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Comparative Quantitative Analysis of Agricultural Chemicals Using a Microplate Mammalian Cell Cytotoxicity Assay

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The production of synthetic chemicals has increased exponentially since World War II. Between 45,000 and 100,000 chemicals are now in commercial use within the United States (Krieger, 2001; NRC, 2000; Thornton, 2000) and a substantial amount of this production is devoted to pesticides. In the late 1930s approximately 32 pesticidal active ingredients were registered with the Federal government (Wayland & Hayes, 1975); this number, however, has now increased to 890 active ingredients, with 20,700 different products (EPA, 1997). Each year 4.5 billion pounds (2.04×10^9 kg) of pesticide are used in the United States (EPA,1997) including wood preservatives, biocides, chlorine, hypochlorites, sulfur, petroleum, and conventional-based pesticides. Current use of conventional pesticides in the United States is estimated at approximately 1.23 billion pounds (5.58×10^8 kg) annually, with approximately 80% consumed for agricultural purposes (EPA, 1997; USGS, 2000).

The National Research Council reported that a large majority of pesticides have not been adequately tested for toxicity (NRC, 1984; NRC, 1993; Silbergeld, 1995; Thornton, 2000). The reevaluation of old pesticides is in progress; however, completion of these mandates are not expected until 2006 (Krieger, 2001). In addition, the work involved in retesting and reevaluating these pesticides by traditional whole animal studies is laborious, costly, and time consuming. Thus, a quantitative mammalian cell assay to test multiple concentrations, with multiple replicates, would be efficient and would prove useful as an initial tool to rank order the cytotoxic effects of pesticides. This assay has utility as a screening tool and measures effects that retard the growth rate or causes the death of cells. This in vitro assay has been used to analyze drinking water disinfection by-products and toxic environmental agents (Plewa et al., 2000; Plewa et al., 2002; Rayburn et al., 2001). The objective of this study was to evaluate the use of a microplate mammalian cell cytotoxicity assay in assessing the cytotoxicity of six pesticides and two metabolites.

MATERIALS AND METHODS

Reagent grade or higher quality pesticides (Table 1), ethyl methanesulfonate (EMS), and dimethylsulfoxide (DMSO) were used. All pesticides were purchased from ChemService, Inc, West Chester, PA. Other 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. Chinese Hamster Ovary (CHO) cell line AS52, clone 11-4-8 was obtained from Dr. Elizabeth Wagner (Wagner et al., 1998). Cells were maintained in Ham's F12 medium with 5% FBS and grown at 37°C and 5% CO_2.

The chronic microplate mammalian cell cytotoxicity assay was used to analyze the impact of the specific pesticides on the CHO cell survivorship. A 96-well microplate

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Table 1 Pesticide characteristics.

Trade Name	Chemical Name	CAS Number	H_2O[a] Solubility @ 25°C mg/L	Annual Use[b] (kg applied)
2,4-D (herbicide)	(2,4dichlorophenoxy) acetic acid	94-75-7	900	1.9×10^7
Alachlor (herbicide)	2-chloro-2',6'-diethyl-N-(methoxymethyl) acetanilide	15972-60-8	242	6.8×10^6
Atrazine (herbicide)	2-chloro-4-ethylamine-6-iso-propylamino-*s*-triazine	1912-24-9	28	3.4×10^7
Atrazine-desethyl	4-amino-2-chloro-6-isopropylamino-*s*-triazine	6190-65-4	3200	NA[c]
Atrazine 2-hydroxy	2-hydroxy-4-ethylamino-6-isopropyl amino-1,3.5 triazine	2163-68-0	NA	NA
Dicamba (herbicide)	3,6-dichloro-O-anisic acid	1918-00-9	6500	4.5×10^6
Oxamyl (insecticide)	N,N-dimethyl-2-methyl-carbamoyloxyimino-2-(methylthio)-acetamide	23135-22-0	280,000	4.3×10^5
Trifluralin (herbicide)	a,a,a-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine	1582-09-8	< 1	9.9×10^6

[a] Source References: Kidd H et al., 1991; U.S. Environmental Protection Agency, 1994b,1995,1987; Weed Science Society of America, 1994; Stevens JT et al.,1991; Baron RL et al., 1991.
[b] Gianessi and Marcelli, 2000
[c] NA, Information not available.

was divided into a series of columns with each column representing one treatment group. Within each treatment group, 2 to 6 replicate wells were prepared for each concentration of test agent. The blank control column had 8 replicate wells consisting of F12 + 5% FBS medium only. Column 2 was a negative control column, with 8 replicate wells consisting of 100 μL of a titered CHO cell suspension (3,000 cells) plus 100 μL F12 + FBS medium. The wells for the remaining columns contained 3,000 CHO cells, F12 + FBS, and a known concentration of each pesticide in a total of 200 μL. Each pesticide was initially diluted in DMSO and final dilutions were made with F12 medium to achieve the desired concentration. Concentration ranges for each pesticide is provided in Table 2. DMSO concentrations with the medium did not exceed a maximum of 0.5%. EMS was selected as a positive control as it is a well characterized cytotoxic and genotoxic agent. After a 72 h incubation at 37°C, each well of the plate was gently aspirated, fixed in 50% methanol, and stained with 1%

crystal violet in 50% methanol. The microplate was gently rinsed, drained, and 50 µL of DMSO was added and allowed to extract the stain from the attached cells for 30 min. The plate was then analyzed in a BioRad, model 550, microplate reader and the data were automatically recorded for future analysis.

Table 2 LD50 and % C½ summary of test agents.

Trade Name	RFD[a]	CR[b] µM	ANOVA Test Statistic[c]	LD_{50}[a] rats mg/kg	%C½ µM	r^2	Rank Order
2,4-D	0.01	0.09-905	$F_{28,290}$ = 22.190; $P \leq 0.001$	375-666	515	0.97	6
Alachlor	0.01	1.8-227.4	$F_{26,172}$ = 42.619; $P \leq 0.001$	930-1350	24	0.99	1
Atrazine	0.035	9-460	$F_{19,246}$ = 16.295; $P \leq 0.001$	1,869-3,090	210	0.95	3
Atrazine-desethyl	NA[d]	9-460	$F_{19,106}$ = 3.576; $P \leq 0.001$	NA[d]	321	0.77	4
Atrazine 2-hydroxy	0.01	9-460	$F_{36,195}$ = 2.569; $P \leq 0.001$	NA	>460	NA	5
Dicamba	0.03	0.09-407	$F_{30,279}$ = 7.984; $P \leq 0.001$	757-1707	2247	0.56	7
Oxamyl	0.025	1-173.2	$F_{22,187}$ = 38.313; $P \leq 0.001$	5.4	81	0.92	2
Triflur-alin	0.008	3-59650	$F_{46,475}$ = 13.152; $P \leq 0.001$	$>10^4$	5936	0.79	8

[a]RFD, Reference Dose mg/kg/day (Kidd H et al., 1991; U.S. Environmental Protection Agency, 1994b,1995,1987; Weed Science Society of America, 1994; Stevens JT et al., 1991; Baron RL et al., 1991)
[b]CR, Selected Concentration Range
[c]Analysis of variance test of the data presented in Figure 1.
[d] NA, Information not available.

The average absorbance of the blank column was subtracted from the absorbance data for each well. The negative control, consisting of the CHO cell density, was set as 100%. The absorbency for each treatment-group well was converted into a percentage of the negative control. The cytotoxicity data for each pesticide were then tested for significant differences from their concurrent negative controls. An analysis of variance test statistic was conducted; if the F value was significant at a $P \leq 0.05$, a Dunnett's Multiple Comparison analysis was conducted (Table 2). The power of

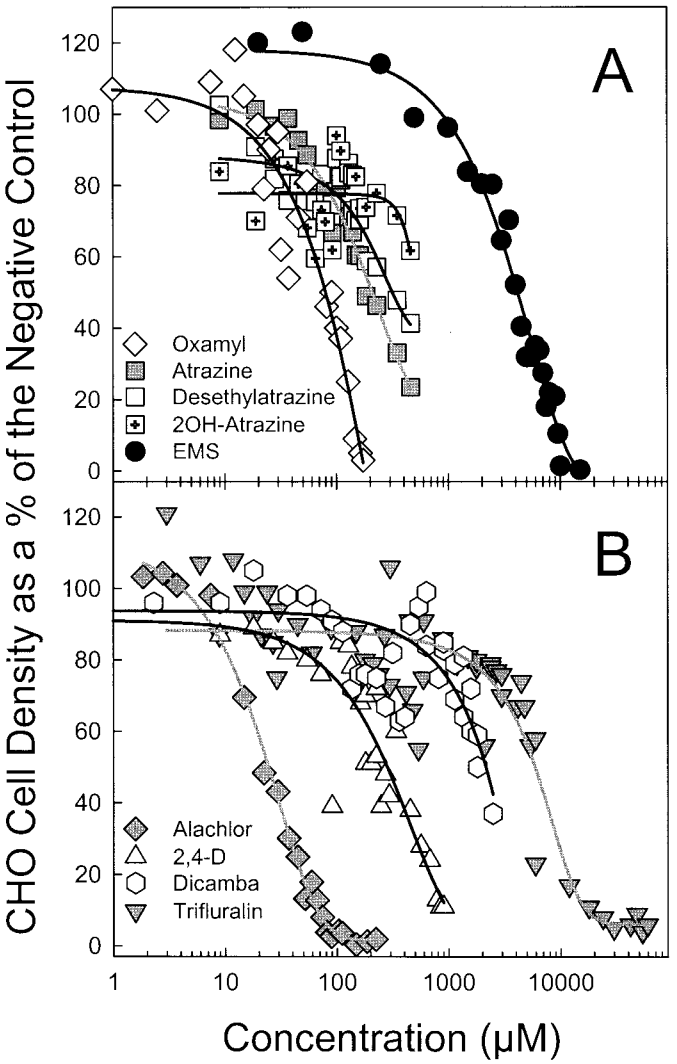

Figure 1 Concentration-response curves of the CHO cell cytotoxicity of (A) oxamyl, atrazine and its metabolites desethylatrazine and 2OH-atrazine, and the positive control EMS, (B) alachlor, 2,4-D, dicamba and trifluralin. The r^2 values of the regression analysis for each curve is presented in Table 2.

the test statistic (β) was ≤ 0.8 at $\alpha = 0.05$. A Pearsons Product Moment Correlation test was used to determine if a significant relationship existed between the calculated %C½ values and the published rat LD_{50} values. The %C½ value is the concentration of the pesticide that induced 50% cell growth repression as compared to its concurrent negative control.

RESULTS AND DISCUSSION

We used a microplate based assay to quantitatively compare the cytotoxicity of agriculturally important pesticides. The usual method of measuring toxic responses

in mammalian cells as a reduction of plating efficiency would be costly, time consuming and would require comparatively large amounts of sample. The microplate mammalian cell cytotoxicity assay was used to determine the cytotoxicity of drinking water disinfection by-products (Plewa et al., 2002). Here we applied this assay to quantitatively compare the cytotoxic response of a series of pesticides.

The cytotoxic response for the concentration range of each pesticide, compared to its negative control, using the analysis of variance test is presented in Table 2. All pesticides showed a significant toxic response as compared to their concurrent negative control. The concentration-response curve for each pesticide and the positive control, EMS, is presented in Figure 1. All of the pesticides, excluding trifluralin, showed a cytotoxic response greater than that of EMS, a potent mutagen and carcinogen. A regression analysis of the data for each pesticide concentration response curve was performed and the %C½ value was calculated (Table 2). Each pesticide showed a reduction in cell density. A reduction in cell density is a reflection of the induced cytotoxicity of the pesticide. However, this assay would register a response if the pesticide disrupted the cell cycle or retarded the growth of the cells. Thus we refer to the pesticide concentration that caused a 50% reduction of the cell density as compared to its concurrent negative control as the %C½ value rather than the LC_{50}. The rank order in declining toxicity of the %C½ values for each pesticide was Alachlor > Oxamyl > Atrazine > Atrazine-desethyl > Atrazine 2-hydroxy > 2,4-D > Dicamba > Trifluralin (Table 2). The mammalian cell cytotoxicity (%C½) values were compared to the lowest concentration of published LD_{50} values for rats. The Pearsons Product Moment Correlation Coefficient indicated that the %C½ and LD_{50} values were significantly and highly correlated ($r = 0.92$; $P \leq 0.01$). Although this comparison is limited by a small sample size it is interesting that a correlation exists between these *in vitro* and *in vivo* toxicity assays. *In vitro* assays have the advantage of generating large amounts of data with large replicate experiments. These assays are very specific and precise. The disadvantage of *in vitro* assays is that they cannot measure the broad toxic impact of agents that only animal tests can resolve. Currently the CHO microplate cytotoxicity assay is being employed as the principal measurement in a U.S. Environmental Protection Agency study on determining the toxicity of individual versus complex mixtures of drinking water disinfection by-products (Dr. J. Simmons, EPA, personal communication, 2002). The results of this study demonstrate that a mammalian microplate assay can quantitatively assess the toxicity of pesticides and can compare these data to standard toxicity measures.

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