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Cytotoxicity of Organophosphorus Ester (OP) Insecticides and Cytotoxic Synergism of 2-Acetoxyacetylaminofluorene (2AAAF) in Chinese Hamster Ovary (CHO) Cells

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An estimated 4.1×10^7 kg of organophosphorus ester (OP) insecticides are used annually in the United States (USEPA, 2002). A substantial proportion of the general population express recent OP exposure. Although OP insecticides have been used for several decades, there is growing concern that the majority of pesticides have not been adequately tested for toxic effects in non-target tissues. To determine cellular responses to environmental agents we developed and calibrated a rapid mammalian cell cytotoxicity assay (Plewa et al., 2002; 2004; Sorensen et al., 2003).

Aromatic amines are another class of chemicals with wide human exposure. Heterocyclic aromatic amines are carcinogens that are generated when meat or fish are cooked and are highly implicated in the induction of cancer. We discovered that the mutagenicity of heterocyclic amines was significantly enhanced in the presence of OP insecticides or their metabolites in *Salmonella typhimurium* (Gichner et al., 1996; Plewa et al., 1997; Wagner et al., 1997; 2003). These synergistic responses may be important in risk assessment. 2AAAF is a representative activated heterocyclic amine and a well-characterized toxin and mutagen. In the present study, a series of insecticides and their metabolites were analyzed for mammalian cell cytotoxicity after a 72-h exposure. The objectives of this study were to assess the individual cytotoxicity of four OP insecticides and their metabolites using the microplate-based mammalian cell assay, and to determine if cytotoxic synergy was expressed by combinations of OP insecticides and 2AAAF.

MATERIALS AND METHODS

General laboratory reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St Louis, MO). Media supplies and fetal bovine serum (FBS) were purchased from HyClone Laboratories (Logan, UT). The insecticides, their CAS numbers and the rat oral LD_{50} values were the following: chlorpyrifos (CAS 2921-88-2, LD_{50} = 135-163 mg/kg), ethyl parathion (CAS 56-38-2, LD_{50} = 3.6-13.0 mg/kg), malathion (CAS 121-75-5, LD_{50} = 1375 mg/kg), malaoxon (CAS 1634-78-2, LD_{50} = 158 mg/kg), methyl parathion (CAS 298-00-0, LD_{50} = 9-25 mg/kg), and methyl paraoxon (CAS 950-35-6) were purchased from ChemService, Inc., (West Chester, PA) and were of 98 – 99.5% purity with the exception of malaoxon (88%

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purity). Ethyl paraoxon (CAS 311-5-5, LD_{50}, mouse = 12.8 mg/kg) was purchased from Sigma Chemical Co. and was of 90% purity. 2-Acetoxyacetylaminofluorene (2AAAF, CAS 6098-44-8) was purchased from Chemsyn Science Co. (Lenexa, KS). Concentrated solutions of the insecticides and 2AAAF were prepared in dimethyl sulfoxide (DMSO). We employed Chinese hamster ovary (CHO) cell line AS52 clone 11-4-8 which were maintained in Ham's F12 medium with 5% FBS at 37°C in a humidified atmosphere of 5% CO_2 (Wagner et al., 1998).

Treatments were conducted in flat bottom 96-well microplates. Dilutions of the concentrated stock solutions were prepared in F12 + FBS. The final DMSO concentration did not exceed 0.5%. Each treatment well contained 3,000 cells, F12 + FBS and the test agent for a total volume of 200 µL. Eight replicate wells provided a blank control of F12 + FBS only. The negative control consisted of 8 wells with 3,000 cells and F12 + FBS. In general, each chemical concentration was replicated 4-8 times per microplate and each experiment was repeated. The wells were covered with AlumnaSeal™. The cells were treated for 72 h at 37°C, 5% CO_2. Each well was aspirated; the cells were fixed in 100% methanol and stained with 1% crystal violet in 50% methanol for 30 min. The wells were gently washed, drained and 50 µl of DMSO was added for 30 min. The microplate was analyzed at 595 nm with a microplate reader; the absorbancy of each well was automatically recorded. The average absorbance of the blank wells (no cells) was subtracted from the absorbance data from each well. The value for the negative control (cells with medium only) was set at 100% and the absorbance for each treatment group well was converted into a percentage of the negative control. From regression analysis of the concentration-response data, a %C½ value was determined (the concentration that reduced the CHO cell density by 50% as compared to its concurrent negative control). To determine significant differences from the negative control, a one-way analysis of variance was conducted. To determine cytotoxic synergy we examined the effect of 2AAAF with chlorpyrifos and malathion. Each column of 8 wells contained the identical concentration of insecticide with 10 concentrations per microplate. The top 4 wells of each column did not contain 2AAAF; the bottom 4 wells of each column contained a constant concentration of 2AAAF (1 µM). This concentration was chosen because of its low cytotoxicity. There were 2 concurrent negative controls. The cells exposed to the insecticide without 2AAAF were compared to a concurrent control of cells with F12 + FBS. The cells exposed to the insecticide and 2AAAF were compared to a concurrent control of cells, F12 + FBS and 1 µM 2AAAF. To determine if cytotoxic synergy occurred, a two-factor analysis of variance with a Holm Sidak multiple comparison test was conducted. The null hypothesis was rejected if a significant difference ($P \leq 0.05$) was obtained and if the power of the test exceeded 0.8 with $\alpha = 0.05$.

RESULTS AND DISCUSSION

The CHO chronic cytotoxicity assay provides a quantitative and comparative analysis for environmental chemicals. It was recently used to analyze alachlor, oxamyl,

atrazine, 2,4-D, dicamba and trifluralin (Sorensen et al., 2003) and a series of drinking water disinfection by-products (Plewa et al., 2002; 2004). In the present study all of the agents showed a significant toxic response (Table 1). The %C½ values of the insecticides (the concentration that reduced CHO cell density by 50%) expressed an approximately 5.7-fold range from a value of 50.11 μM for chlorpyrifos to 285.32 μM for ethyl paraoxon. 2AAAF was the most cytotoxic (%C½ = 3.88 μM). The rank order of the insecticides in declining toxicity was chlorpyrifos > malathion > malaoxon > ethyl parathion > methyl paraoxon > methyl parathion > ethyl paraoxon.

Table 1. Mammalian cell cytotoxicity of chemicals.

Chemical	CR [a] (μM)	ANOVA Test	r^2 [b]	%C½ [c] (μM)
Chlorpyrifos	6.25-250	$F_{16, 248}$=128 $P \leq 0.001$	0.99	50.11
Ethyl Parathion	12.5-350	$F_{11, 236}$=160 $P \leq 0.001$	0.98	178.56
Ethyl Paraoxon	25-1000	$F_{11, 164}$=181 $P \leq 0.001$	0.99	285.32
Malathion	12.5-500	$F_{16, 190}$=160 $P \leq 0.001$	0.99	79.65
Malaoxon	1-500	$F_{12, 213}$=126 $P \leq 0.001$	0.99	115.96
Methyl Parathion	5-500	$F_{11, 276}$=149 $P \leq 0.001$	0.99	235.34
Methyl Paraoxon	25-1000	$F_{12, 169}$=74 $P \leq 0.001$	0.95	220.58
2AAAF	0.1-10	$F_{27, 128}$=25 $P \leq 0.001$	0.95	3.88

[a] Concentration range of the test chemical. [b] The coefficient of determination for nonlinear regression equations that were used to calculate the %C½ value. [c] Calculated concentration of the test agent that reduced the CHO cell density to 50% of the negative control.

From a structure-function comparison, we could not deduce specific features that contributed to cytotoxicity. It is surprising that malathion and ethyl parathion were more cytotoxic than their respective metabolites; we have no explanation for this at present. Cytotoxicity was not correlated with LD_{50} values. However, this is not unexpected. Acute toxicity of OPs are primarily caused by inhibition of acetylcholinesterase, however, OPs also affect cellular structures, activate biochemical pathways including apoptosis and signal transduction and induce

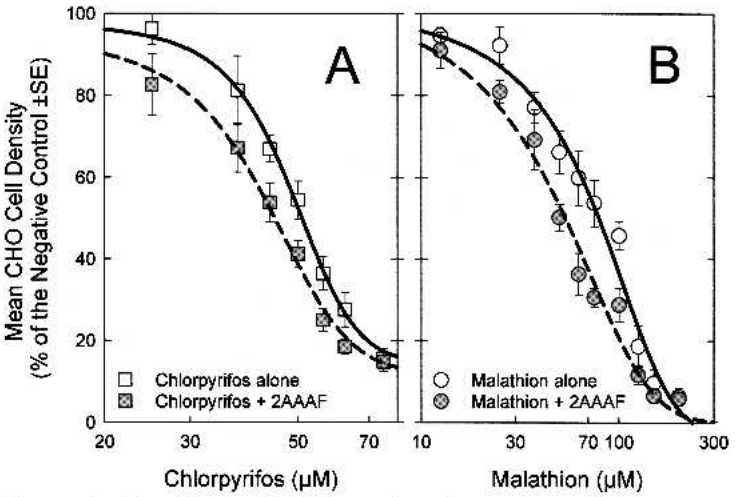

Figure 1. Log-linear plot illustrating the concentration-response CHO cell cytotoxicity curve (solid line) for chlorpyrifos (A) or malathion (B). Cytotoxic synergy with a nontoxic concentration of 2AAAF is illustrated (dashed lines) for chlorpyrifos (A) or malathion (B).

oxidative stress and DNA damage. The cytotoxicity assay presented here measures an overall reduction in cell density that could be attributed to any of these mechanisms.

To determine cytotoxic synergy we examined the effect of 2AAAF with the two most potent insecticides, chlorpyrifos and malathion. As illustrated in Figure 1A, 2AAAF significantly enhanced the cytotoxicity of chlorpyrifos within the concentration range of 25 - 50 μM. The %C½ values for chlorpyrifos alone and with 1 μM 2AAAF were 50.9 μM and 44.8 μM, respectively. 2AAAF (1 μM) significantly enhanced the cytotoxicity of malathion at concentrations of 50 - 100 μM (Figure 1B). The %C½ value for malathion alone was 79.2 μM; with 2AAAF it decreased to 53.7 μM.

Several *in vivo* and *in vitro* studies reported synergistic toxicity between pesticides. The enhanced toxicity was attributed to an increase in metabolism (Belden and Lydy, 2000), an inhibition in detoxication (Johnston et al., 1994), or to enhanced oxidative damage (Hazarika et al., 2003). In neuroblastoma cells, a chronic exposure to diazinon followed by an acute exposure increased the toxicity of some but not all of the pesticides examined (Axelrad et al., 2003). The data presented here demonstrate that genotoxic synergic effects between OP insecticides and aromatic amine carcinogens reported in *S. typhimurium* are also expressed as cytotoxicity in mammalian cells. This paper presents discovery science and is the first report of synergistic cytotoxicity between an arylamine and OP insecticides. The mechanism of action is unknown. The CHO cell microplate cytotoxicity assay is well suited for the analysis of pesticides and other environmental contaminants. It is relatively rapid

and is useful for analyzing test materials where the quantity is limited. This quantitative cytotoxicity assay may also be used to determine toxic synergistic effects of agents with wide human exposure.

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