoring. Apply anytime from 7 days before until 3 days after peak adult emergence.
4. **Sugarbeet root maggot larvae:** Use as a supplemental postemergence treatment following an at-plant insecticide application for control of root maggot larvae. Base application timing upon local field trap monitoring. Apply anytime from 7 days before until 3 days after peak adult emergence.
5. **Sugarbeet root maggot:** To prevent the potential development of insecticide resistance, producers are encouraged to take the following steps: (1) avoid making more than two applications of Lorsban Advanced per season when adults are active; (2) if an organophosphate insecticide was applied at planting, make no more than one postemergence application of Lorsban Advanced when adults are active.

Specific Use Restrictions:

- **Preharvest Interval:** Do not apply within 30 days before harvest of beet roots and tops.
- Do not make more than three applications of Lorsban Advanced or other product containing chlorpyrifos per season.
- Maximum single application rate is 0.94 lb ai chlorpyrifos (2 pints of Lorsban Advanced) per acre.
- Do not apply more than a total of 2.82 lb ai chlorpyrifos (6 pints of Lorsban Advanced) per acre per season.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.
- Do not allow meat or dairy animals to graze in treated areas or harvest treated beet tops as feed for meat or dairy animals within 30 days of last treatment.
- To avoid unacceptable crop injury, do not tank mix Lorsban Advanced with Quadris or Headline or with any EC formulation or any tank mix containing an oil adjuvant.

Sunflower

(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Preplant Incorporation Application

Broadcast apply to soil surface in a minimum spray volume of 10 gpa using suitable ground spray equipment. On the same day of treatment, incorporate the insecticide into the top 2 to 4 inches of soil using a disc, field cultivator, or equivalent equipment. Use a higher rate in the rate range when there is increased pest pressure.

Target Pests	Lorsban Advanced (pint/acre)
cutworms	2 - 4

Postemergence Broadcast Application

Apply as a postemergence broadcast spray using sufficient spray volume to ensure thorough coverage of treated plants, but no less than 15 gpa for ground spray equipment or 2 to 5 gpa for aircraft equipment. Use a higher rate in the rate range when there is increased pest pressure.

Target Pests	Lorsban Advanced (pint/acre)
grasshoppers	1
banded sunflower moth seed weevil (4) stem weevil (2) sunflower beetle larvae and adults (1) sunflower moth (3) woolly bears	1 – 1.5
cutworms	2
tarnished plant bug (*Lygus*) (5)	1 - 2

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:

1. **Sunflower beetle:** For control of larvae or adults, treat when field counts indicate 10 larvae or 1 to 2 adults per seedling.
2. **Stem weevil:** Optimal treatment time is within 5 to 7 days after adult weevils begin to appear.
3. **Sunflower moth:** To control, make first application during early 1% to 5% bloom stage.
4. **Seed weevil:** To control, apply when field counts indicate 10 to 12 adults per plant for oil crop varieties and 1 to 3 adults per plant on confectionery crop varieties.
5. **Tarnished plant bug (*Lygus*):** Use a higher rate in the rate range where populations are heavy. Apply at the onset of pollen spread or approximately 10% bloom (R-5 growth stage). For best protection, make a second application 10 days later. Use sufficient water to ensure thorough coverage of treated plants.

Specific Use Restrictions:

- **Preharvest Interval:** Do not apply within 42 days before harvest.
- Do not make more than three applications of Lorsban Advanced or other product containing chlorpyrifos per season.
- Maximum single application rate is 1.88 lb ai chlorpyrifos (4 pints of Lorsban Advanced) per acre for preplant incorporation and 0.94 lb ai chlorpyrifos (2 pints of Lorsban Advanced) per acre for postemergence broadcast treatment.
- Do not apply more than a total of 2.82 lb ai chlorpyrifos (6 pints of Lorsban Advanced) per acre per season.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.
- Do not allow meat or dairy animals to graze in treated areas.

Sweet Potato

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Apply to the soil surface as a preplant broadcast spray to reduce the feeding damage caused by listed pests. Use a spray volume of 10 gpa or more. Incorporate immediately after application to a depth of 4 to 6 inches using a rotary hoe, disc cultivator, or other suitable incorporation equipment. Plant sweet potatoes in the usual manner no more than 14 days after treatment. Delaying planting more than 14 days after application will reduce the time interval of protection against feeding damage.

Target Pests	Lorsban Advanced (pint/acre)
Conoderus (wireworm) sweet potato flea beetle *Systena* (flea beetle)	4

Specific Use Precaution:
- Lorsban Advanced will not control false wireworms, white fringe beetle or other grubs that attack sweet potatoes.

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 125 days before harvest.
- Do not make more than one application of Lorsban Advanced or other product containing chlorpyrifos per season.
- Maximum single application rate is 1.88 lb ai chlorpyrifos (4 pints of Lorsban Advanced) per acre.
- Do not aerially apply this product in Mississippi.

Tobacco

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Apply as a preplant broadcast spray to reduce the feeding damage caused by listed pests. Apply 24 to 48 hours before bedding and transplanting using a spray volume of 10 gpa or more. Incorporate immediately after application to a depth of 2 to 4 inches using suitable incorporation equipment.

Before broadcast application of Lorsban Advanced onto existing beds, knock down beds to final shape for transplanting. Use PTO-driven implements that will incorporate Lorsban Advanced to a depth of 4 inches.

Target Pests	Lorsban Advanced (pint/acre)
cutworms flea beetles mole crickets root maggots wireworms	2

To control the above listed pests and suppress populations of rootknot nematodes in all tobacco growing regions, use Lorsban Advanced in a tank mix with Nemacur 3 at the rate of 2 pints of Lorsban Advanced plus 4 quarts of Nemacur 3 per acre. Read and carefully follow all applicable directions, restrictions, and precautions on labeling for Nemacur 3 used in combination with Lorsban Advanced. Apply the specified rate(s) to the soil surface in a spray volume of 10 gpa or more 24 to 48 hours before bedding and transplanting. Immediately following application, incorporate into the soil to a depth of at least 4 inches using suitable equipment. Where the nematode species *Meloidogyne arenaria* or *M. javanica* are present, or there are high populations of *M. incognita*, apply Telone® II soil fumigant at the specified label rate.

Specific Use Restrictions:
- Do not make more than one application of Lorsban Advanced or other product containing chlorpyrifos per season.
- Maximum single application rate is 1 lb ai chlorpyrifos (2 pints of Lorsban Advanced) per acre.
- Do not aerially apply this product in Mississippi.

Tree Fruits,[1] Almond, and Walnut (Dormant/Delayed Dormant Sprays)
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 4 days for tree fruits and 24 hours for almond and walnut unless PPE required for early entry is worn.

[1]Apple, cherry, nectarine, peach, pear, plum, prune

Apply as a dormant or delayed dormant spray. While Lorsban Advanced may be used without oil, for best results, use oil to control additional pests, such as European red mite. See precautions for use of oil below. Apply as a concentrate or dilute spray using conventional, power-operated spray equipment. For dilute sprays (greater than 200 gpa), use sufficient spray volume to completely wet tree foliage, but not to point of runoff. For concentrate sprays (less than 200 gpa), uniformly apply an equivalent amount of Lorsban Advanced per acre.

Use a higher rate in the rate range when there is increased pest pressure.

Use Precautions for Tree Fruits, Almond and Walnut:
- Cold or dry conditions may cause Lorsban Advanced plus oil sprays to infuse into trees, resulting in bud damage or bud drop. Do not apply until winter rains or irrigation has replenished soil moisture such that bark and twigs are not desiccated.
- To avoid contamination of irrigation tail waters, do not flood irrigate within 24 hours of application of Lorsban Advanced.

Use Restrictions for Tree Fruits, Almond and Walnut:
- Make only one application of chlorpyrifos during the dormant season.
- For apple, do not make more than one application of Lorsban Advanced to the apple tree trunk per year as either a prebloom or post-bloom application.
- Do not use more than a total of 1.88 lb ai chlorpyrifos (4 pints of Lorsban Advanced) per acre per season as a dormant/delayed dormant application.
- Do not allow meat or dairy animals to graze in treated orchards.

Almond, Cherry, Nectarine, Peach, Pear, Plum, Prune

Target Pests	Lorsban Advanced (pint/acre)
American plum borer brown almond mite climbing cutworms European red mite greater peach tree borer lesser peach tree borer mealy plum aphid peach twig borer pear psylla adults San Jose scale	1.5 - 4

Specific Use Precautions for Almond, Cherry, Nectarine, Peach, Pear, Plum, Prune:
- Avoid contact with foliage in sweet cherries as premature leaf drop may result.

Specific Use Restrictions for Almond, Cherry, Nectarine, Peach, Pear, Plum, Prune:
- Do not make a soil or foliar application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of a dormant/delayed dormant application of chlorpyrifos to the orchard.

Additional Restrictions Specific to California for Almond, Cherry, Nectarine, Peach, Pear, Plum, Prune:
- Do not use more than 1% dormant oil and/or penetrating surfactants in almond orchards less than 4 years old.
- Use a minimum of 100 gpa of total spray volume.
- Use up to 2% supreme oil with no more than 4 gpa on almonds.
- Use up to 2% supreme oil with no more than 6 gpa on peaches and nectarines.
- Refer to the University of California pest management guide for pears, plums, and prunes.
- In orchards with high overwintering populations of European red mite or brown almond mite, use higher spray volumes that allow for the use of higher per acre rates of oil.
- Do not use any adjuvants or surfactants in addition to, or as a substitute for, a petroleum spray oil in a tank mix with Lorsban Advanced.
- Do not apply on almonds in the following counties in California: Butte, Colusa, Glenn, Solano, Sutter, Tehama, Yolo, and Yuba.

Apple

Target Pests	Lorsban Advanced (pint/acre)
climbing cutworms *Lygus* obliquebanded leafroller pandemis leafroller rosy apple aphid San Jose scale	1.5 - 4

Specific Use Restrictions for Apple:
- Only one application of any chlorpyrifos containing product can be made per year. The application can be either a prebloom dormant/delayed dormant spray to the canopy or the trunk, or a post-bloom application to the lower 4 feet of the trunk (for post-bloom application instructions and restrictions on apple, refer to Apple Tree Trunk section of the label).

Additional Restrictions Specific to California for Apple:
- Use a minimum of 100 gpa of total spray volume.
- Refer to the University of California pest management guide for apples.

- In orchards with high overwintering populations of European red mite or brown almond mite, use higher spray volumes that allow for the use of higher per acre rates of oil.
- Do not use any adjuvants or surfactants in addition to, or as a substitute for, a petroleum spray oil in a tank mix with Lorsban Advanced.

Tree Fruits[1] and Almond (Trunk Spray or Preplant Dip)
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 4 days for tree fruits and 24 hours for almond unless PPE required for early entry is worn.

[1]Cherry, nectarine, peach, plum

Apply Lorsban Advanced to tree trunks and lower branches using a coarse, low-pressure spray to control pests listed in the following table. Use a higher rate in the rate range when there is increased pest pressure. Unless otherwise specified, a second application may be made after two weeks and a third application may be made after harvest. Avoid spray contact with foliage in sweet cherries as premature leaf drop may result. Consult your state agricultural experiment station or extension service specialist for proper application timing for your area.

Crops	Target Pests	Lorsban Advanced (quart/100 gal)
cherry	American plum borer greater peach tree borer lesser peach tree borer	1.5 – 3
almond nectarine peach plum	peach tree borers (1) (2)	3

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:
1. **Preplant Dip Application (Peaches and Nectarines Only).** For preplant control of **peachtree borer**, use Lorsban Advanced at the equivalent application rate of 3 quarts per 100 gallons of water. Dip trees several inches above the grafting bud scar and plant immediately or allow them to dry before returning to storage. Do not allow peach trees to remain in contact with the dip solution.
2. **Peach tree borer:** For control in established trees, apply before newly hatched borers enter the tree. Use as a coarse, low-pressure trunk spray and thoroughly wet all bark areas from ground level to scaffold limbs. Do not allow spray to contact fruit. Consult written recommendations provided by your State agricultural experiment station or extension service specialist for proper time to treat in your area.

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 14 days before harvest of almonds, nectarines, peaches and plums or within 21 days before harvest of cherries.
- Do not make more than one chlorpyrifos application per year in nectarines peaches, and no more than three chlorpyrifos applications per year in cherries.
- Do not allow meat or dairy animals to graze in treated orchards.

Tree Nuts[1] (Foliar Sprays)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

[1]Almond, filbert, pecan, walnut

Apply Lorsban Advanced as a foliar spray at the dosages indicated to control pests listed in the following table. Mix the required dosage in sufficient water to ensure thorough and complete coverage of the foliage and crop and apply as a concentrate or dilute spray using conventional, power-operated spray equipment. For dilute sprays applied to tree nut crops, mix the required dosage in sufficient water to allow for spray to runoff. For concentrate sprays, apply an equivalent amount of Lorsban Advanced per acre. Treat when pests appear or in accordance with local conditions. Aerial application may result in less effective insect control because of reduced coverage. Consult your State agricultural experiment station, certified pest control advisor, or extension service specialist for specific use information in your area.

Crops	Target Pests		Lorsban Advanced (pint/acre)
almond	leaf footed plant bug navel orangeworm	peach twig borer San Jose scale	4
filbert	brown marmorated stink bug eye-spotted bud moth filbert aphid filbert leafroller	filbert worm obliquebanded leafroller omnivorous leaftier winter moth	3 – 4
pecan	blackmargined aphid (1) spittlebugs (2)	yellow pecan aphid (1)	1 – 4
	fall webworm	pecan nut casebearer	1.5 – 4
	black pecan aphid brown marmorated stink bug hickory shuckworm (3)	*Phylloxera* spp.(4) pecan leaf scorch mite (suppression) (5)	2 – 4
walnut	codling moth walnut husk fly	walnut scale	4

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:
1. **Yellow pecan aphid** and **blackmargined aphid:** For control, apply in tank mix combination with the specified rate of a pyrethroid insecticide labeled for control or suppression of these aphids.
2. **Spittlebug:** For control, use a dosage of 2 to 4 pint per acre for concentrate sprays.
3. **Hickory shuckworm**, For best results, make two applications, 10 to 14 days apart.
4. ***Phylloxera* spp.:** For best control, make two applications at a 10-day interval using a minimum of 1 pint of Lorsban Advanced per acre starting at bud swell.
5. **Pecan leaf scorch mite:** For suppression, use a preventative program.

Specific Use Precautions:
- Lorsban Advanced is highly toxic to bees exposed to direct treatment and should not be applied when bees are foraging in the treated area.
- To avoid contamination of irrigation tail waters, do not flood irrigate within 24 hours of application of Lorsban Advanced.

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 14 days before harvest of almonds, filberts and walnuts, or 28 days before harvest of pecans.
- Do not make more than three total applications of Lorsban Advanced or other product containing chlorpyrifos per season to almonds, pecans and filberts and no more than two applications per season on walnuts.
- Do not apply more than a total of 3.76 lb ai chlorpyrifos (8 pints of Lorsban Advanced) per acre per season as a foliar spray.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.
- Do not allow meat or dairy animals to graze in treated orchards.
- Do not use on almond, filbert or walnut in Mississippi.
- Do not aerially apply this product in Mississippi.

Tree Nut[1] Orchard Floors
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

[1]Almond, pecan, walnut

Apply as a ground broadcast spray directed to the orchard floor using ground application equipment that will apply the spray uniformly. Do not allow spray to contact foliage or fruit. Treat when ant activity (excluding fire, harvester, carpenter, and pharaoh ants) becomes evident in the orchard. Since worker ants (excluding fire, harvester, carpenter, and pharaoh ants) cease most of their foraging activity at temperatures above 90°F, best results will be achieved if applied at a time of day when temperatures are below 90°F.

Chemigation: Lorsban Advanced may be applied to almond, pecan and walnut orchard floors through sprinkler irrigation systems only if the system uniformly covers the soil surface at the base of the tree. Use specified broadcast application rates to control listed pests. See Chemigation Application section.

Orchard Floor	Target Pests	Lorsban Advanced (pint/acre)
pecan	ants (1)	4
almond walnut		4 – 8

Numbers in parentheses (-) refer to Pest Specific Use Directions.

Pest Specific Use Directions:
1. Excludes fire, harvester, carpenter, and pharaoh ants.

Eliminate weed growth that would prevent uniform coverage of the orchard floor by mowing or herbicide treatment. Foliar applications of Lorsban Advanced may be made in addition to the orchard floor treatment.

Specific Use Precaution:
- To avoid contamination of irrigation tail waters, do not flood irrigate within 24 hours of application of Lorsban Advanced.

Specific Use Restrictions:
- **Preharvest Interval:** Do not apply within 14 days before harvest.
- Do not make more than two applications of Lorsban Advanced or other product containing chlorpyrifos per season to the orchard floor. If the 8 pint per acre rate is used, a second application is not allowed.
- Do not apply more than 3.76 lb ai chlorpyrifos (8 pints of Lorsban Advanced) per acre per season to the orchard floor.
- Do not make a second application of Lorsban Advanced or other product containing chlorpyrifos within 10 days of the first application.
- Do not allow meat or dairy animals to graze in treated orchards.

Turfgrass
(Not for use in Mississippi)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Dilute Lorsban Advanced in water and apply to turfgrass grown for sod using suitable application equipment. For best results, turfgrass should be moist at time of treatment.

Pests		Lorsban Advanced fl oz/ 1000 sq ft	Lorsban Advanced quart/ acre
ants (1) armyworms beet fall yellowstriped centipedes chiggers chinch bugs crickets cutworms deer ticks earwigs European crane fly larvae fiery skipper fleas gnats grasshoppers	greenbug aphids green June beetle grubs leafhoppers Lucerne moth millipedes mites Bermudagrass stunt clover winter grain mosquitoes pillbugs springtails sod webworms (lawn moths) (2) sowbugs ticks	0.75	1
billbug adults (3) bluegrass Denver hunting		0.75 - 1 1/2	1 - 2
annual bluegrass weevil (*Hyperodes*) (4) black turfgrass ataenius adults (5) mole crickets (6)		1.5	2
white grubs (7) black turfgrass ataenius European chafer Japanese beetle larvae northern and southern masked chafers)		1.5 - 3	2 - 4

Numbers in parentheses (-) refer to Pest-Specific Use Directions below.

Pest-Specific Use Directions:
1. Excludes fire, harvester, carpenter, and pharaoh ants.
2. **Sod webworms:** Delay watering or mowing of the treated area for 12 to 24 hours after treatment.
3. **Billbugs:** Spray early in the season just prior to or coinciding with first appearance of adults as recommended by your local Agricultural Extension Service Specialist.
4. **Annual bluegrass weevil:** To control, spray suspected problem areas in mid-April and again in mid-May, or as recommended by your local Agricultural Extension Service Specialist.
5. **Black turfgrass ataenius adults:** Spray early in the season as recommended by your local Agricultural Extension Service Specialist. A repeat application may be needed 1 to 2 weeks later.
6. **Mole crickets:** To control in turfgrass, apply Lorsban Advanced through high-pressure injection or other suitable subsurface placement application equipment. Depending upon the application equipment used, follow the manufacturer's directions for calibration and the volume of spray per acre needed to provide control or as recommended by your local Agricultural Extension Service Specialist. For best results, apply when young nymphs are active.
7. **White grubs:** Spray when grubs are young and actively feeding near the soil surface, usually during late July and August, or as recommended by your local Agricultural Extension Service Specialist. For best results, soil should be moist prior to treatment. For best results, immediately after spraying, irrigate the treated area with 1/2 to 1 inch of water to wash the insecticide into the thatch and underlying soil.

Wheat
(For use only in Arizona, California, Colorado, Idaho, Kansas, Minnesota, Montana, Nebraska, New Mexico, Nevada, North Dakota, Oklahoma, Oregon, South Dakota, Texas, Utah, Washington and Wyoming)

Worker Restricted Entry Interval: Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 24 hours unless PPE required for early entry is worn.

Foliar Application
Apply using aerial (fixed wing or helicopter) or power-operated ground spray equipment. Mix the required dosage with water and apply in a minimum of 2 to 5 gpa finished spray volume for aerial equipment or 15 gpa for ground spray equipment. Apply when field counts indicate damaging pest populations are developing or present.

Chemigation: Lorsban Advanced may be applied through sprinkler irrigation systems at specified broadcast application rates to control listed foliar pests. See Chemigation Application section.

Target Pests	Lorsban Advanced (pint/acre)
aphids (1) English grain aphid greenbug Russian wheat aphid brown wheat mite grasshoppers	0.5 - 1
army cutworms (2) armyworms (3) cereal leaf beetle (4) cutworms (suppression) (2) wheat midge (5)	1

Numbers in parentheses (-) refer to Pest-Specific Use Directions.

Pest-Specific Use Directions:
1. Consult university extension bulletins for local treatment recommendations.
2. Control may be reduced under high temperature conditions (greater than 80°F), under dry soil conditions, or if larvae are more than 1/2 inch long.
3. Expect suppression under conditions of heavy pest populations or large worms.
4. Target application when eggs are near hatching and larvae is emerging as monitored by plant inspection.
5. **Wheat midge:** For control, treat when 75% of the wheat heads have emerged from the boot and when midge adults are found in the crop (1 midge per 4 to 5 heads). If possible, apply in the late afternoon or early evening when temperatures exceed 50°F and wind speed is less than 7 mph.

Specific Use Restrictions:

- **Preharvest Interval:** Do not apply within 14 days before harvest for forage and hay and within 28 days before harvest for grain and straw.
- Do not make more than two applications of Lorsban Advanced or other product containing chlorpyrifos per season.
- Maximum single application rate is 0.47 lb ai chlorpyrifos (1 pint of Lorsban Advanced) per acre.
- Do not allow meat or dairy animals to graze or otherwise feed on treated forage within 14 days of application.
- Do not feed straw from treated wheat within 28 days of application.

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Produced for
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EPA accepted 12/21/12

Revisions:

1. Added buffer zone language based upon application rate and nozzle droplet size.
2. Under Brassica (Cole) Leafy Vegetables[1] and Radish, Rutabaga, and Turnip within the Specific Use Restrictions for Preplant Incorporation and At-Plant or Post Plant Soil Applications section removed 'cauliflower' from the third bullet.
3. Updated trademarking

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND
POLLUTION PREVENTION

MEMORANDUM

Date: November 3, 2016

SUBJECT: Chlorpyrifos: Revised Human Health Risk Assessment for Registration Review

PC Code: 059101
Decision No.: 522687
Petition No.: NA
Risk Assessment Type: Single Chemical Aggregate
TXR No.: NA
MRID No.: NA

DP Barcode: D436317
Registration No.: NA
Regulatory Action: Registration Review
Case No.: NA
CAS No.: 2921-88-2
40 CFR: 40 CFR§180.342

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1.0 Executive Summary

This document presents the revised human health risk assessment for the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Registration Review of the organophosphate (OP) insecticide chlorpyrifos.

Background
A preliminary human health risk assessment (HHRA) for chlorpyrifos was completed on June 30, 2011 (D. Drew *et. al*, D388070, 06/30/2011) as part of the FIFRA Section 3(g) Registration Review program. A revised HHRA was completed in 2014 (D. Drew *et. al*, D424485, 12/29/2014) to address comments received on the preliminary HHRA and to incorporate new information and new approaches that had become available since the June 2011 risk assessment. Most notably, the 2014 revised HHRA incorporated the following: (1) a physiologically-based pharmacokinetic-pharmacodynamic (PBPK-PD) model for deriving toxicological points of departure (PoDs) based on 10% red blood cell (RBC) acetyl cholinesterase (AChE) inhibition; and (2) evidence on neurodevelopmental effects in fetuses and children resulting from chlorpyrifos exposure as reported in epidemiological studies, particularly the results from the Columbia Center for Children's Environmental Health (CCCEH) study on pregnant women which reported an association between fetal cord blood levels of chlorpyrifos and neurodevelopmental outcomes. The 2014 revised HHRA retained the 10X Food Quality Protection Act (FQPA) Safety Factor (SF) because of the uncertainties that neurodevelopmental effects may be occurring at doses lower than those that cause 10% RBC AChE inhibition and used for the PoD.

Based on the aggregate risks identified in 2014 (D. Drew *et. al*, D424485, 12/29/2014), a proposed rule (PR) for revoking all tolerances of chlorpyrifos was published in the Federal Register on November 6, 2015 (80 FR 69079). At that time, the EPA had not completed a refined drinking water assessment or additional analysis of the hazard from chlorpyrifos that was suggested by several commenters to the EPA's 2014 registration review revised HHRA. Those commenters raised the concern that the use of 10% RBC AChE inhibition for deriving PoDs for chlorpyrifos may not provide a sufficiently health protective human health risk assessment given the potential for neurodevelopmental outcomes. Accordingly, following the issuance of the proposed rule, the EPA conducted additional hazard analyses using data on chlorpyrifos levels in fetal cord blood (reported by the CCCEH study investigators) as the source for new PoDs for risk assessment.

The EPA consulted the FIFRA Scientific Advisory Panel (SAP) for scientific advice on the proposed approach of using the CCCEH cord blood data at a meeting on April 19 – 21, 2016. The 2016 SAP did not support using the cord blood data quantitatively for deriving PoDs. However, the Panel concluded that epidemiology and toxicology studies suggest there is evidence for adverse health outcomes associated with chlorpyrifos exposures below levels that result in 10% RBC AChE inhibition, which was used as the PoD in the EPA's 2014 RHHRA and for the 2015 proposed revocation rule. The SAP therefore appears to have rejected both the approach the EPA put forward in its proposed rule derived from the 2014 risk assessment as well as the EPA's initial efforts to address the results of the CCCEH study quantitatively.

The SAP report, however, did present the EPA with a path forward for a third approach to setting the PoDs. First, as a foundation, it is important to note that the SAP was supportive of the EPA's use of the PBPK model as a tool for assessing internal dosimetry from typical Office of Pesticide Programs (OPP) exposure scenarios using peer reviewed exposure assessment approaches (e.g., food, water, residential, occupational). Use of the PBPK model coupled with typical exposure scenarios provides the strongest scientific foundation for chlorpyrifos human health risk assessment and is the approach used in this 2016 assessment. Given that the window(s) of susceptibility are currently not known for the observed neurodevelopmental effects, and the uncertainties associated with quantitatively interpreting the CCCEH cord blood data, the SAP recommended that the agency use a time weighted average (TWA) blood concentration of chlorpyrifos for the CCCEH study cohort as the PoD for risk assessment. The EPA has chosen to follow that advice in this assessment. Thus, for this assessment, the PBPK model was used to determine the TWA blood level expected from post-application exposures from the chlorpyrifos indoor crack and crevice use scenario. This scenario was selected as it represents the most appropriate exposure for the women in the CCCEH cohort (i.e., crack and crevice was the predominant application type during the time of the CCCEH study and is considered protective of other possible exposures for the women in the cohort). In order to derive a TWA of chlorpyrifos concentrations in blood for a predicted risk assessment endpoint, the dose reconstruction analysis assumed exposures for 2 hours per day with a daily shower, for a total of 30 days. Additionally, chlorpyrifos residues were assumed to dissipate 10% daily; that is, the total amount of residue available for transfer from the treated floor is assumed to reduce by 10% for each subsequent day of exposure until the end of the 30th day prior to the next application.

The TWA blood level was used as the internal dose for determining separate PoDs for infants, children, and adults exposed to chlorpyrifos. These separate PoDs have been calculated by PBPK modeling for dietary (food, drinking water), residential, and occupational exposures. With the exception of the acute (single day) exposure assessment for non-occupational bystander post-application inhalation exposures, only steady state[1] (repeat) exposure durations are considered in this assessment as assessing the steady state exposure duration most closely matches the TWAs calculated for the PoDs. The PoDs derived from the TWA blood level are protective of any additional acute exposures to chlorpyrifos.

The TWA blood level resulting from chlorpyrifos exposure from the crack and crevice scenario is considered a lowest-observed-adverse-effect-level (LOAEL) rather than a no-observed-adverse-effect-level (NOAEL), since this is the exposure level likely to be associated with neurodevelopmental effects reported in the CCCEH study. In situations where the agency selects a PoD from a study where a NOAEL has not been identified, the EPA generally will retain the FQPA SF of 10X to account for the uncertainty in using a LOAEL. Therefore, the 10X FQPA SF has been retained in this revised risk assessment for chlorpyrifos. The revised risk assessment also applies a 10X uncertainty factor for intraspecies variability because of the lack

[1] Organophosphates (OPs), including chlorpyrifos, exhibit a phenomenon known as steady state AChE inhibition. After repeated dosing at the same level, the degree of inhibition comes into equilibrium with the production of new, uninhibited enzyme. At this point, the amount of AChEI at a given dose remains relatively consistent across duration. In general, OPs reach steady state within 2-3 weeks. Therefore, for OPs it is appropriate to assess steady state exposure durations (up to 21 days) instead of longer term exposures. The steady state point of departure is protective of any longer exposure duration, including chronic exposure.

of sufficient information to reduce or remove this factor. Typically, the agency uses animal studies for selection of PoDs and, as such, retains a 10X interspecies factor for extrapolation of the animal data to assess human health. However, with use of the PBPK-PD model which accounts for the pharmacokinetic and pharmacodynamic differences between animals and humans to derive PoDs, it is appropriate to reduce the interspecies factor to 1X. Therefore, the total uncertainty factor for chlorpyrifos in this 2016 risk assessment is 100X.

For the dietary assessment, PoDs are divided by the total uncertainty factor (100) to derive a population adjusted dose (PAD). The chlorpyrifos exposure values resulting from dietary modeling are compared to the PAD. There are potential risks of concern when estimated dietary risk exceeds 100% of the PAD.

For the residential and occupational assessments, margins of exposure (MOEs) are calculated by comparing the PoDs to the calculated exposures for each scenario. The resulting MOEs are then compared to the level of concern (LOC) of 100 (the total uncertainty factor is the LOC). If calculated MOEs are less than 100 then a risk of concern is identified for that exposure scenario.

This 2016 human health risk assessment only provides limited summary information and substantially relies on the following previous documents developed for chlorpyrifos, and the updated drink water assessment, which contain more detailed evaluations of the risk assessment approach, scientific literature, and the PBPK model:

- D. Drew *et al.,* Chlorpyrifos: Revised Human Health Risk Assessment for Registration Review, December 29, 2014, D424485;
- U.S. Environmental Protection Agency, Literature Review on Neurodevelopment Effects & FQPA Safety Factor Determination for the Organophosphate Pesticides, September 15, 2015, D331251;
- R. Bohaty and J. Hetrick. Chlorpyrifos Registration Review Drinking Water Assessment, April 14, 2016, D432921
- U.S. Environmental Protection Agency, Chlorpyrifos Issue Paper: Evaluation of Biomonitoring Data from Epidemiology Studies, March 11, 2016 and supporting analyses presented to the FIFRA Scientific Advisory Panel's (SAP) meeting on April 19-21, 2016, (EPA-HQ-OPP-2016-0062).

Use Profile

Chlorpyrifos is a broad-spectrum, chlorinated OP insecticide. Registered use sites include a large variety of food crops, and non-food use settings. Public health uses include aerial and ground-based fogger adulticide treatments to control mosquitoes. There is a wide range of registered formulations, application rates, and application methods. Registered labels generally require that handlers use normal work clothing (i.e., long sleeved shirt and pants, shoes and socks) and coveralls, chemical resistant gloves, and dust/mist respirators. Also, some products are marketed in engineering controls such as water soluble packets. The restricted entry intervals (REIs) on the registered chlorpyrifos labels range from 24 hours to 5 days. The pre-harvest intervals (PHIs) range from 0 days (Christmas trees) to 365 days (ginseng).

Dietary Risk Assessment

This assessment indicates that steady state dietary exposure analysis is highly refined. The large

majority of food residues used were based upon U. S. Department of Agriculture's Pesticide Data Program (PDP) monitoring data. Percent crop treated information and food processing factors were included, where available. All commodities with U.S. tolerances for residues of chlorpyrifos are included in the assessment.

The steady state dietary (food only) exposures for chlorpyrifos are of risk concern (> 100% steady state PAD for food ($ssPAD_{food}$)) at the 99.9th percentile of exposure for all population subgroups analyzed. Children (1-2 years old) is the population subgroup with the highest risk estimate at 14,000% of the $ssPAD_{food}$.

For chlorpyrifos, a drinking water level of comparison (DWLOC) approach is used to calculate the amount of exposure available in the dietary 'risk cup' for chlorpyrifos in drinking water after accounting for chloropyrifos exposure from food. This DWLOC is then compared to the estimated drinking water concentration (EDWC) to determine if there is a risk of concern for drinking water exposures. However, because this assessment indicates that dietary risks from food alone are of concern it is not possible to calculate a DWLOC; essentially the steady state DWLOC is '0' after accounting for food exposures.

Hypothetically, if there were no exposure to chlorpyrifos from food and the entire dietary 'risk cup' was available for drinking water, the resulting steady state DWLOC for infants (the most highly exposed population subgroup for water) would be 0.014 ppb. An EDWC at or exceeding this concentration would be considered a risk of concern for exposures to chlorpyrifos in drinking water. The refined chlorpyrifos EDWCs are presented in the revised drinking water assessment (DWA) (Bohaty, R., 4/14/2016, D432921, Chlorpyrifos Revised Drinking Water Assessment for Registration Review).

Residential (Non-occupational) Risk Assessment
Residential post-application exposures can occur for adults and children golfing on chlorpyrifos-treated courses. The residential post-application assessment considered and incorporated all relevant populations and chemical-specific turf transferable residue (TTR) data. This assessment indicates that all residential post-application exposures are of concern (i.e., MOEs are < 100) on the day of application (Day 0); all MOEs < 1 (LOC = 100). Further, all residential post-application exposure scenarios assessed following aerial and ground Ultra Low Volume (ULV) mosquitocide applications result in risks of concern; MOEs ranged from < 1 to 68 (LOC = 100).

Non-Occupational Spray Drift Exposure and Risk Assessment
A quantitative non-occupational spray drift (from treatment of agricultural fields) assessment was conducted for this assessment. Adult dermal and children's (1 < 2 year old) dermal and incidental oral risk estimates from indirect exposure to chlorpyrifos from spray drift result in risk estimates of concern at the field edge. All scenarios require buffer distances of > 300 feet to be below the level of concern.

Non-Occupational Bystander Post-Application Inhalation Exposure and Risk Assessment
In the 2014 risk assessment, the agency did not include a quantitative assessment of post-application inhalation exposure to bystanders. This assessment was not included since two vapor-phase AChE inhibition inhalation toxicity studies were submitted and reviewed which

demonstrated that no inhibition of AChE occurred even at the saturation concentration. Therefore, it was assumed that there were no anticipated risks of concern from exposure to the volatilization of either chlorpyrifos or chlorpyrifos oxon. However, in the current assessment, the points of departure for risk assessment have been chosen to be protective of potential neurological effects that occur below levels where AChE inhibition could occur. For that reason, a quantitative bystander/volatilization assessment has been included in this update.

The EPA has assessed residential bystander exposure from field volatilization of applied chlorpyrifos based on available *ambient* (five studies/11 locations) and *application site* (one study/2 locations) air monitoring data. Of the 11 acute *ambient* air concentrations assessed, six resulted in risk estimates that are of concern (i.e., MOEs < 100). Only one steady-state *ambient* air concentration resulted in a risk estimate not of concern (i.e., MOEs > 100). For the *application site* air concentrations assessed, all resulted in risk estimates of concern (i.e., MOEs < 100).

Aggregate Risk Assessment
For the chlorpyrifos aggregate assessment, the EPA has traditionally used a DWLOC approach to calculate the amount of exposure available in the total 'risk cup' for chlorpyrifos in drinking water after accounting for any chloropyrifos exposures from food and residential use. This DWLOC is then compared to the EDWC to determine if there is an aggregate risk of concern. However, because the dietary risks from food exposure alone and from residential exposure alone are of concern, it is not possible to calculate a DWLOC; essentially, the steady state aggregate DWLOC is '0' after accounting for food and residential exposures. Quantitatively aggregating (combining) residential, food, and drinking water exposures would result in risks of concern.

Occupational Risk Assessment
Steady state occupational handler and post-application exposure analyses were previously completed for the registered uses of chlorpyrifos. However, occupational exposures and risk estimates have been updated to incorporate the revised PBPK-derived PoDs. The scenarios, assumptions, and exposure inputs have not changed since the previous assessment.

Using the updated PBPK-derived steady state PoDs and uncertainty factors (dermal and inhalation LOC = 100), all agricultural occupational handler scenarios, all primary seed treatment handler scenarios, and all secondary seed treatment (planter) scenarios are of concern with label-specified and maximum levels of personal protective equipment (PPE) or engineering controls (MOEs < 100).

Using the updated PBPK-derived steady state PoDs and uncertainty factors (dermal LOC = 100), all occupational dermal post-application scenarios were of concern on Day 0. The REIs on the registered chlorpyrifos labels range from 24 hours to 5 days. On average, scenarios were not of concern ≥ 18 days after treatment.

2.0 Use Profile

Chlorpyrifos (0,0-diethyl-0-3,5,6-trichloro -2-pyridyl phosphorothioate) is a broad-spectrum,

chlorinated OP insecticide. Registered use sites include a large variety of food crops (including fruit and nut trees, many types of fruits and vegetables, and grain crops), and non-food use settings (e.g., golf course turf, industrial sites, greenhouse and nursery production, sod farms, and wood products). Public health uses include aerial and ground-based fogger adulticide treatments to control mosquitoes. There are also residential uses of roach bait products and ant mound treatments. Permanent tolerances are established (40 CFR§180.342) for the residues of chlorpyrifos in/on a variety of agricultural commodities, including meat, milk, poultry and eggs. There are also tolerances for use in food handling/service establishments (FHE or FSE). Chlorpyrifos is manufactured as granular, microencapsulated liquid, soluble concentrate liquid, water dispersible granular in water soluble packets (WSP), wettable powders in WSPs, impregnated paints, cattle ear tags, insect bait stations and total release foggers. There is a wide range of application rates and methods. The residues of concern for risk assessment purposes are chlorpyrifos and chlorpyrifos oxon under some circumstances.

3.0 Tolerance Considerations

See Section 2.0 and Appendix 8 of D22485 (D. Drew *et al.,* 12/29/2014) for details regarding the analytical enforcement method, U.S. tolerances and international residue levels for chlorpyrifos.

4.0 Chemical Identity and Physical/Chemical Properties

See Sections 3.1 and 3.2 and Appendix 7 of D22485 (D. Drew *et al.,* 12/29/2014) for details regarding the chemical identity and physical/chemical characteristics of chlorpyrifos.

5.0 Hazard Characterization and Dose-Response Assessment

5.1 Introduction & Background

Historically, the EPA has used AChE inhibition as the critical effect for deriving risk assessment PoDs for OP pesticides, including chlorpyrifos. However, there is a breadth of information available on the potential adverse neurodevelopmental effects in infants and children as a result of prenatal exposure to chlorpyrifos. Over the last several years, the agency has taken a stepwise, objective, and transparent approach to evaluate, interpret, and characterize the strengths and uncertainties associated with the available neurodevelopmental information. This effort has involved extensive collaboration across the EPA and also within the Federal government.

The stepwise evaluation began with the September 2008 FIFRA SAP. The SAP evaluated the agency's preliminary review of available literature and research on chlorpyrifos, with a particular focus on effects seen in women and children following chlorpyrifos exposures (USEPA, 2008). Subsequently, the agency has developed approaches for risk assessment of semi-volatile pesticides (USEPA, 2009), and developed the draft "Framework for Incorporating Human Epidemiologic & Incident Data in Health Risk Assessment" to better integrate epidemiology data with other types of experimental data in pesticide risk assessments (USEPA, 2010; FIFRA SAP 2010a,b). In early 2011, the FIFRA SAP reviewed the chlorpyrifos physiologically based pharmacokinetic – pharmacodynamic (PBPK-PD) model to conduct quantitative risk assessment.

The model estimates AChE inhibition in humans following exposure to chlorpyrifos and/or the oxon from a variety of exposure pathways (FIFRA SAP 2011).

In 2012, the agency convened another FIFRA SAP to review the latest experimental data related to AChE inhibition, cholinergic and non-cholinergic adverse outcomes, including neurodevelopmental studies on behavior and cognition effects (FIFRA SAP 2012[2]). Similarly, the agency also performed an in-depth analysis of the available chlorpyrifos biomonitoring data and of the available epidemiologic studies from three major children's health cohort studies in the U.S., including those from the Columbia University. The agency also explored plausible hypotheses on mode of actions/adverse outcome pathways (MOAs/AOPs) leading to neurodevelopmental outcomes seen in the biomonitoring and epidemiology studies.

Following the 2012 SAP meeting, the agency solicited additional input from federal experts in the areas of Magnetic Resonance Imaging (MRI) and neurobehavioral testing in children to further clarify results obtained by examination of the epidemiological cohorts.[3] Also, the agency evaluated the potential for chlorpyrifos exposure to lead to the neurobehavioral outcomes seen in the cohorts, and the ability of other environmental exposures to affect the interpretation of the results from the Columbia University studies.

In December, 2014, the agency released "Chlorpyrifos: Revised Human Health Risk Assessment for Registration Review" (herein called "HHRA", D. Drew *et al.,* D424485, 12/29/2014). The 2014 assessment used a PBPK-PD model (Appendix 2) to derive human PoDs based on 10% RBC AChE inhibition; for more information see Appendix 2 of D424485 (D. Drew *et al.,* 12/29/2014). In accordance with the recommendation of the FIFRA SAP (2012), the agency conducted a dose reconstruction analysis based on registered uses available for use in indoor residential areas prior to the year 2000. The highest exposures resulted from the registered broadcast use in residential homes. Based on the output from the PBPK-PD model, for the highest exposure considered (i.e., contact with hard floors following indoor broadcast use of a 1% chlorpyrifos formulation), <10% RBC AChE inhibition in pregnant women and young children would be expected from residential uses. It is noteworthy that all estimates of exposure based on conservative assumptions lead to predicted AChE inhibition levels < 10%. The chlorpyrifos 2014 revised HHRA included retention of the 10X FQPA SF for all populations assessed; including infants, children, youths, and women of childbearing age. The 10X FQPA safety factor was retained based on the conclusion that, given the totality of evidence, chlorpyrifos likely played a role in the neurodevelopmental outcomes reported by the Columbia University investigators but uncertainties, such as the lack of an established MOA/AOP for neurodevelopmental effects and the exposure to multiple AChE-inhibiting pesticides, precluded definitive causal inferences. As a result, there is sufficient uncertainty in the human dose-response relationship for neurodevelopmental effects which prevents the agency from reducing or removing the statutory 10X FQPA SF (D. Drew *et al.,* D424485, 12/29/2014).

In 2013, the EPA sought to obtain the original raw data used to support certain epidemiological analyses of *in* utero exposure to chlorpyrifos and subsequent adverse neurodevelopmental health outcomes in children generated by the CCCEH. While the researchers did not agree to provide

[2] https://www.regulations.gov/docket?D=EPA-HQ-OPP-2012-0040
[3] http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2008-0850-0170

these data to the EPA, agency staff gained valuable insight into the conduct of the study and the data that were collected in a visit to Columbia University in April 2013. The agency wrote a summary of the 2013 meeting with researchers from Columbia University which can be found in "Appendix 6 Columbia Center for Children's Environmental Health (CCCEH) Epidemiology Data Acquisition "Raw Data Request" of Drew *et. al*, D424485, 12/29/2014. In the summer of 2015, Dr. Dana Barr of Emory University (formerly of CDC) provided the EPA with limited raw urine and blood data in her possession from the three cohorts. However, the files provided from Dr. Barr are not useful for the EPA's current purpose of assessing risk to chlorpyrifos (D. Vogel, Record of Correspondence, 10/2016). The EPA does not have any of the other measurements of the children in the cohort (e.g., chlorpyrifos blood data, interviews, test or IQ scores).

In a 2016 white paper, the agency proposed using data on cord blood reported from the investigators at the Columbia Center for Children's Environmental Health (CCCEH) as the source for new PoDs for risk assessment. This 2016 white paper was reviewed by the FIFRA SAP in April, 2016[4]. The 2016 Panel did not support using the CCCEH chlorpyrifos concentrations in cord blood quantitatively to derive PoDs for risk assessment. The Panel noted a number of uncertainties, including: the use of results from a single longitudinal study without replication from another cohort; the lack of verification and replication of the analytical chemistry results that reported very low levels of chlorpyrifos (pg/g); and the lack of raw data available for independent evaluation. Importantly, however, the Panel agreed that "both epidemiology and toxicology studies suggest there is evidence for adverse health outcomes associated with chlorpyrifos exposures below levels that result in 10% red blood cell (RBC) acetylcholinesterase (AChE) inhibition (i.e., toxicity at lower doses)." Moreover, the Panel did support the use of the PBPK model to assess internal dosimetry from various exposure scenarios. The SAP specifically stated that PBPK modelling "is a valuable tool to interpret the biomonitoring data in circumstances where multiple routes of exposure occur and when based on best available information as inputs."

Therefore, based on the evidence collected from 2014 to date, as summarized above, the agency has updated its HHRA for the existing uses of chlorpyrifos. This 2016 human health risk assessment provides limited, summary information and substantially relies on previous documents developed for chlorpyrifos which contain more detailed evaluations of scientific literature and the PBPK model:

- D. Drew *et al.,* Chlorpyrifos: Revised Human Health Risk Assessment for Registration Review, December 29, 2014, D424485; and
- U.S. Environmental Protection Agency, Literature Review on Neurodevelopment Effects & FQPA Safety Factor Determination for the Organophosphate Pesticides, September 15, 2015, D331251.

5.2 Summary of the Literature Review on Neurodevelopmental Effects

Detailed summaries of the epidemiological studies used in this literature review can be found either in the 2014 chlorpyrifos HHRA (D. Drew *et al.,* D424485, 12/29/2014), the 2015 literature review for other organophosphates (OPP/USEPA, D331251, 09/15/2015), and reviews of newer studies (E. Holman, D432184, 03/25/2016). Only brief summaries of the literature reviews are

[4] https://www.regulations.gov/docket?D=EPA-HQ-OPP-2016-0062

provided below.

Newer lines of research on OPs have raised some uncertainty about the agency's risk assessment approach of using AChE inhibition for deriving PoDs. These uncertainties are in the areas of potential AOPs; *in vivo* animal studies; and notably results seen in epidemiological studies in mothers and children, with regard to the potential for neurodevelopmental effects in fetuses and children. Many of these studies have been the subject of review by the agency over the last several years as part of the development of the 2014 chlorpyrifos HHRA (D. Drew *et al.*, D424485, 12/29/2014).

A review of the scientific literature on potential MOAs/AOPs[5] leading to effects on the developing brain was conducted for the 2012 FIFRA SAP meeting (USEPA, 2012) and updated for the December 2014 chlorpyrifos HHRA (D. Drew *et al.*, D424485, 12/29/2014). In short, multiple biologically plausible hypotheses and pathways are being pursued by researchers that include targets other than AChE inhibition, including cholinergic and non-cholinergic systems, signaling pathways, proteins, and others. However, no one pathway has sufficient data to be considered more credible than the others. Published and submitted guideline developmental neurotoxicity (DNT) laboratory animal studies have been reviewed for OPs (D. Drew *et al.*, D424485, 12/29/2014 and USEPA, D331251, 09/15/2015). Neurobehavioral alterations in laboratory animals were often reported; however, at AChE inhibiting doses. Moreover, there was generally a lack of consistency in pattern, timing, and dose-response for these effects; and a number of studies were of low quality. However, the information on neurobehavioral effects as a whole provides evidence of long-lasting neurodevelopmental disorders in rats and mice following gestational exposure to OPs.

Initially, the agency focused on epidemiological studies from three US cohorts: 1) The Mothers and Newborn Study of North Manhattan and South Bronx performed by the CCCEH at Columbia University; 2) the Mt. Sinai Inner-City Toxicants, Child Growth and Development Study or the "Mt. Sinai Child Growth and Development Study;" and 3) the Center for Health Assessment of Mothers and Children of Salinas Valley (CHAMACOS) conducted by researchers at University of California Berkeley. The agency has evaluated these studies and sought external peer review (FIFRA SAP reviews in 2008 and 2012; federal panel, 2013[6]) and concludes they are of high quality. In the three US epidemiology cohort studies, mother-infant pairs were recruited for the purpose of studying the potential health effects of environmental exposures during pregnancy on subsequent child development. Each of these cohorts has evaluated the association between prenatal chlorpyrifos and/or OP exposure with adverse neurodevelopmental outcomes in children through age 7-11 years. For the 2014 chlorpyrifos HHRA (D. Drew *et al.*, D424485, 12/29/2014), the EPA included epidemiologic research results from these three US prospective birth cohort studies but primarily focused on the results of CCCEH since this cohort has published studies on the association between cord blood levels of chlorpyrifos and neurodevelopmental outcomes. The agency retained the FQPA 10X SF in the 2014 chlorpyrifos revised risk assessment, in large part, based on the findings of these studies.

[5] Mode of action (MOA) and adverse outcome pathways (AOPs) describe a set of measureable key events that make up the biological processes leading to an adverse outcome and the causal linkages between such events.

[6] http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2008-0850-0170

In the 2015 updated literature review (USEPA, D331251, 09/15/2015), the agency conducted a systematic review expanding the 2012/2014 review which was focused only on US cohort studies with particular emphasis on chlorpyrifos. The expanded 2015 review includes consideration of the epidemiological data on any OP pesticide, study designs beyond prospective cohort studies, and non-U.S. based studies. The updated literature review identified seven studies which were relevant (Bouchard *et al.,* 2010; Fortenberry *et al.,* 2014; Furlong *et al.,* 2014; Guodong *et al.,* 2012; Oulhote and Bouchard, 2013; Zhang *et al.,* 2014; Shelton *et al.,* 2014). These seven studies have been evaluated in context with studies from the 2012/2014 review (D. Drew *et al.,* D424485, 12/29/2014). In addition, the agency has also reviewed more recent studies from CCCEH (Rauh *et al.*, 2015) and a pooled analysis of U.S. cohort studies (Engel *et al.*, 2015) (E. Holman, D432184, 03/25/2016). As discussed below, Rauh *et al.* (2015) provides further evidence of neurodevelopmental outcomes in the CCCEH study. The Engel *et al.* (2015) study shows relatively consistent results compared to previous studies conducted at 24 months (Engel *et al.*, 2011; Rauh *et al.*, 2006). Only a brief summary of this review is provided below. The agency continues to conclude that the 3 U.S. cohort studies (CCCEH, CHAMACOS, and Mt. Sinai) provide the most robust available epidemiological evidence.

The agency acknowledges the lack of established MOA/AOP pathway, the inability to make strong causal linkages, and the unknown window(s) of susceptibility. These uncertainties do not undermine or reduce the confidence in the findings of the epidemiology studies. The epidemiology studies reviewed in the 2012/2014 and 2015 literature reviews represent different investigators, locations, points in time, exposure assessment procedures, and outcome measurements. Despite differences in study design, with the exception of two negative studies in the 2015 literature review (Guodong *et al.,* 2012; Oulhote and Bouchard, 2013) and the results from the more recent Engel *et al.* (2015) study[7], all other study authors have identified associations with neurodevelopmental outcomes associated with OP exposure; these conclusions were across four cohorts and twelve study citations. Specifically, there is evidence of delays in mental development in infants (24-36 months), attention problems and autism spectrum disorder in early childhood, and intelligence decrements in school age children who were exposed to OPs during gestation. Investigators reported strong measures of statistical association across several of these evaluations (odds ratios 2-4 fold increased in some instances), and observed evidence of exposures-response trends in some instances, *e.g.*, intelligence measures.

The CCCEH study primarily tested for the presence of chlorpyrifos in cord blood, and therefore remains the most relevant for the purposes of chlorpyrifos risk assessment. As summarized above, when comparing high to low exposure groups at 3 years of age in the CCCEH study (Rauh *et al.*, 2006), there were increased odds of:

- Mental delay (odds ratio; OR=2.4; 95% Confidence interval (CI): 1.1–5.1);
- Psychomotor delay (OR=4.9; 95% CI: 1.8–13.7);
- Attention disorders (OR=11.26; 95% CI: 1.79–70.99);
- Attention deficit hyperactivity disorder (ADHD) (OR=6.50; 95% CI: 1.09–38.69); and
- Pervasive Developmental Disorders (PDD) (OR=5.39; 95% CI: 1.21–24.11).

In a follow-up study at age 11, CCCEH study authors observed increased odds of mild to

[7] It is noted that the CCCEH study participants included in the Engel et al (2015) study are women enrolled from 2000-2001, i.e. after the cancellation of the residential uses of chlorpyrifos.

moderate tremor when comparing high to low exposure groups (Rauh *et al.*, 2015). Rauh *et al.*, (2011) evaluated relationship between prenatal chlorpyrifos exposure and neurodevelopment in 265 of the CCCEH cohort participants at age 7 years. They described the log of Working Memory Index (WMI) of children as linearly associated with concentration of chlorpyrifos (CPF) in cord blood: Slope = -0.006 (95% CI = -0.01, -0.002). For each standard deviation increase in exposure (4.61 pg/g), they observed a 1.4% reduction in Full-Scale IQ and a 2.8% reduction in Working Memory.

In summary, the EPA's assessment is that the CCCEH study, with supporting results from the other 2 U.S. cohort studies and the seven additional epidemiological studies reviewed in 2015, provides sufficient evidence that there are neurodevelopmental effects occurring at chlorpyrifos exposure levels below that required for AChE inhibition.

5.3 Dose-Response Assessment

5.3.1 Conceptual Approach

As noted above, the agency has historically used 10% inhibition of RBC AChE as the critical effect for deriving PoDs for chlorpyrifos and other OPs. For example, the 2014 HHRA on chlorpyrifos used the PBPK-PD model to derive PoDs that could result in 10% RBC AChE inhibition for multiple exposure scenarios (e.g., worker, dietary, residential). While significant uncertainties remain about the actual exposure levels experienced by mothers and infant participants in the children's health cohorts, it is unlikely that these exposures resulted in RBC AChE inhibition at or above the 10% AChE inhibition response level. For example, as part of the CHAMACOS study, Eskenazi *et al.*, (2004) measured AChE activity and showed that no inhibition in AChE activity were observed. Additionally, following the recommendation of the FIFRA SAP in 2012, the agency conducted a dose reconstruction analysis for pregnant women and young children based on registered residential chlorpyrifos uses available prior to 2000 inside the home (D. Drew *et al.*, D424485, 12/29/2014). The PBPK-PD model using this dose reconstruction analysis indicates that for the highest exposure considered (i.e., indoor broadcast use of a 1% chlorpyrifos formulation), <1% RBC AChE inhibition was produced in pregnant women. While uncertainty exists as to actual chlorpyrifos exposure at (unknown) critical windows of exposure, the agency believes it is unlikely individuals in the epidemiology studies experienced RBC AChE inhibition from their exposure to chlorpyrifos. The 2016 SAP concluded that "epidemiology and toxicology studies suggest there is evidence for adverse health outcomes associated with chlorpyrifos exposures below levels that result in 10% RBC AChE inhibition (i.e., toxicity at lower doses)." As such, the use of 10% RBC AChE inhibition for deriving PoDs for chlorpyrifos may not provide a sufficiently protective human health risk assessment. Therefore, the agency has endeavored to derive PoDs and uncertainty/safety factors for risk assessment that are protective of both the AChE inhibition and any adverse effects that could occur at lower doses.

As noted, however, the 2016 SAP did not support using the CCCEH cord blood quantitatively in deriving revised PoDs. In their verbal comments, multiple panelists suggested a 'hybrid' approach. In the written report, the SAP did not provide a suggested approach for how the EPA might continue to use the epidemiology data results in a quantitative risk assessment without

attempting to derive the PoD from cord blood data. Specifically, the SAP stated that, given the absence of a particular key window of exposure for the effects shown in the CCCEH study, the EPA should use estimated peak blood concentrations or TWA blood concentrations within the prenatal period as the PoD rather than blood concentrations at delivery. The Panel was also positive and supportive of the agency's use of the PBPK model as a tool for assessing internal dosimetry from the typical OPP exposure scenarios using peer reviewed exposure assessment approaches (e.g., food, water, residential, worker). As such, use of the PBPK model coupled with the typical OPP exposure scenarios to derive PoDs based on TWA blood concentrations, as recommended by the SAP, provide the strongest scientific foundation for moving forward in human health risk assessment for chlorpyrifos. This approach:

- incorporates peer reviewed and accepted inputs for both chlorpyrifos and standard pesticide risk assessment, including: the Residential SOPs[8], the EPA Exposure Factors Handbook 2011 Edition, chlorpyrifos-specific residential exposure modeling inputs and others;
- does not directly rely on quantitative measures of chlorpyrifos in cord blood obtained from the CCCEH, which were the source of uncertainty identified by the 2016 SAP, while still accepting the qualitative findings that chlorpyrifos contributed to the outcomes reported by the CCCEH, which were supported by the 2008 and 2012 SAPs; and
- does not directly rely on quantitative measures of chlorpyrifos in cord blood obtained from the CCCEH, and thus, the lack of access to the raw data from the CCCEH is less of an uncertainty.

The following sections describe the use of the PBPK model to 1) predict TWA of blood concentrations from an exposure scenario likely to be experienced by women in the CCCEH study (indoor use of chlorpyrifos-containing products), and 2) determine the external doses (PoDs for risk assessment) for infants, children, youths, and adults using current exposure assumptions and methodologies (i.e., The 2012 Residential SOPs, and chemical-specific exposure data, etc.) that result in the predicted TWA of blood concentration. The likely indoor use scenario which was experienced by the women in the CCCEH study was derived from the indoor crack and crevice uses of chlorpyrifos; reasoning for selecting this specific scenario is detailed below.

5.3.2 Deriving Internal Concentrations of Chlorpyrifos from Indoor, Crack & Crevice Use

In order to derive a protective PoD for risk assessment from the internal concentrations of chlorpyrifos, the agency reviewed the chlorpyrifos registered uses that would have been available to the CCCEH cohort. The following two risk mitigation actions were the basis for the agency's conclusion that the crack and crevice uses of chlorpyrifos was the most appropriate scenario to assess exposure to the women in the CCCEH cohort in the approximate 1998-2000 timeframe:

- In January 1997, the technical registrants agreed to cancel all broadcast and total release/aerosol foggers containing chlorpyrifos in order to reduce indoor exposures, especially to children and other sensitive groups. The following chlorpyrifos uses were

[8] https://www.epa.gov/sites/production/files/2015-08/documents/usepa-opp-hed_residential_sops_oct2012.pdf

also cancelled: all direct application of pet products including sprays, shampoos, and dips (pet collars not included); and all insecticidal paint additives. Further, all concentrates which required mixing were eliminated, limiting the household consumer's access to only ready-to-use products. Although the above uses were cancelled in 1997, existing stocks could be phased out, or applied until depleted. Indoor crack and crevice (perimeter) and spot treatment as a termiticide uses of chlorpyrifos continued to be registered.

- In June 2000, the technical registrants of chlorpyrifos, agreed to eliminate or phase out nearly all remaining uses that resulted in residential exposure, including: home lawn, crack and crevice, and other indoor uses. Non-residential uses where children could be exposed, such as schools and parks, were also cancelled, with the exception of roach and ant baits in child resistant packaging, and mosquito and fire ant control. For uses that were cancelled, retailers had a stop sale date of December 31, 2001. A phase out of existing stocks was allowed following the 2001 stop sale.

Additionally, in the summer of 2016, OPP contacted several professional pesticide applicators working in New York City apartment buildings around the time of the CCCEH cohort. These professional pesticide applicators recalled that the crack and crevice[9] use was the predominant use around 1998-2000 (D. Friedman, Record of Correspondence, 10/2016). Based on this input, and the mitigation rationale outlined above, the agency has focused on crack and crevice exposures for the 2016 risk assessment.

The 2012 FIFRA SAP (2012) recommended that the EPA conduct a "dose reconstruction" analysis of indoor residential uses to assess potential for RBC AChE inhibition. The dose reconstruction analysis was conducted and presented in the 2014 HHRA[10]. The goal of the dose reconstruction exercise was to estimate upper limit, bounding level exposures, to test the hypothesis of whether RBC AChE at or above the 10% inhibition level used by the agency for typical AChE PoDs may have occurred in the CCCEH cohort. For example, in the dose reconstruction analysis, exposure to the women was assumed to occur 24 hours a day without adjustments for bathing, showering, or leaving the residence for 14 consecutive days. For the 2014 HHRA, residential handler and post-application exposures from indoor broadcast applications resulted in the highest risk estimates and, therefore, were the only exposure estimates presented. The purpose of 2016 analysis for this risk assessment is to predict typical product usage and behaviors thereby deriving more accurate and realistic estimates of exposure compared to the 2014 analysis.

For the 2016 risk assessment, the agency has assessed chlorpyrifos exposures resulting from post-application exposures only. Whyatt *et al.* (2002) reported that many women applied pesticide products themselves, and that majority who reported using pesticide products used them at least once per month. However, as the agency has shown in the 2014 dose reconstruction analysis, post-application exposures are greater in magnitude than exposures which occur during an application. Therefore, the assessment of post-application exposure ensures that the highest potential exposures are evaluated. Specifically, the 2016 risk assessment

[9]Per the 2012 Residential SOPs, a crack and crevice application is defined as application of pesticides with the use of a pin stream nozzle, into cracks and crevices in which pests hide or through which they may enter a building. Such openings commonly occur at expansion joints, between different elements of construction, and between equipment and floors.

[10] The methods, algorithms, and exposure data used to conduct the dose reconstruction analysis can be referenced in Appendix 10 of the 2014 HHRA.

focuses on the post-application exposures from the chlorpyrifos in crack and crevice use since this was the predominant application type during the time of the CCCEH cohort.

The dose reconstruction in the 2016 risk assessment is based on the methods outlined in the 2012 Residential SOPs[11] which describe specific algorithms and inputs, on a scenario-specific basis.[12] Appendix 10 of the 2014 HHRA (D. Drew *et al.,* D424485, 12/29/2014) can be referenced for a description of the methods, algorithms, and inputs used. Specifically, the 2012 Residential SOPs[13] have been used to predict the range of potential exposures which could have occurred to individuals in the cohort for crack and crevice hard surface and carpet treatments. The present analysis uses the same chemical-specific exposure data inputs recommended in the 2012 Residential SOPS (*i.e.*, the fraction of chlorpyrifos residues transferred from treated carpet and hard surfaces to the exposed individual; and exposure data used to derive the liquid formulation transfer coefficient (TC)). Additionally, chemical-specific exposure data were used to define the concentrations of chlorpyrifos present in air following indoor applications. The differences between the previous dose reconstruction and the present analysis are: (1) the exposure duration was 24 h/day for the 2014 dose reconstruction analysis, and 2 h/day for the present analysis; (2) predicted endpoint for the dose reconstruction analysis was the peak RBC AChE inhibition level during the 14 days post-application, and the predicted endpoint for the present analysis was time-weighted average of chlorpyrifos concentrations in blood; (3) no shower was assumed to occur over the 14-day exposure period for the dose reconstruction analysis, whereas a daily shower is assumed to occur for the present analysis; (4) the total exposure duration was 14 days in the dose reconstruction analysis, and 30 days in the present analysis. The assumption that women followed in the CCCEH cohort showered immediately after exposure leads to significantly more conservative estimates of risk assessment PoDs (i.e., neurodevelopmental effects may have occurred at lower exposure levels when assuming that the women showered after daily exposure vs. when it is assumed that the women did not shower after daily exposure); however, since other inputs (e.g., 50% of the body exposed) lead to less conservative PoD estimates, the combination of inputs used to estimate exposures is expected to reasonably approximate exposures to these women resulting in reasonable risk assessment PODs.

For the 2016 risk assessment, the agency assumed a once daily shower occurred immediately following exposure activities. The PBPK model simulation were conducted for a 30-day post-application in the crack & crevice scenario. Daily exposure durations for post-application dermal contact with carpets and hard surfaces were selected based on the recommendation in the 2012 Standard Operating Procedures for Residential Pesticide Exposure Assessment[14] (herein referred to as the 2012 Residential SOPs). Specifically, for adults, the recommended exposure durations for post-application dermal contact are 8 and 2 hours daily for carpets and hard surfaces, respectively. These values are based on the EPA Exposure Factors Handbook 2011[15] Edition that provides information on the total time spent in a residence and time spent in various rooms within a residence. The hard surface exposure scenario resulted the highest estimated exposures and, therefore, was selected for PBPK model PoD derivation. Additionally, chlorpyrifos residues were assumed to dissipate 10% daily; that is, the total amount of residue

[11] https://www.epa.gov/sites/production/files/2015-08/documents/usepa-opp-hed_residential_sops_oct2012.pdf
[12] The 2012 Residential SOPs were subjected to peer review by FIFRA SAP in October 2009. http://www.regulations.gov/#!docketBrowser;rpp=50;po=0;D=EPA-HQ-OPP-2009-0516
[13] https://www.epa.gov/sites/production/files/2015-08/documents/usepa-opp-hed_residential_sops_oct2012.pdf
[14] http://www.epa.gov/pesticides/science/residential-exposure-sop.html
[15] http://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=236252

available for transfer from the treated floor is assumed to reduce by 10% for each subsequent day of exposure until the end of the 30th day prior to the next application. The 10% value was based on an evaluation of all available chlorpyrifos-specific floor residue data. For all post-application exposure scenarios a female bodyweight reflective of all trimesters of pregnancy, 75 kg, was assumed to reflect the population of interest from the CCCEH cohort. This value was derived from the EPA Exposure Factors Handbook 2011 Edition (adult female: Tables 8-3 through 8-5; body weight of pregnant women: Table 8-29).

The results of the 2016 dose reconstruction assessment of the post-application exposures following contact with hard surfaces following indoor chlorpyrifos crack and crevice treatment is presented in Table 5.3.2.

Table 5.3.2. Residential Post-application Exposures to Women in the CCCEH Cohort Following Indoor Chlorpyrifos Crack and Crevice Treatment.

Exposure Scenario	Formulation	Deposited Residue[1] ($\mu g/cm^2$)	Fraction Transferred[2]	Transferable Residue[3] ($\mu g/cm^2$)	Transfer Coefficient (cm^2/hr)	Exposure Time (hr/day)	Dermal Dose[4] (mg/kg/day)	Airborne Concentration of Chlorpyrifos[5] (mg/m^3) - Day of Application
Crack and Crevice (Hard Surfaces)	1% PCO Crack and Crevice Application	0.30	0.13	0.039	6,800	2	0.00707	0.00089

1 Estimated based on the recommendations of the 2012 Residential SOPs: Indoor Environments SOP.
2 Chlorpyrifos-specific fraction transfer as recommended in the 2012 Residential SOPs: Indoor Environments SOP (Table 7-9; Arithmetic Mean).
3 Transferable Residue ($\mu g/cm^2$) = Deposited Residue ($\mu g/cm^2$) * Fraction Transferred (unitless)
4 Dermal Dose (mg/kg/day) = Transferable Residue ($\mu g/cm^2$) * Transfer Coefficient (cm^2/hr) * Exposure Time (hr/day) * Conversion Factor (0.001 $mg/\mu g$)
5 Average airborne concentration of chlorpyrifos from crack and crevice on the day of product application as determined from 3 literature studies and 1 registrant submitted study.

The PBPK model-predicted time course of chlorpyrifos concentrations in blood based on the crack and crevice scenario is provided in Figure 1. The predicted TWA of chlorpyrifos concentration in blood from this scenario was 0.004 µg/L, shown as the solid horizontal line in Figure 1.

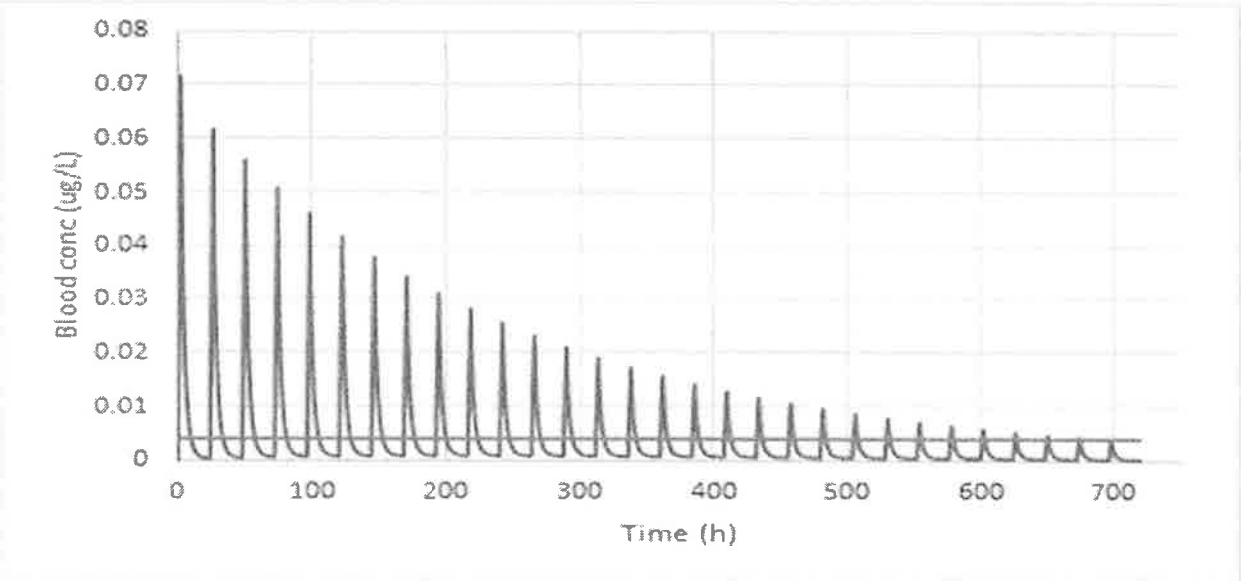

Figure 1: The PBPK model-predicted time course of chlorpyrifos concentrations in blood based on the crack and crevice scenario. The predicted TWA of chlorpyrifos concentration in blood (0.004 µg/L) is shown by the solid line.

5.3.3 Determining PoDs

In typical risk assessments, PoDs are derived directly from laboratory animal studies and inter- and intra-species extrapolation is accomplished by use of 10X factors. In the case of chlorpyrifos, the PBPK model for chlorpyrifos was used as a data-derived extrapolation approach to estimate individual PoDs for pregnant women and children. As noted above, the PBPK model was first used to predict, from the crack and crevice post-application scenario, the TWA of chlorpyrifos concentration in blood as the internal dose metric for deriving PoDs in the subsequent analyses.

For the 2014 HHRA (D. Drew *et. al*, D424485, 12/29/2014), the EPA developed PoDs based on AChE inhibition to protect against cholinergic toxicity; such cholinergic toxicity could occur to any lifestage if exposure is sufficiently high. As such, in 2014, the EPA evaluated the spectrum of lifestages from the fetus through adulthood. Fetuses may be exposed to chlorpyrifos through the mother while infants and children may be exposed directly. Studies in laboratory animals do not suggest any specific critical period or lifestage, but instead suggest pre- and post-natal periods of susceptibility. The EPA acknowledges that the epidemiology literature regarding associations between post-natal (infancy, childhood) biomarker metrics and neurodevelopmental outcomes is limited to the Bouchard *et al.,* (2010) study, a cross-sectional study that observed positive association between attention and behavior problems and total dialkyl phosphate metabolites (DAPs) and dimethyl alkylphosphate metabolites (DMAPs), using urinary National Health and Nutrition Examination Survey (NHANES) data in children 8–15 years old. The other studies which evaluated postnatal biomarker metrics and neurodevelopment outcomes have found no statistically significant associations. Specifically, postnatal exposure to OPs (measured as DAPs) has been assessed in the CHAMACOS cohort (Eskenazi *et al.*, 2007; Young *et al.*,

2005; Bouchard *et al.*, 2011), two other cross-sectional studies (Guodong *et al.*, 2012; Oulhote and Bouchard, 2013) and Engel *et al.,* (2016). Despite the limited epidemiological evidence from postnatal exposure, the EPA is proposing to use the TWA as the most relevant source of information for deriving a PoD specific for chlorpyrifos for fetuses, infants, and children. Consistent with the advice from the 2016 SAP, the EPA believes that the CCCEH results are directly relevant to fetal exposure and newborns; however, the EPA acknowledges they may be less relevant to older infants, toddlers, and children. The EPA has conducted exposure assessments for all typical age groups for completeness and acknowledges that the exposure and risk assessment results for females 13-49 years old are the most relevant to the CCCEH data.

The PBPK model accounts for pharmacokinetic characteristics to derive age, duration, and route specific PoDs (Table 5.3.3.3). Separate PoDs have been calculated for dietary (food, drinking water), residential, and occupational exposures by varying inputs on types of exposures and populations exposed to obtain a predicted time-weighted average of 0.004 µg/L chlorpyrifos in blood using inputs specific to each scenario (i.e., duration exposed, amount consumed, etc). Specifically, the following characteristics have been evaluated: route (dermal, oral, inhalation); body weights which vary by life-stage; exposure duration (hours per day, days per week); and exposure frequency [events per day (eating, drinking)].

To derive a PoD for each non-dietary and dietary exposure scenario and subpopulation, the appropriate body weight for each age group or sex was taken from the Exposure Factors Handbook (USEPA, 2011) (for occupational exposures) or from the NHANES/What We Eat in America (WWEIA) Survey[16] (for dietary exposures). All body weights used are consistent with those assumed for typical pesticide dietary, occupational, and residential exposure assessments and shown in Table 5.3.3.1.

Table 5.3.3.1. Body Weight Assumptions Incorporated into PBPK Model for Chlorpyrifos.

Exposure Scenario	Exposure Pathway	Population & Body Weight (kg)				
		Infants (< 1 yr old)	Young Children (1 - 2 years old)	Children (Residential:6-11 years old; Dietary:6-12 years old)	Youths (Residential:11-16 years old; Dietary:13-19 years old)	Females (13 – 49 years old)
Dietary	Food and Drinking Water	4.8[1]	12.6[2]	37.1[2]	67.3[2]	72.9[2]
Residential (Golfers)	Dermal			32[5]	57[6]	69[4]
Residential (Mosquitocide Application)	Dermal, Oral, Inhalation		11[3]			
Residential (Bystander/ Volatilization Assessment)	Inhalation		11[3]			
Occupational	Dermal, Inhalation					

1 For infants from birth to < 1 year old, the agency has selected the body weight for the youngest age group, birth to < 1 month old, 4.8 kg (Exposure Factors Handbook, Table 8-3, mean body weight for the birth to < 1 month age group).
2 NHANES/WWEIA
3 Exposure Factors Handbook, Table 8-3, mean body weight for the 1 to < 2 year old age group.

[16] http://www.ars.usda.gov/Services/docs.htm?docid=13793

4 Exposure Factors Handbook, Table 8-5, mean body weight for females 13 to < 49 years old.
5 Exposure Factors Handbook, Table 8-3, mean body weight for the 6 to < 11 year old age group.
6 (Exposure Factors Handbook, Table 8-3, mean body weight for the 11 to < 16 year old age group).

Table 5.3.3.2 shows the durations (days) of exposure included in the PBPK model to derive PoDs.

Table 5.3.3.2. Days of Exposure Assumptions Incorporated into PBPK Model for Chlorpyrifos.

Exposure Scenario	Exposure Pathway	Population & Days of Exposure				
		Infants (< 1 yr old)	Young Children (1 - 2 years old)	Children (Residential:6-11 years old; Dietary:6-12 years old)	Youths (Residential:11-16 years old; Dietary:13-19 years old)	Females (13 – 49 years old)
Dietary	Food and Drinking Water	21	21	21	21	21
Residential (Golfers)	Dermal			21	21	21
Residential (Mosquitocide Application)	Dermal, Oral, Inhalation		21			
Residential (Bystander/ Volatilization Assessment)	Inhalation		1 & 21			
Occupational	Dermal, Inhalation					

To derive the dietary exposure PoDs, dietary exposure was estimated daily for 21 days. For drinking water exposures, the daily water consumption volume was set to 0.688557 L for infants, children between 1-2 year old, and children 6-12 years old; 1.71062 L for youths 13-19 years old and female adults. Infants and children were assumed to consume water six times a day; youths and female adults were assumed to consume water four times a day. For food exposures, the eating event was set to one meal per day. The daily volumes consumed and number of daily consumption events for all populations are mean values by age group based on USDA's WWEIA. The mean daily water consumption amounts for children 1- 2 years old (0.35 L) and children 6-12 years old (0.58 L), were less than that for infants (0.688557 L); the infant daily water consumption volume was selected for all child sub-populations to be protective. For youths 13-19 years old, the mean daily water consumption amount (0.93 L) was less than that for the female adults (1.71062 L); therefore, the adult daily water consumption was selected for both subpopulations to be protective.

For all residential dermal exposures to chlorpyrifos, the fraction of skin in contact with chlorpyrifos was set to 50% to reflect uncovered skin areas for adults and children wearing shorts and a tee shirt. A daily shower (i.e., washing off the chlorpyrifos) was assumed immediately following chlorpyrifos exposure. All residential exposures were set to be continuous for 21 days. For residential exposures via golfing on treated turf, the daily exposure time is assumed to be 4 hours/day; for residential exposures via contact with turf following public health mosquitocide application, the daily exposure duration is assumed to be 1.5 hours for ground applications and 1 hour for aerial applications. For residential inhalation exposures following public health mosquitocide application, the exposure duration was set to 1 hour per

day. These exposure times selected were based on those recommended in the 2012 Residential SOPs. For residential bystander exposures from volatilization following treatment of nearby fields, the inhalation exposure time was set to 24 hours per day. For inhalation exposures following mosquitocide application and from volatilization, the inhalation rates were set to 0.33 m^3/hour for children 1 to < 2 years old and 0.64 m^3/hour for adults.

In addition to dietary and residential exposures, the PBPK model was also used to estimate PoDs resulting in a time-weighted average of 0.004 µg/L chlorpyrifos in blood following occupational exposures (Table 5.3.3.3). Dermal exposures for workers assumed even distribution across the entire body surface area. A daily shower (i.e., washing off the chlorpyrifos) was assumed following chlorpyrifos exposure. The worker was assumed to be a female adult between the ages of 13 to 49, and had a body weight of 69 kg. This worker is exposed to chlorpyrifos either via inhalation or skin for 8 hours/day, 5 days/week, for a total of 21 days.

Table 5.3.3.3. PBPK Model-Predicted Chlorpyrifos Point of Departures (PoDs) Corresponding to a Time-Weighted Average of 0.004 µg/L Chlorpyrifos in Plasma*.

Exposure Scenario	Exposure Pathway	Infants (< 1 year old)	Young Children (1 - 2 years old)	Children (Residential:6-11 years old; Dietary:6-12 years old)	Youths (Residential:11-16 years old; Dietary:13-19 years old)	Females (13 – 49 years old)
Dietary	Drinking Water (µg/kg/day)	1.4	3.2	7.1	4.8	5.1
	Food (µg/kg/day)	0.2	0.17	0.13	0.12	0.12
Residential (Golfers)	Dermal (µg/kg/day)			2.2	1.4	1.3
Residential (Mosquitocide Application)	Dermal (µg/kg/day)		14.9			3.4
	Oral (µg/kg/day)		0.17			
	Inhalation (concn. in air mg/m^3)[1]		*Aerial:* 0.00165 *Ground:* 0.0011			*Aerial:* 0.0051 *Ground:* 0.0034
Residential (Bystander/ Volatilization Assessment)	Inhalation (concn. in air mg/m^3)		*Steady State:* 0.00068 *Acute:* 0.0013			*Steady State:* 0.00021 *Acute:* 0.004
Occupational	Dermal (µg/kg/day)					0.47
	Inhalation (concn. in air mg/m^3)					0.0011

*PoDs and exposure and risk estimates for females 13-49 yrs covers all youths >13 yrs.

1. PBPK model inputs for inhalation mosquitocide scenarios differ based on the exposure scenario being assessed. Since the AgDISP (v8.26) model predicts the 1 hour average air concentration following aerial applications, the PBPK-PD was model was run assuming 1 hr of inhalation exposure/day, 7 days/week, and 21 days of exposure. For ground based ULV applications, risks are estimated based on the inhalation exposure duration for time spent outdoors (1.5 hours/day) and, therefore, the PBPK-PD model was run assuming 1.5 hours of inhalation exposure/day, 7 days/week, 21 days of exposure.

5.3.4 Uncertainty, Extrapolation, & FQPA Safety Factors

The TWA blood level resulting from chlorpyrifos exposure from the crack and crevice scenario

is considered a LOAEL rather than a NOAEL, since this is the exposure level likely to be associated with neurodevelopmental effects reported in the CCCEH study. In situations where the agency selects a PoD from a study where a NOAEL has not been identified, the EPA generally will retain the FQPA SF of 10X to account for the uncertainty in using a LOAEL. In the 2016 revised risk assessment this is being done for chlorpyrifos. The 2016 revised risk assessment also applies a 10X uncertainty factor for intraspecies variability because of the lack of sufficient information to reduce or remove this factor. Typically, the agency uses animal studies for selection of PoDs and, as such, retains a 10X interspecies factor for extrapolation of the animal data to assess human health. However, with use of the PBPK-PD model which accounts for the pharmacokinetic and pharmacodynamic differences between animals and humans to derive PoDs, it is appropriate to reduce the interspecies factor to 1X. Therefore, the total uncertainty factors for chlorpyrifos in this 2016 risk assessment are 100X (10x for intra-species extrapolation and 10x for the FQPA 10 safety factor).

6.0 Dietary Exposure and Risk Assessment

HED had previously conducted both acute and steady state dietary (food only) exposure analyses for chlorpyrifos using DEEM and Calendex software with the Food Commodity Intake Database (FCID) (D. Drew *et al.,* D424486, 11/18/2014), respectively.

For the current assessment, the steady state exposure values resulting from the 2014 dietary assessment are compared to the updated PBPK-derived steady state Population Adjusted Dose (ssPAD). When the dietary exposure exceeds 100% of the ssPAD there is a potential risk concern.

Since the steady state dietary assessment is protective of any acute food exposures, only the results of the steady state assessment are discussed herein. The steady state analysis calculated exposures for the sentinel populations of infants <1 year old, children 1-2 years old, youth 6-12 years old, and females 13-49 years old.

All details pertaining to the assumptions, data inputs, and exposure outputs for the dietary analysis may be found in the 2014 dietary assessment memorandum (D. Drew *et al.,*D425586, 11/18/2014).

6.1 Food Residue Profile

The residue of concern for tolerance expression and risk assessment in plants (food and feed) and livestock commodities is the parent compound chlorpyrifos. Based on the available crop field trials, metabolism studies, and PDP monitoring, the cholinesterase inhibiting metabolite, chlorpyrifos oxon, would be not be present in edible portions of the crops, or in livestock tissue or milk and, therefore, is not included in the food assessment.

The steady state dietary exposure analysis is highly refined. The large majority of food residues used were based upon USDA's PDP monitoring data except in a few instances where no appropriate PDP data were available. In those cases, field trial residues or tolerance level residues were assumed. The Biological & Economic Analysis Division (BEAD) provided

percent crop treated information in the Screening Level Usage Analysis (SLUA; May 1, 2014). Food processing factors from submitted studies were used as appropriate. All commodities with current U.S. tolerances for residues of chlorpyrifos are included in this assessment (40 CFR§180.342).

6.2 Steady State Dietary (Food Only) Exposure and Risk Estimates

The steady state dietary (food only) exposures for chlorpyrifos are of concern at the 99.9th percentile of exposure for all population subgroups analyzed. Children (1-2 years old) is the population subgroup with the highest risk estimate at 14,000% of the $ssPAD_{food}$.

Table 6.2. Steady State Dietary (Food Only) Exposure and Risk Estimates for Chlorpyrifos.

Population Subgroup	ss PoD_{food}[1] (µg/kg/day)	$ssPAD_{food}$[2] (µg/kg/day)	Food Exposure[3] (µg/kg/day)	% of $ssPAD_{food}$
Infants (< 1 yr)	0.20	0.002	0.186	9,300
Children (1-2 yrs)	0.17	0.0017	0.242	14,000
Youths (6-12 yrs)	0.12	0.0012	0.128	11,000
Adults (Females 13-49 yrs)	0.12	0.0012	0.075	6,200

1 Steady state point of departure; daily dose predicted by PBPK-PD for steady state (21 day) dietary (food) exposures (see Table 5.3.3.3 for PoDs).
2 ssPAD= Steady state population adjusted dose = PoD (Dose predicted by PBPK model ÷ total UF; Total uncertainty factor =100X (10X intraspecies factor and 10X LOAEL to NOAEL extrapolation factor).
3 Steady state (21 day) food-only exposure estimates from Calendex (at 99.9th percentile).

6.3 Steady State Dietary (Food Service/Food Handling Establishments) Exposure and Risk Estimate

There are chlorpyrifos uses in food handling establishments (FHE) where food and food products are held, processed, prepared or served. These may include areas such as boxcars, shipping containers, and warehouses. FHE uses in restaurants, or similar service areas where food is prepared and served, may also be referred to as *food service establishment* (FSE) uses. There are no tolerances for the chlorpyrifos uses in FHEs except for the specific use of chlorpyrifos in FSEs as stated in the 40 CFR§180.342 (a) (3):

A tolerance of 0.1 part per million is established for residues of chlorpyrifos, per se, in or on food commodities (other than those already covered by a higher tolerance as a result of use on growing crops) in food service establishments where food and food products are prepared and served, as a result of the application of chlorpyrifos in microencapsulated form.

Typically, where there are established tolerances for FSE (or FHE) uses, anticipated residues for *all* foods would be included in the dietary assessment along with the residues on the foods with crop tolerances. The food only exposures in Section 6.2do not incorporate potential exposure from residues that may result on foods from FSE uses and, therefore, may underestimate actual exposures. A previous dietary risk assessment included a chronic analysis for FSE uses (D. Soderberg, D388166, 6/11/2011). This analysis was based on a BEAD estimate of < 2% of

establishments treated with chlorpyrifos and half the analytical limit of detection (½ LOD; 0.01 ppm) based on all nondetectable residues in a chlorpyrifos FHE study. That analysis resulted in a chronic dietary exposure of 0.009 µg/kg for children ages 1-2 years old (highest exposed population subgroup). HED has used this exposure value to compare to the ssPAD for children ages 1-2 years old. For the FSE uses alone, the children ages 1-2 years old steady state dietary (food only) exposures for chlorpyrifos are of concern, with an estimated risk of 530% of the ssPAD.

6.4 Dietary Drinking Water Risk Assessment

The total dietary exposure to chlorpyrifos is through both food and drinking water. EFED has provided a revised drinking water assessment (DWA) for chlorpyrifos (R. Bohaty, D432921, 04/14/2016) which includes the updated EDWCs for dietary risk assessment. A DWLOC approach is used to calculate the amount of exposure available in the total dietary 'risk cup' for chlorpyrifos in drinking water after accounting for chloropyrifos exposure from food. This DWLOC is then compared to the EDWC to determine if there is a risk of concern for drinking water exposures (See D. Drew, D424485, 12/29/2014 for details on the DWLOC approach and calculations). However, because the dietary risks from food alone are of concern (exceed the ssPAD), it is not possible to calculate a DWLOC; essentially the steady state DWLOC is '0' after accounting for food exposures.

Hypothetically, if there were no exposure to chlorpyrifos from food, and the entire dietary 'risk cup' was available for drinking water, the resulting steady state DWLOC for infants (the most highly exposed population subgroup for water) would be 0.014 ppb. An EDWC at or exceeding this concentration would be considered a risk of concern for exposures to chlorpyrifos in drinking water.

7.0 Residential (Non-Occupational) Exposure/Risk Characterization

Residential exposures to chlorpyrifos are currently expected from homeowner use. Formulations/use sites registered for homeowner use include a granular ant mound use and roach bait in child-resistant packaging. Additionally, chlorpyrifos is labeled for public health aerial and ground-based fogger ULV mosquito adulticide applications and for golf course turf applications. All residential exposures and risks were previously assessed in support of the 2014 HHRA (W. Britton, D424484, 12/29/2014). The previous assessment included evaluation of residential post-application risks from playing golf on chlorpyrifos-treated courses and from exposures which can occur following aerial and ground-based ULV mosquito adulticide usage. The potential for residential exposures from the roach bait product was determined to be negligible. Further, residential exposures from the ant mound use were also determined to be negligible since these products can only be applied professionally and direct exposure with treated ant mounds is not anticipated.

In addition to the assessment of residential exposure, the potential for post-application exposures to residential bystanders who live on, work in, or frequent areas adjacent to treated fields from spray drift and volatilization were also evaluated and presented in the 2014 HHRA.

The previously assessed residential post-application, residential bystander/volatilization, and non-occupational spray drift risk estimates have been updated to incorporate the approach applied for PBPK-derivation of PoDs for infants, children, and adults based on the exposures estimated from the indoor crack and crevice uses of chlorpyrifos during the time of the CCCEH cohort.

7.1 Residential Handler Exposure/Risk Estimates

HED uses the term "handlers" to describe those individuals who are involved in the pesticide application process. HED believes that there are distinct tasks related to applications and that exposures can vary depending on the specifics of each task. Residential handlers are addressed somewhat differently by HED as homeowners are assumed to complete all elements of an application without use of any protective equipment.

Based upon review of all chlorpyrifos registered uses, only the roach bait products can be applied by a homeowner in a residential setting but the application of roach bait products has not quantitatively assessed because these exposures are negligible. The roach bait product is designed such that the active ingredient is contained within a bait station which eliminates the potential for contact with the chlorpyrifos containing bait material. Therefore, updated residential handler risks are not required for these uses.

7.2 Residential Post-application Exposure/Risk Estimates

Residential post-application exposures are likely from being in an environment that has been previously treated with chlorpyrifos. Chlorpyrifos can be used in areas frequented by the general population including golf courses and as an aerial and ground-based ULV mosquito adulticide applications made directly in residential areas. Post-application exposure from residential ant mound treatment was assessed qualitatively as addressed above because negligible exposures are anticipated.

All of the residential post-application exposure scenarios, data and assumptions, and algorithms used to assess exposures and risks from activities on golf course turf following chlorpyrifos application are the same as those used in the 2014 HHRA and ORE assessment. Additionally, this updated assessment makes use of the same chemical-specific turf transferable residue (TTR) data used previously to assess exposures and risks from golfing. Only the PoDs and LOCs have changed.

The residential post-application exposures and risks resulting from aerial and ground-based ULV mosquito adulticide applications have also been updated to reflect the updated PoDs and LOCs. However, the risks from the exposure scenarios have also been updated to reflect 1) the current default deposition fraction recommended for ground applied ULV mosquitocides (i.e., 8.7 percent of the application rate vs the previous 5 percent) and 2) several iterations of aerial applications modeled assuming differing winds speeds and release heights allowed by chlorpyrifos mosquitocide ULV labels. All other inputs and algorithms used for assessment of these exposure scenarios in 2014 remain the same, including the use of the chemical-specific TTR data. The AgDISP (v8.2.6) model input parameters, outputs, and the algorithms used to estimate residential post-application exposures following aerial and ground-based ULV

mosquitocide application can be found in Appendix A.

Default deposition fraction for ground applied ULV mosquitocides: Previously, an off-target deposition rate of 5 percent of the application rate was used by HED to evaluate ground-based ULV applications (i.e., 5 percent of the target application rate deposits on turf). This recommendation was based on data from Tietze *et al.,* and Moore *et al.* In a 2013 analysis (C. Peck, D407817, 3/28/2013), the Environmental Fate and Effects Division (EFED) reviewed eight published studies on ground ULV application in which deposition was measured. The studies varied in collection media (i.e., grass clippings and coupons), distance from application or spray head (ranging from 8 meters to 500 meters), and chemical measured (i.e., fenthion, malathion, naled, and permethrin). The analysis included the Moore *et al.,* and Tietze *et al.*, studies cited above. After considering the available data, HED has determined that an off-target deposition rate of 8.7 percent of the application rate may be used by HED to evaluate ground-based ULV applications (i.e., 8.7 percent of the target application rate deposits on turf). This value is the 90 percent upper confidence limit on the mean and is slightly higher than the mean values from all the data points observed in the studies (7.1%, n= 94). The adjusted application rate was then used to define TTR levels by scaling the available TTR data as appropriate.

Aerial application wind speed, volume median diameter, and release height: Previously, HED used the AgDISP (v8.2.6) model to assess deposition and air concentrations from aerial ULV applications assuming a 1 mph wind speed, volume median diameter is less than 60 µm (Dv 0.5 < 60 µm), and 300 foot release height. For this updated assessment, bounding risks have been estimated using the model based on a range of labeled application parameters. Lower spray height and lower wind speeds, and a greater Dv 0.5, results in the worst case potential exposures, or reduced potential for spray drift and, as a result, a greater deposition fraction and 1 hour average concentration. Therefore, estimated dermal and inhalation risks would be greater under these application conditions. The reverse is true for the best-case modeling scenario.

- Worst-case - 1 mph wind speed, Dv 0.5 = 60 µm, and 75 foot release height; and
- Best-case - 10 mph wind speed, Dv 0.5 = 40 µm, and 300 foot release height.

The following inputs were used for AgDISP (v8.26) modeling of chlorpyrifos ULV aerial applications.

Table 7.2.1. AGDISP Inputs (v8.26): Chlorpyrifos Mosquitocide ULV Aerial Application.

Input Parameters	Inputs to include in the AgDISP model	Notes/Comments
Application Method	Aerial	Default
Aircraft	Air Tractor AT-401	Default
Release Height	75, 300 Feet minimum release	Label allows a release height ranging from 75 to 300 feet.
Spray Lines	20 Reps	Default
Application Technique	Liquid	Default
Application Technique *Nozzles*	3; Extent 76.3%; Spacing 18.7 ft	Default
Application Technique *Drop Size Distribution*	User defined Parametric; $D_{V0.5}$: 40, 60 µm; and relative span: 1.4.	A $D_{v0.5}$ value of < 60 µm is allowable on the label. A $D_{v0.5}$ value of < 40 µm was modeled to estimate a lower droplet size

Table 7.2.1. AGDISP Inputs (v8.26): Chlorpyrifos Mosquitocide ULV Aerial Application.		
Input Parameters	**Inputs to include in the AgDISP model**	**Notes/Comments**
	no conversion to Malvern Drop Size Distribution	as is typically used for ULV aerial application.
Swath Width	500 feet	Default
Swath Displacement	Worst case application parameters: -130 feet Best case application parameters: 3,729 feet	The modeled spray deposition shows the peak deposition to be at a distance other than 0 feet. Therefore, the swath displacement w changed to the horizontal distance from the y axis where the peak deposition occurred and then the air concentration value was selected at this distance.
Meteorology	Wind type: single height Wind speed: 1, 10 mph Wind direction: -90 deg Temperature: 85 F° Relative humidity: 50%	No wind speed was identified on the label. The wind speeds of 1 and 10 mph were modeled to represent a reasonable range of wind speeds typical of ULV aerial applications.
Spray Material	Name: Oil Spray Material Evaporates: Yes Spray volume rate: 1.5 (gal/A) Active Fraction: 0.1936 Nonvol Fraction: 1	Spray material criteria as defined by the product label.
Atmospheric Stability	Overcast	Default
Surface	Upslope angle: 0 deg Sideslope angle: 0 deg Canopy: None	Default
Transport	Distance: 0 feet	Default
Advanced	Default Swatch offset: 0 Swath Specific Gravity carrier: Oil Specific Gravity active and additive= 0.929 Evaporation Rate: 84.76	Inputs based on criteria as defined by the product label.

Summary of Residential Post-application Non-Cancer Exposure and Risk Estimates

A summary of risk estimates is presented in Tables 7.2.2 through 7.2.8 below.

All residential post-application exposure scenarios assessed for playing golf on chlorpyrifos-treated courses, including all relevant populations and in consideration of all TTR data state sites, result in risks of concern (i.e., MOEs are < 100). Further, all residential post-application exposure scenarios assessed following aerial and ground ULV mosquitocide application result in risks of concern. All risk estimates are provided in Appendix B.

Table 7.2.2. Residential Post-application Non-cancer Exposure and Risk Estimates from Playing Golf on Chlorpyrifos-Treated Courses.						
Lifestage	**Post-application Exposure Scenario**		**Application Rate[1]**	**State (TTR Data)**	**Dose (mg/kg/day)[2]**	**MOEs[3]**
	Use Site	**Route of Exposure**				
Adult	Golf Course	Dermal	1.0	CA	0.010	0.13

Table 7.2.2. Residential Post-application Non-cancer Exposure and Risk Estimates from Playing Golf on Chlorpyrifos-Treated Courses.

Lifestage	Post-application Exposure Scenario		Application Rate[1]	State (TTR Data)	Dose (mg/kg/day)[2]	MOEs[3]
	Use Site	Route of Exposure				
(Females)	Turf		(Emulsifiable Concentrate)	IN	0.0069	0.19
				MS	0.012	0.11
				Mean	0.0095	0.14
Youths 11 to < 16 years old				CA	0.010	0.14
				IN	0.0070	0.20
				MS	0.012	0.12
				Mean	0.0096	0.15
Children 6 to < 11 years old				CA	0.012	0.19
				IN	0.0082	0.27
				MS	0.014	0.16
				Mean	0.011	0.20
Adult (Females)			1.0 (Granular)	CA	0.0088	0.15
Youths 11 to < 16 years old					0.0088	0.16
Children 6 to < 11 years old					0.010	0.21

1 Based on the maximum application rates registered for golf course turf use.
2 Dose (mg/kg/day) equations for golfing are provided in Appendix B of the 2014 HHRA. For dose estimation from exposures to golfing on treated turf TTR data was used. Doses have been presented for all State sites, including the mean of all State sites.
3 MOE = PoD (mg/kg/day) ÷ Dose (mg/kg/day). See Table 5.3.3.3 for PODs.

Table 7.2.3. Residential Post-application Inhalation Steady State Exposure Estimates from Chlorpyrifos ULV Aerial Mosquitocide Application - AgDISP Model.

Application Parameters	Population	Air Concentration Estimate (mg/m^3)[1]	MOE[2]
1 mph Wind Speed Dv 0.5 = 60 µm 75 Foot Release Height	Adults	0.0047	**1.1**
	Children 1 to <2 years old		**0.35**
10 mph Wind Speed Dv 0.5 = 40 µm 300 Foot Release Height	Adults	0.00070	**7.3**
	Children 1 to <2 years old		**2.4**

1 Air concentration estimate modeled using AGDISP v8.2.6 at breathing height of adults and children.
2 MOE = PoD (mg/m^3) ÷ Dose (mg/m^3). See Table 5.3.3.3 for PODs.

Table 7.2.4. Residential Post-application Inhalation Steady State Exposure Estimates from Chlorpyrifos ULV Ground Mosquitocide Application - WMB Model.

Population	Air Concentration Estimate (mg/m^3)[1]	MOE[2]
Adults	0.0013	**0.66**
Children 1 to <2 years old		**0.21**

1 Air concentration estimate modeled using the well mixed box model. The inputs and algorithms used are presented in Appendix C of the 2014 HHRA.
2 MOE = PoD (mg/m^3) ÷ Dose (mg/m^3). See Table 5.3.3.3 for PODs.

Table 7.2.5. Residential Post-application Dermal Steady State Exposure Estimates Resulting from Chlorpyrifos Aerial ULV Mosquitocide Application.

Application Parameters	Lifestage	Application Rate (lb ai/A)	AgDISP Deposition Fraction[1]	Adjusted TTR[2] ($\mu g/cm^2$)	Dermal Dose[3] (mg/kg/day)	MOE[4]
1 mph Wind Speed Dv 0.5 = 60 µm 75 Foot Release Height	Adults	0.010	1.0	0.00038	0.0015	**2**
	Children 1 to < 2 Years Old				0.0026	**6**
10 mph Wind Speed Dv 0.5 = 40 µm 300 Foot Release Height	Adults	0.010	0.086	0.000033	0.00013	**27**
	Children 1 to < 2 Years Old				0.00022	**68**

1 Aerial fraction of mosquitocide application rate deposited on turf as determined using AgDISP model v8.2.6.
2 TTR_t ($\mu g/cm^2$) = [(Day 0 Residue from MS TTR study ($\mu g/cm^2$) x Application Rate (0.010 lb ai/A)) / Application Rate of MS TTR Study (3.83 lb ai/A))] * AgDISP Deposition Fraction
3 Dermal Dose (mg/kg/day) = [(TTR_t ($\mu g/cm^2$) * CF1 (0.001 mg/µg) * Transfer Coefficient (180,000 cm^2/hr, adults; 49,000 cm^2/hr, children) * ET (1.5 hrs))] ÷ BW (kg)
4 MOE = PoD (mg/kg/day) ÷ Dose (mg/kg/day). See Table 5.3.3.3 for PODs.

Table 7.2.6. Residential Post-application Dermal Steady State Exposure Estimates Resulting from Chlorpyrifos ULV Ground Mosquitocide Application.

Lifestage	Application Rate (lb ai/A)	Deposition Fraction[1]	Adjusted TTR[2] ($\mu g/cm^2$)	Dermal Dose[3] (mg/kg/day)	MOE[4]
Adults	0.010	1.0	0.00038	0.0015	**26**
Children 1 to < 2 Years Old				0.0026	**67**

1. Ground fraction of mosquitocide application rate deposited on turf as determined using eight published studies on ground ULV application in which deposition was measured.
2. TTR_t ($\mu g/cm^2$) = [(Day 0 Residue from MS TTR study ($\mu g/cm^2$) x Application Rate (0.010 lb ai/A)) / Application Rate of MS TTR Study (3.83 lb ai/A))] * AgDISP Deposition Fraction
3. Dermal Dose (mg/kg/day) = [(TTR_t ($\mu g/cm^2$) * CF1 (0.001 mg/µg) * Transfer Coefficient (cm^2/hr - 180,000, adults; 49,000, children) * ET (1.5 hrs))] ÷ BW (kg)
4. MOE = PoD (mg/kg/day) ÷ Dose (mg/kg/day). See Table 5.3.3.3 for PODs.

Table 7.2.7. Residential Post-application Steady State Incidental Oral Exposure Estimates Resulting from Chlorpyrifos ULV Aerial Mosquitocide Application.

Application Parameters	Lifestage	Application Rate (mg ai)	Dermal Exposure (mg/day)[1]	Incidental Oral Dose (mg/kg/day)[2]	MOE[3]
1 mph Wind Speed Dv 0.5 = 60 µm 75 Foot Release Height	Children 1 to < 2 Years Old	0.010	0.028	$5.2x10^{-5}$	**3**
10 mph Wind Speed			0.0022	$4.5x10^{-6}$	**38**

Dv 0.5 = 40 µm 300 Foot Release Height					

1 Dermal exposure (mg/day) as calculated for children's aerial based ULV applications using the algorithms described in Table 6.2.4 above, and as described in Appendix C of the 2014 HHRA.
2 Incidental Oral Dose estimated using the algorithms as described below in Appendix C of the 2014 HHRA.
3 MOE = PoD (mg/kg/day) ÷ Dose (mg/kg/day). See Table 5.3.3.3 for PODs.

Table 7.2.8. Residential Post-application Steady State Incidental Oral Exposure Estimates Resulting from Chlorpyrifos ULV Ground Mosquitocide Application.

Lifestage	Application Rate (mg ai)	Dermal Exposure (mg/day)[1]	Incidental Oral Dose (mg/kg/day)[2]	MOE[3]
Children 1 to < 2 Years Old	0.010	0.0024	4.5×10^{-6}	**37**

1 Dermal exposure (mg/day) as calculated for children's ground based ULV applications using the algorithms described in Table 6.2.5 above, and as described below in Appendix C of the 2014 HHRA.
2 Incidental Oral Dose estimated using the algorithms as described in Appendix C of the 2014 HHRA.
3 MOE = PoD (mg/kg/day) ÷ Dose (mg/kg/day). See Table 5.3.3.3 for PODs.

7.3 Residential Risk Estimates for Use in Aggregate Assessment

All residential risks assessed with the updated PBPK-derived PODs are of concern (i.e., all MOEs are < the LOC of 100). Therefore, quantitatively aggregating residential exposures with food and drinking water exposures would also result in risks of concern.

8.0 Non-Occupational Spray Drift Exposure and Risk Estimates

Spray drift is a potential source of exposure to those nearby pesticide applications. This is particularly the case with aerial application, but, to a lesser extent, spray drift can also be a potential source of exposure from the ground application methods (e.g., groundboom and airblast) employed for chlorpyrifos. Sprays that are released and do not deposit in the application area end up off-target and can lead to exposures to those it may directly contact. They can also deposit on surfaces where contact with residues can eventually lead to indirect exposures (*e.g.*, children playing on lawns where residues have deposited next to treated fields). The potential risk estimates from these residues can be calculated using drift modeling coupled with methods employed for residential risk assessments for turf products.

In the 2011 occupational and residential exposure assessment, the potential risks to bystanders from spray drift and exposure from volatilization were identified as possible concerns. Spray drift is the movement of aerosols and volatile components away from the treated area during the application process. The potential risks from spray drift and the impact of potential risk reduction measures were assessed in July 2012 (J. Dawson *et al.,* D399483, 07/13/2012). This evaluation supplemented the 2011 assessment where limited monitoring data indicate risks to bystanders. To increase protection for children and other bystanders, chlorpyrifos technical registrants voluntarily agreed to lower application rates and to other spray drift mitigation measures (R. Keigwin, 2012). As of December 2012, spray drift mitigation measures and use restrictions appear on all chlorpyrifos agricultural product labels. For the 2014 HHRA, spray drift risks were updated due to the use of the PBPK-PD model which impacted the PoDs, and

thus spray drift risk estimates. This assessment updates chlorpyrifos risks once more to incorporate the approach applied for PBPK-derivation of PoDs for infants, children, and adults based on the exposures estimated from the indoor crack and crevice uses of chlorpyrifos during the time of the CCCEH cohort.

With a dermal and incidental oral LOC of 100, all non-occupational spray drift risk estimates are of concern at the field edge with the use of certain application rates, nozzle droplet sizes, and application methods. Buffer distances > 300 feet are needed for MOEs to be not of concern. The estimated buffer distances are in excess of those agreed to by the technical registrants in July 2012. All drift risk estimates are presented in Appendix C.

9.0 Non-Occupational Bystander Post-Application Inhalation Exposure and Risk Estimates

In January 2013, a preliminary assessment of the potential risks from volatilization was conducted (R. Bohaty *et al.,* D399484 and D400781, 01/31/2013). The assessment evaluated the potential risks to bystanders, or those who live and/or work in proximity to treated fields, from inhalation exposure to vapor phase chlorpyrifos and chlorpyrifos-oxon emitted from fields following application of chlorpyrifos. The results of the January 2013 assessment indicated that offsite concentrations of chlorpyrifos and chlorpyrifos-oxon may exceed the target concentration based on the toxicological endpoints used at that time (J. Hotchkiss *et al.,* EPA MRID 48139303).

In June 2014, a re-evaluation of the 2013 preliminary volatilization assessment was conducted since the Registrant had conducted and submitted two, high quality nose-only vapor phase AChE inhibition inhalation studies for both chlorpyrifos and chlorpyrifos-oxon (W. Irwin, D411959, 06/25/2014) to address the uncertainty surrounding exposure to aerosol versus vapor phase chlorpyrifos. In the vapor studies, female rats were administered a saturated vapor, meaning that the test subjects received the highest possible concentration of chlorpyrifos or chlorpyrifos-oxon which can saturate the air in a closed system. At these saturated concentrations, no statistically significant inhibition of AChE activity was measured in RBC, plasma, lung, or brain at any time after the six-hour exposure period in either study. Under actual field conditions, indications are that exposures to vapor phase chlorpyrifos and its oxon would be much lower as discussed in the January 2013 preliminary volatilization assessment. Since the studies demonstrated that no toxicity occurred even at the saturation concentration, the agency concluded that there was no risk potential, as risk is a function of both exposure and hazard.

However, in the current risk assessment for chlorpyrifos, the PoDs for risk assessment have been chosen to be protective of potential neurological effects below levels where AChE inhibition could occur. For that reason, a quantitative bystander/volatilization assessment has been included in this update. This assessment is an update to the 2013 assessment and has been updated to reflect air monitoring data collected since 2006, and the updated PoDs for chlorpyrifos.

There are six available chlorpyrifos air monitoring studies that were conducted since 2006 (brief study summaries available in W. Britton, D388165, 06/27/2011). These include:

- One application site study conducted in North Central and Yakima Valley, OR by the University of Washington Department of Environmental and Occupational Health Sciences, and
- Five ambient air studies
    - one conducted in North Central and Yakima Valley, by the University of Washington Department of Environmental and Occupational Health Sciences;
    - two conducted by Pesticide Action Network North America (PANNA) in Washington and Minnesota; and
    - two conducted by CalDPR.

Application site air monitoring refers to the collection of air samples around the edges of a treated field during and after a pesticide application. Samples are generally collected for short intervals (e.g., < 8 hours), for at least the first day or two after application with subsequent samples increasing in duration. In this type of study, it is typically known when an application occurred, the equipment used for the application, and the application rate. Application site monitoring data represents an exposure to vapors at or near the field edge resulting from an application.

Ambient air monitoring typically is focused on characterizing the airborne pesticide levels within a localized airshed or community structure of some definition (e.g., city, township, or municipality). This type of monitoring effort also can be focused on capturing chronic background levels or other temporal characteristics of interest such as focusing on seasonal pesticide use patterns. Typically, samples are taken for 24 consecutive hours and collected at the same site over an extended period of time (e.g., several weeks or months). In contrast to application site air monitoring, information on the precise timing and location of pesticide applications are rarely collected in ambient air monitoring studies. However, this does not mean that an application did not occur near an ambient sampler during the monitoring period

The EPA has assessed residential bystander exposure to chlorpyrifos based on the available ambient and application site air monitoring data (Tables 9.1 and 9.2). The chlorpyrifos bystander volatilization inhalation exposure assessment includes acute and steady state exposure scenarios. The acute scenario compares the maximum air concentration detected in the monitoring studies to the acute PoD. The steady state scenario compares the arithmetic mean chlorpyrifos air concentration from several monitoring studies to the steady state PoD.

The EPA has assessed residential bystander exposure from field volatilization of applied chlorpyrifos based on available *ambient* (five studies/11 locations) and *application site* (one study/2 locations) air monitoring data. For adults, of the 11 acute *ambient* air concentrations assessed, six resulted in risk estimates that are of concern (i.e., MOEs < 100). Only one steady state *ambient* air concentration resulted in a risk estimate not of concern (i.e., MOEs > 100). For the *application site* air concentrations assessed, all resulted in risk estimates of concern (i.e., MOEs < 100). For children 1 to <2 years old, of the 11 acute *ambient* air concentrations assessed, all resulted in risk estimates that are of concern (i.e., MOEs < 100). Only four steady state *ambient* air concentration resulted in a risk estimate not of concern (i.e., MOEs > 100). For the *application site* air concentrations assessed, all resulted in risk estimates of concern (i.e.,

MOEs < 100). All bystander risk estimates are presented in Appendix D.

Table 9.1. Chlorpyrifos Preliminary Volatilization Risk Analysis for Residential Adult Bystanders.					
Study, Year	**Sampler/ Site Location**	**Maximum Air Concentration (ng/m³)**	**Arithmetic Mean Air Concentration (ng/m³)**	**Acute MOEs[1] (LOC = 100)**	**Steady State MOEs[2] (LOC = 100)**
Application Site Data					
WA DOH, 2008	North Central District Perimeter Site	1145	153	**3.5**	**1.4**
	Yakima Valley Perimeter Site	1002	294	**4**	**0.71**
Ambient Air Data					
WA DOH, 2008	North Central District Ambient	21	7	190	**31**
	North Central District Receptor	606.8	33	**6.6**	**6.4**
	Yakima Valley Ambient	30	9	130	**23**
	Yakima Valley Receptor	243	30	**16**	**6.9**
Parlier, CA (CalDPR) 2009		150	96	**27**	**2.2**
Cowiche PANNA 2006		462	155	**8.7**	**1.4**
PANNA MN Drift Study (2006-2009)	Browerville Site B	15	2.7	270	**79**
	Perham Site C	47	1.9	**85**	110
CDPR 2014 Air Monitoring Network	Salinas, CA	14.1	5.4	280	**39**
	Shafter, CA	337.9	92.1	**12**	**2.3**
	Ripon, CA	14.1	14.1	280	**15**

1 Acute MOE = Acute PoD (4,000 ng/m³) / Study maximum air concentration (ng/m³).
2 Steady State MOE = Steady State PoD (210 ng/m³) / Study arithmetic mean air concentration (ng/m³).

Table 9.2. Chlorpyrifos Preliminary Volatilization Risk Analysis for Residential Children (1 to <2 Years Old) Bystanders.					
Study, Year	**Sampler/ Site Location**	**Maximum Air Concentration (ng/m³)**	**Arithmetic Mean Air Concentration (ng/m³)**	**Acute MOEs[1] (LOC = 100)**	**Steady State MOEs[2] (LOC = 100)**
Application Site Data					
WA DOH, 2008	North Central District Perimeter Site	1145	153	**1.1**	**4.4**
	Yakima Valley Perimeter Site	1002	294	**1.3**	**2.3**
Ambient Air Data					
WA DOH, 2008	North Central District Ambient	21	7	**62**	100
	North Central District Receptor	606.8	33	**2.1**	**21**
	Yakima Valley Ambient	30	9	**43**	**73**
	Yakima Valley Receptor	243	30	**5.3**	**22**

Table 9.2. Chlorpyrifos Preliminary Volatilization Risk Analysis for Residential Children (1 to <2 Years Old) Bystanders.					
Study, Year	**Sampler/ Site Location**	**Maximum Air Concentration (ng/m^3)**	**Arithmetic Mean Air Concentration (ng/m^3)**	**Acute MOEs[1] (LOC = 100)**	**Steady State MOEs[2] (LOC = 100)**
Parlier, CA (CalDPR) 2009		150	96	**8.7**	**7.1**
Cowiche PANNA 2006		462	155	**2.8**	**4.4**
PANNA MN Drift Study (2006-2009)	Browerville Site B	15	2.7	**87**	260
	Perham Site C	47	1.9	**28**	350
CDPR 2014 Air Monitoring Network	Salinas, CA	14.1	5.4	**92**	130
	Shafter, CA	337.9	92.1	**3.8**	**7.4**
	Ripon, CA	14.1	14.1	**92**	**48**

1 Acute MOE = Acute PoD (1,300 ng/m^3) / Study maximum air concentration (ng/m^3).
2 Steady State MOE = Steady State PoD (680 ng/m^3) / Study arithmetic mean air concentration (ng/m^3).

Characterization of Bystander Risk Assessment/Uncertainties

Some of the limitations and considerations that have been identified that should be considered in the interpretation of these results include:

- Most of the data utilized in this preliminary assessment are 24-hour air samples. When these data are used, an assumption is made that an individual is exposed to the same air concentration for 24-hours every day. However, this is not always the case as real world time-activity data indicate that many parts of the population move from site to site on a daily basis (e.g., go to work and back).

- This assessment is only representative of outdoor concentrations (i.e., the exposure and risk estimates assume an individual is outdoors all the time). It does not take into account potential effects of air conditioning systems and similar air filtration systems which could potentially reduce air concentrations indoors. The agency believes that indoor concentrations will be at worst equivalent to outdoor concentrations and may potentially be lower.

- All of the data used for this analysis have been generated in California and Washington; however, chlorpyrifos is used in many regions throughout the country. Therefore, the results based on the limited available air monitoring data were used to represent the rest of the country due to a lack of adequate information for any other region. It is unclear what potential impacts this extrapolation might have on the risk assessment. Factors such as meteorology and cultural practices may impact the overall amounts of chlorpyrifos that volatilize from a treated field as well as the rate at which it volatilizes.

- As part of the December 2009 SAP, the agency presented their analysis of several models that could be used as screening tools to predict the air concentration and volatilization flux based on intrinsic properties and transport behaviors of pesticides. These models would allow the agency to better represent the potential volatilization of semi-volatile

pesticides across various regions of the country and thus would provide refinement to this assessment over using straight air monitoring data. The SAP provided a number of comments regarding the agency's model analysis, including the recommendation to evaluate some additional models. The agency is currently in the process of evaluating the SAP's comments. As appropriate, the agency will revise the modeling approach presented to the SAP for determining the rate of volatilization (flux) for semi-volatile pesticides and for estimating air concentrations of applied pesticides in the atmosphere under varying environmental conditions. After any policies or procedures are put into place, the agency may revisit the residential bystander exposure and risk assessment.

10.0 Aggregate Exposure/Risk Characterization

In accordance with the FQPA, HED must consider and aggregate (add) pesticide exposures and risks from three major sources: food, drinking water, and residential exposures. In an aggregate assessment, exposures from relevant sources are added together and compared to quantitative estimates of hazard, or the risks themselves can be aggregated. The steady state aggregate assessment includes food, drinking water, and residential exposures.

For chlorpyrifos aggregate assessment, a DWLOC approach is used to calculate the amount of exposure available in the total 'risk cup' for chlorpyrifos in drinking water after accounting for any chloropyrifos exposures from food and residential uses. This DWLOC is then compared to the EDWC to determine if there is an aggregate risk of concern. However, because the dietary risks from food exposure alone and from residential exposure alone are of concern, it is not possible to calculate a DWLOC; essentially, the steady state aggregate DWLOC is '0' after accounting for food and residential exposures.

[See the December 2014 chlorpyrifos HHRA for details of the DWLOC approach and calculations. See the April 2016 DWA for the EDWCs.]

11.0 Occupational Exposure and Risk Estimates

HED had previously conducted both steady state occupational handler and post-application exposure analyses for chlorpyrifos (W. Britton, D424484, 12/29/2014). However, occupational exposures and risks have been updated to incorporate the approach applied for PBPK-derivation of PoDs for infants, children, and adults based on the exposures estimated from the indoor crack and crevice uses of chlorpyrifos during the time of the CCCEH cohort. The scenarios, assumptions, and exposure inputs have not changed since the previous assessment; the assessment below estimates occupational handler exposures using the updated PBPK-derived steady state PoDs. Details on the exposure inputs, scenarios, and assumptions can be found in the 2014 ORE assessment (W. Britton, D424484, 12/29/2014).

It is agency policy to use the best available data to assess exposure. The same chemical-specific dislodgeable foliar residue (DFR) studies were used for the 2014 assessment of occupational post-application exposure to chlorpyrifos have been used for this update, including: emulsifiable concentrate formulations on sugarbeets, pecans, citrus, sweet corn, cotton, and turf; wettable powder formulations on almonds, apples, pecans, cauliflower, tomato and turf; granular

formulations on sweet corn and turf; a total release aerosol formulation on ornamentals; and a microencapsulated liquid formulation on ornamentals.

Several sources of generic data were used in this assessment as surrogate data including: Pesticide Handlers Exposure Database Version 1.1 (PHED 1.1); the Agricultural Handler Exposure Task Force (AHETF) database; the Outdoor Residential Exposure Task Force (ORETF) database; the Agricultural Reentry Task Force (ARTF) database; ExpoSAC Policy 14 [Standard Operating Procedures (SOPs) for Seed Treatment]; HED's 2012 Residential SOPs for Residential Pesticide Exposure Assessment: Lawns/Turf, Outdoor Fogging/Misting Systems, registrant-submitted exposure monitoring studies MRIDs 44180401, 44301301, 44793301, 44829601, 42974501, 43062701, 44748101, 44748102, 46722701, and 46722702, and published literature studies. Some of these data are proprietary, and subject to the data protection provisions of the *Federal Insecticide, Fungicide, and Rodenticide Act (*FIFRA).

In the 2011 HHRA (D. Drew *et al.,* D388070, 06/30/2011), additional studies were recommended to address uncertainties regarding the formation of chlorpyrifos oxon and its decay following applications in greenhouses. To date, no additional data have been submitted.

11.1 Steady State Occupational Handler Risk

The term handlers is used to describe those individuals who are involved in the pesticide application process. There are distinct job functions or tasks related to applications and exposures can vary depending on the specifics of each task. Job requirements (amount of a chemical used in each application), the kinds of equipment used, the target being treated, and the level of protection used by a handler can cause exposure levels to differ in a manner specific to each application event. Based on the anticipated use patterns and current labeling, types of equipment and techniques that can potentially be used, occupational handler exposure is expected from chlorpyrifos use. For purpose of occupational handler assessment, the parent chlorpyrifos is the relevant compound.

Current labels generally require that handlers use normal work clothing (i.e., long sleeved shirt and pants, shoes and socks) and coveralls, chemical resistant gloves, and dust/mist respirators. Also, some products are marketed in engineering controls such as water soluble packets. In order to determine what level of personal protection is required to alleviate risk concerns and to ascertain if label modifications are needed, steady state exposure and risk estimates were updated for occupational handlers of chlorpyrifos for a variety of scenarios at differing levels of personal protection including engineering controls.

The occupational handler scenarios, assumptions, and exposure inputs have not changed since the previous assessment.

Summary of Occupational Handler Non-Cancer Exposures and Risk Estimates

Using the updated PBPK-derived steady state PODs and uncertainty factors (dermal and inhalation LOC = 100), all agricultural occupational handler scenarios, all primary seed treatment handler scenarios, and all secondary seed treatment (planter) scenarios are of concern with label-specified and maximum levels of personal protective equipment (PPE) or engineering

controls (MOEs < 100). Detailed result tables are provided in Appendix E.

11.2 Steady State Occupational Post-Application Risk Estimates

HED uses the term, post-application, to describe exposures that occur when individuals are present in an environment that has been previously treated with a pesticide (also referred to as reentry exposure). Such exposures may occur when workers enter previously treated areas to perform job functions, including activities related to crop production, such as scouting for pests or harvesting. Post-application exposure levels vary over time and depend on such things as the type of activity, the nature of the crop or target that was treated, the type of pesticide application, and the chemical's degradation properties. In addition, the timing of pesticide applications, relative to harvest activities, can greatly reduce the potential for post-application exposure. Chlorpyrifos parent compound is the residue of concern for occupational post-application dermal exposures; however, it may be possible that the formation of the oxon is greater and its deactivation slower in greenhouses when compared to the outdoor environment and that an assessment may be needed for exposure to the oxon in greenhouse settings.

11.2.1 Occupational Post-application Inhalation Exposure/Risk Estimates

There are multiple potential sources of post-application inhalation exposure to individuals performing post-application activities in previously treated fields. These potential sources include volatilization of pesticides and resuspension of dusts and/or particulates that contain pesticides. Previously, a quantitative post-application inhalation risk assessment was not conducted for chlorpyrifos or chlorpyrifos oxon due to the lack of toxicity seen in the available nose-only vapor phase AChE inhibition inhalation studies (W. Britton, D424484, 12/29/2014). The studies did not demonstrate inhalation toxicity, or inhibition of AChE activity measured in RBC, plasma, the lungs, and the brain following exposure to chlorpyrifos or chlorpyrifos oxon vapor, even at the saturation concentration. However, since the previous assessment, the PODs have been updated to reflect the PBPK-derived steady state PoD based on a TWA of blood concentrations corresponding to levels likely to have occurred in the CCCEH cohort, as discussed in Section 5.3.3. Therefore, the agency will be assessing occupational post-application inhalation from the registered uses of chlorpyrifos.

The agency has sought expert advice and input on issues related to volatilization of pesticides from its Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel (SAP) in December 2009, and received the SAP's final report on March 2, 2010 (https://www.regulations.gov/document?D=EPA-HQ-OPP-2009-0687-0037). The agency has evaluated the SAP report and has developed a Volatilization Screening Tool and a subsequent Volatilization Screening Analysis (https://www.regulations.gov/document?D=EPA-HQ-OPP-2014-0219-0001). During Registration Review, the agency will utilize this analysis, and take into consideration the risks identified from the residential bystander assessment, to determine if data (i.e., flux studies) or further analysis is required for chlorpyrifos.

In addition, the agency is continuing to evaluate the available post-application inhalation exposure data generated by the Agricultural Reentry Task Force. Given these two efforts, the agency will continue to identify the need for and, subsequently, the way to incorporate

occupational post-application inhalation exposure into the agency's risk assessments.

The Worker Protection Standard for Agricultural Pesticides contains requirements for protecting workers from inhalation exposures during and after greenhouse applications through the use of ventilation requirements.[40 CFR 170.110, (3) (Restrictions associated with pesticide applications)].

11.2.2 Occupational Post-application Dermal Exposure/Risk Estimates

Occupational post-application assessments were previously performed for: 1) exposures to the parent compound chlorpyrifos in outdoor environments (uses other than greenhouse), 2) exposures to the parent chlorpyrifos (only) in greenhouses and 3) exposures to both the parent and the oxon metabolite in greenhouses; and incorporated: 1) a PBPK modeled dermal PoD specific for occupational assessment 2) the updated master use summary document, 3) the updated adult (female) default body weight, and 4) the changes relating to agricultural transfer coefficients (TC) as described in the *Science Advisory Council for Exposure (ExpoSAC) Policy 3 – Revised March 2013*[17] (W. Britton, D424484, 12/29/2014).

However, the steady state PODs and uncertainty factors have changed since the previous assessment. Therefore, the occupational post-application exposure assessment has been revised. The scenarios, assumptions, and exposure inputs have not changed since the previous assessment; the assessment below estimates occupational post-application dermal exposures using the updated PBPK-derived steady state PODs. Details on the exposure inputs, scenarios, and assumptions can be found in W. Britton, D424484, 12/29/2014. Detailed result tables are provided in Appendix F.

Summary of Occupational Post-application Non-Cancer Exposures and Risk Estimates

263 total occupational post-application scenarios were evaluated. The restricted entry intervals (REIs) on the registered chlorpyrifos labels range from 24 hours to 5 days. All scenarios were of concern on Day 0 with a dermal LOC of 100. On average, scenarios were not of concern $\geq$ 18 days after treatment.

[17] http://www.epa.gov/opp00001/science/exposac-policy-3-march2013.pdf

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13.0 List of Appendices

Appendix A: Non-occupational exposure estimates following mosquitocide applications
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Appendix C: Non-occupational spray drift exposure and risk estimates
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