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In vitro Cytotoxicities of Inorganic Lead and Di- and Trialkyl Lead Compounds to Fish Cells

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Although there has been much research on the effects of inorganic lead (Pb^{2+}) on the aquatic biota (Wong et al. 1978), less is known on the environmental impact of organolead compounds (Jarvie 1988). Organolead compounds are primarily of anthropogenic origin, with the largest consumption of organolead being the use of tetraalkyl Pb as an antiknock additive in gasoline. Once deposited into aquatic environments the tetraalkyl Pb compounds may be degraded to tri- and dialkyl Pb species and then finally to Pb^{2+}. Di- and trialkyl Pb species are used as biocides and may enter aquatic ecosystems through runoff from terrestrial environments (Chau and Wong 1984). This study evaluated the relative cytotoxicities of di- and trialkyl Pb compounds to fish cells in culture.

MATERIALS AND METHODS

Procedures for the establishment of the epithelioid cell line, designated BG/F, from fin tissue of bluegill sunfish (Lepomis macrochirus) fingerlings have been described elsewhere (Babich and Borenfreund 1987a). Cells were propagated in Dulbecco's minimal essential medium (DMEM), supplemented with 10% Serum Plus (Hazelton Labs.), 2% fetal bovine serum (FBS), 100 units/ml penicillin G, 100 ug/ml streptomycin, and 2.5 ug/ml amphotericin B and maintained in a humidified 5.5% CO_2 atmosphere at 34°C. The cells were dissociated with a solution of 0.05% trypsin-0.02% EDTA for subculture.

Individual wells of a 96-well tissue culture microtiter plate were inoculated with 0.2 ml medium containing 2.6 x 10^4 cells. The plates were incubated at 34°C for 24 hr, after which the medium was removed and the cells were exposed to medium (DMEM +2% FBS) unamended or amended with varied concentrations of test agents. Six to 8 wells were used per concentration of test agents. After 24 hr of incubation in the presence of the test agents, the neutral red in vitro cytotoxicity assay was performed.

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The neutral red assay quantitates the number of viable, uninjured cells after their exposure to toxicants and is based on the uptake and accumulation in lysosomes of the supravital dye, neutral red. Spectrophotometric quantitation of the extracted dye has been shown to be linear with cell numbers, both by direct cell counts and by protein determination (Borenfreund and Puerner 1984).

After 24 hr of incubation in the presence of test agents, the medium was removed and replaced with 0.2 ml medium containing 40 ug/ml neutral red. Two wells at the top of the first row of the microtiter plate received medium without neutral red and served as blanks for the subsequent photometric analysis. After 3 hr of incubation, the neutral red containing medium was removed and the cells were washed and fixed rapidly with a solution of 0.5% formaldehyde-1% $CaCl_2$. The dye, incorporated into the lysosomes of viable cells, was then extracted into the supernatant with a solution of 1% acetic acid-50% ethanol. The plates were placed for a few seconds on a microtiter plate shaker and 10 min later were transferred to a microtiter plate reader where absorbance was measured at 540 nm.

All experiments were performed at least 4 times and the relative cytotoxicities of the test agents were compared to untreated controls by computing the concentration needed to reduce absorbance by 50% (NR_{50}). Data for the concentration-response cytotoxicity curves were presented as the arithmetic mean $\pm$ standard error of the mean.

The test agents included lead nitrate [$Pb(NO_3)_2$] (Sigma), dimethyl lead dichloride [$(CH_3)_2PbCl_2$], diethyl lead dichloride [$(C_2H_5)_2PbCl_2$], trimethyl lead acetate [$(CH_3)_3PbOOCCH_3$], and triethyl lead acetate [$(C_2H_5)_3PbOOCCH_3$] (kindly provided by P.T.S. Wong, Canada Centre for Inland Waters, Ontario).

RESULTS AND DISCUSSION

Figure 1 illustrates the concentration-response cytotoxicity curves generated by the Pb species to the BG/F cells after 1 day of exposure. The sequence of cytotoxicity was $(C_2H_5)_3Pb^+ > (CH_3)_3Pb^+ > (CH_3)_2Pb^{2+} > (C_2H_5)_2Pb^{2+} > Pb^{2+}$. The organolead compounds were more toxic than inorganic Pb^{2+} and the trialkyl Pb compounds were more toxic than the dialkyl Pb compounds. Whereas the triethylated Pb was more toxic than the trimethylated Pb, the dimethylated Pb was more toxic than the diethylated Pb. Grundt and Neskovic (1989) noted triethyl lead to be more toxic than lead acetate to rat glial cells in primary culture.

The sequence of *in vitro* cytotoxicity of the organolead compounds to the BG/F cells was similar to that noted for the acute 96-hr toxicity to the marine fish, *Pleuronectes platessa* (Table 1).

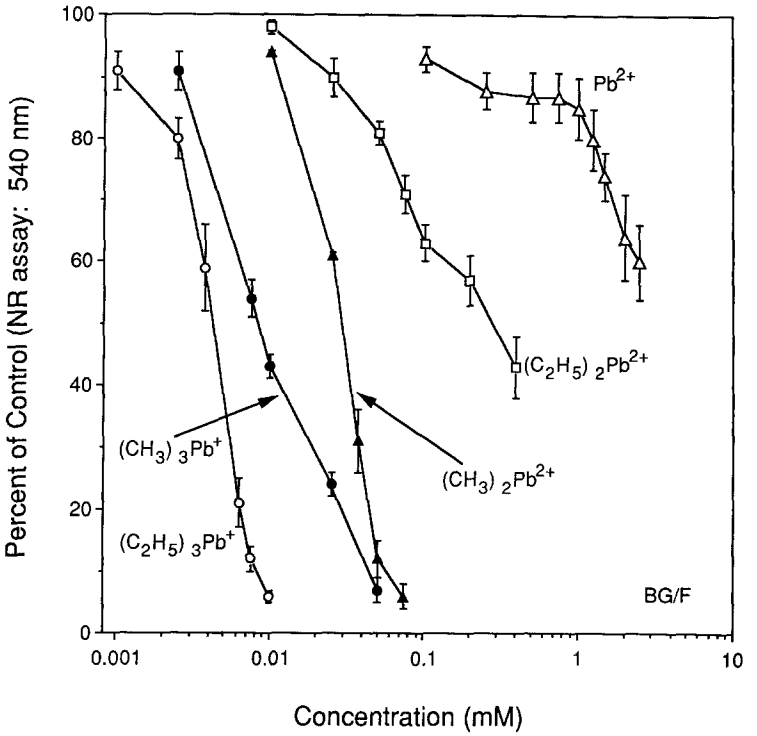

Figure 1. Comparative cytotoxicities of inorganic lead and di- and trialkyl organolead compounds to BG/F cells derived from bluegill sunfish. The data are presented as the mean percent of control ± standard error of the mean.

Table 1. Comparative toxicities of organolead compounds tested in vitro and in vivo.

Organolead	BG/F cells NR-50 (ug Pb/l)[a]	Pleuronectes platessa LC-50 (mg Pb/l)[b]
diethyl lead	62.2	300.0
dimethyl lead	6.2	75.0
trimethyl lead	2.3	24.6
triethyl lead	0.9	1.7

a Midpoint cytotoxicity in the 24-hr neutral red assay
b Midpoint toxicity in a 96-hr LC-50 assay; data from Chau and Wong (1984)

Similar in vitro cytotoxicity studies were performed with a series of mercury-containing compounds (Babich et al. 1989). The sequence of cytotoxicity to the BG/F cells was phenylmercuric chloride > methylmercuric chloride > ethylmercuric chloride > mercuric chloride. Table 2 lists the NR50 values for both series of organometals. Inorganic Hg^{2+} was more cytotoxic than Pb^{2+} and the organomercurials were more cytotoxic than the organolead compounds. Similar results were obtained in a 72-hr acute toxicity assay with the freshwater alga, Poterioochromonas malhamensis (Roderer 1982).

Table 2. Comparative cytotoxicities of organolead compounds and organomercurials to BG/F cells.

Test agent	NR-50 (uM)	Test agent	NR-50 (uM)[a]
lead nitrate	400	mercuric chloride	17.5
diethyl lead chloride	300	ethyl mercuric chloride	1.7
dimethyl lead chloride	30	methyl mercuric chloride	0.8
trimethyl lead acetate	11	phenyl mercuric chloride	0.4
triethyl lead acetate	4.4		

a Data for the organomercurials are from Babich et al. 1989

In addition to good correlations between the in vitro cytotoxicity data obtained with the neutral red assay and the in vivo toxicity data, the neutral red assay has been applied to establishing structure-activity relationships for a variety of chemical groups. Thus, correlations were noted between the sequences of cytotoxicity for a series of inorganic metal cations with their softness parameters (Babich et al. 1986), of diorganotins with their Hansch π parameters (Babich and Borenfreund 1988), and of chlorinated phenols, toluenes (Babich and Borenfreund 1987b), anilines, and benzenes (Babich and Borenfreund 1988) with their logarithmic octanol/water partition coefficients (log P values). Thus, the neutral red assay has proven to be a useful tool in in vitro studies with various chemical test agents.

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