Ultraviolet Photolysis of Chlorpyrifos: Developmental Neurotoxicity Modeled in PC12 Cells

Theodore A. Slotkin,1 Frederic J. Seidler,1 Changlong Wu,2 Emiko A. MacKillop,1 and Karl G. Linden3

1Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina, USA; 2Department of Civil and Environmental Engineering, Duke University, Durham, North Carolina, USA; 3Department of Civil, Environmental, and Architectural Engineering, University of Colorado at Boulder, Boulder, Colorado, USA

BACKGROUND: Ultraviolet photodegradation products from pesticides form both in the field and during water treatment.

OBJECTIVES: We evaluated the photolytic breakdown of the organophosphate pesticide chlorpyrifos (CPF) in terms of both the chemical entities generated by low-pressure ultraviolet C irradiation and their potential as developmental neurotoxicants.

METHODS: We separated by-products using high-performance liquid chromatography and characterized them by gas chromatography/mass spectrometry. We assessed neurotoxicity in neuronyotypic PC12 cells, both in the undifferentiated state and during differentiation.

RESULTS: Photodegradation of CPF in methanol solution generated CPF oxon and trichloropyridinol, products known to retain developmental neurotoxicant actions, as well as a series of related organophosphate and phosphorothionate derivatives. Exposure conditions that led to 50% degradation of CPF thus did not reduce developmental neurotoxicity. The degradation mixture inhibited DNA synthesis in undifferentiated cells to the same extent as native CPF. In differentiating cells, the products likewise retained the full ability to elicit shortfalls in cell number and corresponding effects on cell growth and neurite formation. When the exposure was prolonged to the point where 70% of the CPF was degraded, the adverse effects on PC12 cells were no longer evident; however, these conditions were sufficiently severe to generate toxic products from the methanol vehicle.

CONCLUSIONS: Our results indicate that field conditions or remediation treatments that degrade a significant proportion of the CPF do not necessarily produce inactive products and, indeed, may elicit formation of even more toxic chemicals that are more water soluble and thus have greater field mobility than CPF itself.

KEY WORDS: chlorpyrifos, neurotoxicity, organophosphate insecticides, photolysis products, ultraviolet light. Environ Health Perspect 117:338–343 (2009). doi:10.1289/ehp.11592 available via http://dx.doi.org/117:338–343 (2009)

The nearly ubiquitous exposure of the human population to organophosphate pesticides has raised increasing concern about their propensity to elicit developmental neurotoxicity at exposures that go undetected because of the absence of systemic signs of intoxication (Colborn 2006; Costa 2006; Landrigan 2001; Milesen et al. 1998; Slotkin 2005; Weiss et al. 2004). Chlorpyrifos (CPF), the most extensively studied of the organophosphates, disrupts neural cell replication and differentiation, axonogenesis, and synaptic function, culminating in behavioral deficits that have been noted both in animal models of developmental CPF treatment and in children in settings with high environmental CPF exposures (Rauh et al. 2006; Slotkin 1999, 2004, 2005). Although registration of CPF for use in the home has been withdrawn, it remains widely applied in agriculture, where issues remain about potential toxicity from runoff into natural water bodies and sources of drinking water or residues in food. In the field, CPF and other pesticides are exposed to conditions that lead to degradation, notably, photolysis from ultraviolet (UV) light. In water, these chemicals also can be degraded via environmental exposure to UV light and further transformed in water treatment processes. Recent studies of CPF photolytic products, CPF metabolites, and other organophosphates indicate that under these conditions, degradation involves the formation of a variety of known and unknown derivatives, some of which may retain developmental neurotoxicant features (Bavcon Kralj et al. 2007; Shemer and Linden 2006; Shemer et al. 2005).

In the present study, we evaluated the effects of different degrees of UVC irradiation of CPF and evaluated the potential neurotoxicity of the products in PC12 cells, a neurodevelopmental model derived from pheochromocytoma (Teng and Greene 1994) and recapitulates the major mechanisms and outcomes of CPF effects on the developing brain in vivo (Bagchi et al. 1995, 1996; Crompton et al. 2000a, 2000b; Das and Barone 1999; Flaskos et al. 1994; Jameson et al. 2006b; Li and Casida 1998; Nagata et al. 1997; Qiao et al. 2001, 2005; Slotkin 1999, 2004, 2005; Song et al. 1998; Tuler et al. 1989; Yanai et al. 2002). PC12 cells enable detection of adverse effects on the cell cycle, an important target for CPF and other organophosphates (Slotkin 1999, 2004, 2005), whereas primary neurons do not maintain their mitotic ability in culture and differentiate in a heterogeneous fashion. PC12 cells undergo coordinated differentiation into distinct neuronal phenotypes when nerve growth factor (NGF) is added, exiting the mitotic cycle and growing neuritic projections (Fujita et al. 1989; Song et al. 1998; Teng and Greene 1994). In the present study, we examined the effects of CPF before and after photolysis on both undifferentiated and differentiating PC12 cells with regard to DNA synthesis and indices of cell number and neurite outgrowth. Unlike hepatocytes or myocytes, each neural cell contains a single nucleus, so measuring DNA content evaluates the number of cells (Winick and Noble 1965). We also assessed total protein, which increases with cell growth and membrane protein, which rises with the formation of neurites (Abreu-Villaça et al. 2005; Jameson et al. 2006a; Slotkin et al. 2007b; Song et al. 1998).

Materials and Methods

Photolysis. We dissolved CPF (purity > 99%); Chem Service, West Chester, PA) in methanol (VWR Scientific, West Chester, PA) and carried out photodegradation experiments in a collimated-beam low-pressure UV bench reactor, configured with four mercury vapor germicidal lamps (ozone-free, General Electric no. G15T8), emitting monochromatic light at 254 nm. We placed the test solution in a 70 × 50 mm crystallization dish, which was sealed with a quartz cover to prevent evaporation, and exposed it to UVC irradiation. Temperature in the dish was maintained at 24°C by a cooled water cooling system, and the solution was mixed on a stir plate to mix.
ensure complete mixing and consistent batch conditions. We determined incident irradiance using a calibrated radiometer (IL1700, SED 240/W; International Light, Peabody, MA) and calculated delivered UVC fluence with a spreadsheet program that included lamp spectrum, solution absorbance, exposure time, and incident irradiance (Bolton and Lindén 2003). At specific delivered UVC fluence intervals, about 10 mL of sample was collected with a syringe and sealed in a borosilicate vial for the biological determinations. We determined the final CPF concentrations by high-performance liquid chromatography (HPLC) and conducted control experiments with pure methanol vehicle under the same irradiation conditions.

**Chemical analysis.** We measured concentrations of CPF during photolysis with a Varian Pro Star HPLC (Varian, Inc., Palo Alto, CA) equipped with a polychromatic diode array detector and a 4.6 × 150 mm C18 reverse-phase column (Alltech Associates, Deerfield, IL). We used isocratic elution with a mobile phase of acetonitrile and water (80/20 vol/vol) at a flow rate of 1 mL/min. Under these conditions, the retention time for CPF was 7.9 min. For by-product identification, we used a Shimadzu GC/MS-QP2010 gas chromatograph/mass spectrometer (GC/MS) equipped with a 15-m RTX-5MS column (film thickness, 0.25 μm; i.d., 0.25 mm).

**Cell cultures.** Because of the clonal instability of the PC12 cell line (Fujita et al. 1989), we performed the experiments on cells that had undergone fewer than five passages and repeated all studies several times with different batches of cells. As described previously (Crumpton et al. 2000a; Qiao et al. 2003; Song et al. 1998), we seeded 3 × 10^5 cells and 24 hr later changed the medium to include 50 ng/mL of 2.5 S mouse NGF (Invitrogen); we examined each culture under a microscope to verify the subsequent outgrowth of neurites. CPF or CPF photolysis products were added concurrently with the start of NGF treatment, and cultures were maintained for 6 days, with the test agents included with every medium change.

We chose the CPF concentrations on the basis of previous work. Given our objective to evaluate the loss of activity upon photolysis, our strategy was to elicit both threshold and robust responses for each of the effects evaluated. Accordingly, we used 10 or 30 μM CPF in undifferentiated cells, concentrations that span the range from low to high effects on DNA synthesis, and 30 μM CPF for studies in differentiating cells (Bagchi et al. 1995; Crumpton et al. 2000b; Das and Barone 1999; Jameson et al. 2006b; Qiao et al. 2001, 2003; Slotkin et al. 2007b; Song et al. 1998).

**DNA synthesis.** We introduced CPF or CPF photolysis products for 1 hr in undifferentiated cells, and then, to initiate the measurement of DNA synthesis, we changed the medium to include 1 μCi/mL [3H]thymidine (specific activity, 2 Ci/mmol; GE Healthcare, Piscataway, NJ) along with the continued inclusion of the test substances. After 1 hr, the medium was aspirated and cells were harvested in ice-cold water. Duplicate aliquots of each sample were treated with 10% trichloroacetic acid (TCA) and precipitated with ice-cold 5% trichloroacetic acid and water (80/20 vol/vol) at 37°C. The resulting pellet was washed once with additional trichloroacetic acid and then with 75% ethanol. The final pellet was resuspended in 1 M KOH overnight at 37°C and neutralized with 6 M HCl; the DNA was then precipitated with light microscopy, and counted to verify that reduced DNA content connoted fewer cells. We then washed and resuspended. The membrane pellets were then resuspended and analyzed for protein. We selected representative samples for each experiment, examined them with light microscopy, and counted cells to verify that reduced DNA content connoted fewer cells.

**Data analysis.** We conducted CPF photolysis in three separate sets of samples, with each sample prepared several weeks apart, and then tested them in multiple batches of PC12 cells. Results are reported as mean ± SE. Because of the multiple treatments in each experiment, significant differences were first established by a global analysis of variance (ANOVA) incorporating all treatments, followed by Fisher’s protected least significant difference to evaluate differences between specific treatment groups. The initial test included sample batch and cell batch; however, because the treatment effects were the same, we then combined the results across batches after normalization to account for the differing absolute values for each batch of cells. Significance was assumed at p < 0.05.

**Results**

**Photolysis.** Direct photolysis of CPF can be described as a pseudo-first-order reaction following these two equations (Schwarzenbach et al. 2003)

\[
\frac{d[M]}{dt} = k_d[M] = k_{s,M} \Phi_M[M]
\]

\[
\Phi_M = \frac{k_d}{k_{s,M}},
\]

where

\[
k_{s,M} = \frac{E_0 \varepsilon_M [1 - 10^{-aZ}]^2}{aZ}
\]

is the specific rate of light absorption (E mol⁻¹ sec⁻¹); \(\Phi_M\) is the quantum yield; \(k_d\) is the pseudo-first-order rate constant; \([M]\) is the concentration of target compound; \(E_0\) is incident photon irradiance (10⁻³ E cm⁻² sec⁻¹); \(\varepsilon_M\) is molar absorbance (M⁻¹ cm⁻¹) of the

**DNA and protein content.** We evaluated DNA content, total protein, and membrane protein after 6 days of continuous exposure to NGF and either CPF or CPF photolysis products. The medium was aspirated and the culture was rinsed with a buffer consisting of 154 mM NaCl and 10 mM sodium phosphate (pH 7.4). Cells were harvested in ice-cold buffer and homogenized (Polytron, Brinkmann Instruments, Westbury, NY), and aliquots were withdrawn for measurements of DNA and protein using dye-binding methods (Smith et al. 1985; Trauth et al. 2000). To prepare the cell membrane fraction, we sedimented another aliquot of the homogenate at 40,000 × g for 10 min; and the pellet was then washed and resuspended. The membrane pellets were then resuspended and analyzed for protein. We selected representative samples for each experiment, examined them with light microscopy, and counted cells to verify that reduced DNA content connoted fewer cells.
target compound; \( Z \) is the solution depth; and \( a \) is the light absorbance by the solution. Using a spectrophotometer, we confirmed that the UVC lamp emitted monochromatically at 254 nm, with insignificant contributions from other wavelengths (Figure 1A). CPF had strong absorbance bands at 230 nm and 290 nm, but a local absorption minimum at 254 nm, indicating limited susceptibility to direct photodegradation by low-pressure UVC irradiation. Therefore, the direct photolysis of CPF in methanol progressed slowly because of the low absorbance, combined with the fact that CPF has a low quantum yield. The initial stock CPF concentration was 15.83 mM, and the final concentrations after short-term and long-term UVC treatments were 8.30 mM and 4.84 mM, respectively, corresponding to about 50% and 70% reduction of the initial CPF. Applying the photodegradation data to the integrated form of Equation 1 confirmed the adherence to the predicted pseudo-first-order reaction (Figure 1B).

The application of GC/MS made it possible to determine the major photodegradation by-products by comparing mass spectra of the unknowns with a National Institute of Standards and Technology mass spectral library search (NIST 2008) and through analysis of the mass fragmentation patterns (Table 1). The products included agents whose potential for developmental neurotoxic actions have been characterized in the PC12 model, such as CPF oxon and trichloropyridinol (2-hydroxy-3,5,6-trichloropyridine) (Qiao et al. 2001), as well as novel products that retain organophosphate or phosphorothionate characteristics. In comparing the products obtained in methanol solution to those obtained in water, most of the products were identical: \( O,O \)-diethyl-methyl thiophosphate, \( O,O \)-diethyl thiophosphate, 2-hydroxy-3,5,6-trichloropyridinol (trichloropyridinol), 3,6-dichloro-2-[pyridinyl-\( O, O \)-ethyl thiophosphate, and CPF oxon. Whereas photolysis in methanol solution also produced dimethyl ethyl phosphate, water solution produced the similar (but not identical) product, \( O,O \)-diethyl phosphate. Photolysis in water also produced 3,5,6-trichloro-2-pyridinyl acetic acid, which was not found with the methanol solution, although this is obviously quite similar to trichloropyridinol. We found none of these products in untreated CPF solutions.

### Undifferentiated cells

In agreement with earlier work (Qiao et al. 2001; Slotkin et al. 2007a, 2007b; Song et al. 1998), exposure of undifferentiated PC12 cells to 10 or 30 \( \mu \)M CPF produced concentration-dependent reduction in DNA synthesis, with the effect ranging from about 15% inhibition at the lower concentration (left) to nearly 50% inhibition at the higher concentration (right) (Figure 2). The shorter UVC exposure reduced the CPF concentration (left) to nearly 50% inhibition at the higher concentration (right) (Figure 2).

### Differentiating cells

When CPF was introduced simultaneously with NGF and differentiation allowed to proceed for 6 days, we observed a substantial reduction in the total number of cells as monitored by DNA content (Figure 3). Short-term UVC exposure, which

### Table 1. Structure and mass spectra of photolysis by-products of CPF.

| Name, RT | Proposed structure | Spectral data (m/z) |
|----------|--------------------|--------------------|
| Dimethyl ethyl phosphate* (RT = 5.28 min) | \( \text{H}_3\text{C-O-}\text{O-CH}_3 \) | 153 (M+), 127, 113, 109, 96 |
| \( O,O \)-Diethyl phosphate* (RT = 5.45 min) | \( \text{H}_2\text{O-}\text{O-CH}_3 \) | 154 (M+), 127, 109, 95, 79 |
| \( O,O \)-Diethyl-methyl thiophosphate (RT = 6.51 min) | \( \text{H}_2\text{C-O-P-}\text{O-CH}_3 \) | 184 (M+), 156, 129, 107, 95, 79 |
| \( O,O \)-Diethyl thiophosphate (RT = 6.97 min) | \( \text{H}_2\text{C-O-P-}\text{O-CH}_3 \) | 170 (M), 141, 113, 95, 81 |
| 2-Hydroxy-3,5,6-trichloro pyridine (RT = 10.21 min) | \( \text{ClN-CH}_2-\text{OH} \) | 197 (M+), 169, 134, 107 |
| 3,5,6-Trichloro-2-pyridinyl acetic acid* (RT = 10.27 min) | \( \text{ClN-CH}_2-\text{CH}_3 \) | 226 (M+), 210, 182, 146, 110 |
| 3,6-Dichloro-2-[pyridinyl-\( O, O \)-ethyl thiophosphate (RT = 15.14 min) | \( \text{ClN-CH}_2-\text{CH}_3 \) | 315 (M+), 280, 252, 224, 163, 97 |
| CPF (RT = 17.7 min) | \( \text{ClN-CH}_2-\text{CH}_3 \) | 349 (M+), 314, 288, 258, 259, 197, 125, 97 |
| CPF oxon (RT = 20.88 min) | \( \text{ClN-CH}_2-\text{CH}_3 \) | 333 (M+), 310, 254, 203, 193 |

*Found in methanol solution only. **Found in water solution only.
reduced the effective CPF concentration by about half, failed to alter the deficit, but longer UVC exposure (70% destruction) completely eliminated the cell loss. Again, UVC irradiation of methanol had a minor but significant effect by itself. We obtained similar results for total cell protein: CPF elicited a significant decrease that was not prevented by the shorter UVC irradiation but was prevented by the longer UVC treatment (Figure 4A). However, the effects on total protein were significantly smaller than those on DNA content (CPF × measure, $p < 0.0001$; CPF × UV × measure, $p < 0.002$), reflecting a greater effect of CPF on cell number than on cell growth (Qiao et al. 2001; Slotkin et al. 2007a, 2007b; Song et al. 1998). Accordingly, we also evaluated the total protein:DNA ratio as an index of relative cell size. By itself, CPF evoked an increase in the ratio, and again, short UV irradiation did not prevent the effect (Figure 4B); longer irradiation eliminated the effect of CPF, whereas the methanol vehicle alone more showed a significant effect by itself.

CPF exposure also reduced membrane protein (Figure 5) but to a lesser extent than the effect on DNA content. The effect was maintained or even exacerbated by short-term UVC exposure, whereas long-term exposure eliminated the deficit.

**Discussion**

The main finding of these studies is that photo-degradation of CPF does not necessarily lead to biological inactivation of adverse effects directed at developing neurons, one of the major concerns for CPF as well as other organophosphates (Colborn 2006; Costa 2006; Pope 1999; Slotkin 2005; Weiss et al. 2004). Although UV irradiation is a characteristic of both field conditions and engineered water purification, a number of features make CPF more problematic than several other organophosphates that have been evaluated under these conditions. CPF photodegradation products include the oxon and other bioactive molecules not necessarily shared by degradation of several other organophosphates (Bavcon Kralj et al. 2007). Although CPF is less water soluble and therefore less likely to run off than, for example, parathion, several of the degradation products identified here are far more soluble and thus may elicit effects on aquatic populations. For example, trichloropyridinol is the main CPF metabolite found in humans and other organisms and is known to impair neurite outgrowth (Howard et al. 2005), elicits gliotoxicity (Zurich et al. 2004), affect nuclear transcription factors involved in neurodifferentiation (Schuh et al. 2002), and inhibit mitotic activity in both neurontypic and gliotypic cells (Qiao et al. 2001). In combination with CPF, trichloropyridinol increases the net impact on aquatic organisms (Caceres et al. 2007), emphasizing how photodegradation mixtures may actually show augmented adverse effects.

Although we have already characterized the ability of both CPF oxon and trichloropyridinol to act as developmental neurotoxics (Qiao et al. 2001), it is likely that several other breakdown products are also biologically active. In particular, both CPF oxon and methanol residues were shown to be toxic to PC12 cells in a previous study (Qiao et al. 1998). Accordingly, we also evaluated the lower-order tests, as shown in Table 2 and Figures 2 and 3. These lower-order tests, ANOVA $p$-values across all treatments and UV exposures appear within the panel. For lower-order tests, $p < 0.05$ for *CPF compared with controls; and **UV-exposed control compared with control/none. lower-order tests, ANOVA $p$-values across all treatments and UV exposures appear within the panel. For lower-order tests, $p < 0.05$ for *CPF compared with controls; and **UV-exposed control compared with control/none.
In light of earlier studies, however, it is highly likely that CPF oxon and trichloropyridinol represent a large proportion of the total (Barcelo et al. 1993; Racke 1993; Walia et al. 1988), and as discussed above, there is no question of the role of these agents as contributors to developmental neurotoxicity. The fact that other products retain phosphorothionate and organophosphate characteristics suggests strongly that the other components in the photolytic mixture are also likely to participate in the net outcome.

The present findings indicate that photodegradation of CPF, and potentially other environmental toxicants, does not necessarily produce inactive products, and indeed, some of the resultant compounds and mixtures may actually exhibit equal or greater toxicity than the parent chemical. These factors should be taken into account in determining whether the disappearance of an agent in the field or during remediation treatments actually connotes a safer outcome.

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