Cytotoxicity and Transcriptional Activation of Stress Genes in Human Liver Carcinoma (HepG$_2$) Cells Exposed to Iprodione

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Abstract: Iprodione (C$_{13}$H$_{13}$Cl$_2$N$_3$O$_3$) is a broad spectrum dicarboximide fungicide used on a wide variety of crop diseases. It is used on vegetables, ornamentals, pome and stone fruit, root crops, cotton and sunflowers, to control a variety of fungal pests. Iprodione inhibits the germination of spores and the growth of the fungal mycelium. Experimental studies with mice have indicated that exposure to iprodione at dose levels 5 to 15 folds greater than the LOAEL for liver injury, induces microsomal enzyme activities, hepatocyte proliferation, hepatomegaly, centrilobular hypertrophy, diffuse hypertrophy, and an increase in lauric acid hydroxylation. Currently, there is no toxicological data available on human health effects associated with exposure to iprodione. In this research, we performed the MTT Assay for cell viability to assess the cytotoxicity of iprodione, and the CAT-Tox (L) assay to measure the induction of stress genes in thirteen recombinant cell lines generated from human liver carcinoma cells (HepG$_2$). The cytotoxicity data indicated a strong concentration - response relationship with regard to iprodione toxicity. The percentages of cell viability were $100 \pm 0\%$, $128.0 \pm 41.4\%$, $97.5 \pm 37.7\%$, $70.1 \pm 35.4\%$, $33.5 \pm 16.1\%$, and $5.1 \pm 3.7\%$ in 0, 31.3, 62.5, 125, 250, and 500 μg/mL, respectively. The LC$_{50}$ was 208.3 ± 83.3 μg/mL. Data obtained from the CAT-Tox (L) assay showed that iprodione is able to induce a significant number of stress genes in HepG$_2$ cells. At 250 ug/mL exposure, the induction levels were 1.2 ± 0.4, 50.1 ± 17.8, 3.9 ± 1.2, 16.8 ± 7.2, 10.7 ± 0.7, 1.8 ± 0, 26.3 ± 10.0, 7.2 ± 2.4, 1.8 ± 0.0, 6.8 ± 1.3, 6.7 ± 0.5, and 4.3 ± 1.8 for CYP1A1, GSTYa, XRE, HMTIIA, *c-fos*, NF-kBRE, HSP70, CRE, RARE, GADD153, GADD45, and GRP78, respectively. These results indicate that the metabolism of iprodione involves Phase II biotransformation in the liver (XRE, GSTYa), and that this chemical has the potential to cause cell proliferation and/or inflammatory reactions (*c-fos*, NF-kB), proteotoxic effects (HSP70, GRP78), metabolic disruption (CRE), and DNA damage (GADD45, GADD153).

Key words: Iprodione, cytotoxicity, gene expression, HepG$_2$ cells

Introduction

Iprodione, also known as Rovral, Kidan and Chipco 26019, is a contact fungicide used to control stem and root rots, molds, and mildews in conifers. It is used on agricultural crops such as almonds, apricots, peaches, broccoli, carrots grapes, and lettuce; ornamentals such as flowering trees, vines, evergreens, shade trees, and non-flowering plants; turfgrass such as golf courses, sod farms, institutional and residential lawn areas; and vegetable garden crops such as beans (dried, lima, and snap), garlic, ginseng, mustard cabbage, and strawberries [1,2]. The target pests that iprodione is used to prevent, treat, and control include (but is not limited to) dollar

spot, gray and pink snow mold and brown patch on turfgrass; aerial leaf blight, ink spot fusarium corn rot on ornamentals; brown spot and sheath spot on rice; fruit brown rot scab and cherry leaf spot on stone fruit; gray and whit mold on beans; and black leg and black crown rot on carrots [1,2]. Studies done by the Environmental Working Group have reported iprodione residues in baby food products marketed by Gerber (Fremont, MI), Heinz (Pittsburgh, PA), and Beech-Nut (St. Louis, MO). These studies have also pointed out that iprodione was the most frequently found pesticide residue out of 16 other detections [3].

Studies with laboratory animals have shown iprodione to be of low acute toxicity. However, iprodione is classified as a Group B_2 "probable" human carcinogen, based on evidence of liver tumors in both sexes of mice and in male rats [2]. A cancer potency factor of 4.39 x 10^{-2} estimates the carcinogenic risk in rats' Leydig cells. A safety factor of 10X had been previously determined by the Food Quality Protection Agency (FQPA) for the protection of infants and children, however this safety factor was later changed to 3X due to the following reasons: 1) no enhanced susceptibility seen on its developmental effects on rats and rabbits in a two generation reproduction study; 2) the critical endpoint for acute dietary risk assessment decreased anogenital distance (AGD) was seen at a dose of 120 mg/kg/day and only a marginal difference of decreased AGD was seen among the doses of 20 mg/kg/day, 120 mg/kg/day, and 250 mg/kg/day; 3) the proposed action of testosterone biosynthesis disruption; and 4) the use of realistic dietary exposure data (monitoring data and percent crop treated). The acute dietary risk of iprodione was assessed from a developmental NOEL of 20 mg/kg/day, which was based on anogenital distances in male fetuses. The acute reference dose (RfD) for iprodione is 0.06 mg/kg/day, while its chronic RfD for iprodione is 0.02 mg/kg/day. Based on histopathological lesions in the male reproductive system and adrenal gland effects following chronic dietary exposure to iprodione, a toxicity/ carcinogenicity NOEL of 6.1 mg/kg/day has been computed [4].

Dietary exposure to residues of iprodione is through the diet and drinking water. Tolerance levels have been established and published in the Code of Federal Regulations (40 CFR 180.399) for almonds, apricots, beans, blueberries, broccoli, carrots, sweet and sour cheeries, garlic, peaches, peanuts, plums, potatoes, and strawberries, as well as for eggs, milk, and meat products (fat, liver, kidney, meat) from cattle, goats, hogs, horses, poultry, and sheep [4].

Iprodione is active in the soil and persists for varying periods of time. The half-life ranges from 2 to 160 days depending on the soil type, clay content and acidity. The primary agents of degradation in neutral soil are bacteria and ultraviolet light. Iprodione is moderately soluble in water (13 mg/L at 20^0C). It has the potential to leach into ground water due to its high mobility in loamy sand. In air, iprodione does not evaporate easily. When burned iprodione may decompose to hazardous products including oxides of carbon, nitrogen, sulfur, and hydrogen chloride [1,5].

Handlers can be exposed to iprodione via occupational and residential usage by mixing, loading, and applying the compound as a liquid or wetable powder [4].

Target organs for iprodione identified in animal studies include the reproductive system (prostate and uterus), liver, and kidneys. Experimental studies with mice have indicated that exposure to iprodione causes microsomal enzyme activities, hepatocyte proliferation, and hepatomegaly. Other findings have suspected iprodione of being an endocrine disrupter causing reproductive malformations in male Sprague Dawley rats [6,7]. The Environmental Working Group (EWG) also lists iprodione as an endocrine disrupter that is commonly found along with DDT and its metabolites DDE, PCB's and DEHP; the insecticide endosulfan and metabolites; and other fungicides such as vinclozolin, ethylene thiourea, and dioxin [8].

Iprodione, vinclozolin, and procymidone are members of the imide group of dicarboximide class fungicides. These fungicides appear to be anti-androgenic, and the mechanistic base of anti-androgenic properties has been studied at different degrees. According to EPA's National Health and Environmental Effects Laboratory, vinclozolin and procymidone bind and compete for the androgen receptor. Iprodione disrupts the endocrine system by inhibiting androgen synthesis rather than competing for the androgen receptor. The only known metabolites that these three chemicals have in common are 3,5-dichloraniline (DCA), which is structurally and toxicologically different from the parent compounds and not likely to be anti-androgenic [9].

Previous studies have indicated that dicarboximide fungicides such as iprodione and vinclozolin induce lipid peroxidation by means of oxygen activation in fungi but their action on mammalian cells is not clear [10]. Using human liver carcinoma cells (HepG2), as a model, this research was designed to determine the toxic effects of iprodione at the cellular level.

Materials and Methods

Chemicals

Iprodione, 3-(3,5-dichlorophenyl)-N-(1-methylethyl) 2,4-dioxo-1-imidazoline-carboximide ($C_{13}H_{13}Cl_2N_3O_3$), CAS No. 36734-19-7 with a purity of 99.0% was purchased from Chem Service Inc. in West Chester, Pennsylvania. Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM), Lot No. 1016511 was

purchased from Life Technologies in Grand Island, New York.

Cell Culture

Human liver carcinoma cells (HepG$_2$), and thirteen recombinant constructs generated by creating stable transfectants of different mammalian promoter–chloramphenicol acetyltransferase (CAT)-gene fusions were obtained from Xenometrix, Inc. in Boulder, Colorado. For each construct, a unique stress gene promoter or response element was fused to the CAT reporter gene.

In the laboratory, cells were stored in liquid nitrogen until use. Next, they were thawed in a water bath at 37^{0}C by gentle agitation of their vials for 2 minutes. When thawed, the content of each vial was transferred to a 75cm^2 tissue culture flask, diluted with DMEM supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin and incubated for 24 hours at 37^{0}C in a 5% CO$_2$ incubator to allow the cells to grow and form a monolayer in the flask. Cells grown to 80-95% confluence were washed with phosphate buffer saline (PBS), trypsinized with 10 mL of 0.25% (w/v) trypsin -0.03 (w/v) EDTA, diluted, counted, and seeded (5 x 10^5 cells/well) in two sets of 96-well microtiter tissue culture plates.

Gene Profile and Cytotoxicity Assays

Table 1 represents the promoter/response element-fusion constructs and their respective biological functions [11,12]. The seeded plates were incubated for 24 hours at 37^{0}C in a 5% incubator, followed by a replacement of the old medium by a fresh one containing the appropriate amount of iprodione (0, 32.25, 62.5, 125, 250, and 500 µg/mL respectively of iprodione) using 1% DMSO as a solvent. For quality assurance/quality control purposes, positive control plates were also made using known inducers including 3-methyl cholathrene (3MC-10 µM) for cytochrome P450 1A1 - CYP1A1, cyclic AMP response element - CRE, 45-kDa growth arrest and DNA damage - GADD45, 53-kDa tumor suppressor protein - p53RE, and xenobiotic response element - XRE; methyl methane sulfonate (MMS-100 µg/mL) for glutathione-s-transferase Ya subunit - GSTYa, metallothionein - HMTIIA, proto-oncogene - *c-fos,* 70-kDa heat shock protein - HSP70, NF-kB response element - NFk-BRE, 153-kDa growth arrest and DNA damage - GADD153, and 78-kDa glucose-regulated protein - GRP78; and all-trans retinoic acid (RA-10 µM) for retinoic acid response element - RARE.

Chemicals exposures involved polypropylene 96-well microtiter plates for the purpose of chemical dilutions. A constant volume of 20 µL was transferred from each well

of the chemical dilution plate to the plate containing the cells to give each cell line five chemical doses and a zero control dose, each in triplicate.

After chemical exposure, the cells were re-incubated for 48 hours at 37°C and 5% CO$_2$. Following the incubation period, the total protein was measured by the Bradford method, at 600 nm using a microtiter plate reader [13]. A standard sandwich ELISA was performed, and in the final step, a horseradish peroxidase catalyzed a color change reaction that was measured at 405 nm [11,12].

The HepG$_2$ parental cell line was dosed in the same manner as the recombinant cell lines and was used to perform the MTT [3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based cell viability assay using a microtiter plate reader with the wavelength set at 550 nm [14].

Statistical Analysis

Fold inductions of the CAT-gene for each recombinant cell line at each iprodione concentration were calculated using the CAT-Tox computer software based on the optical density readings at 600 nm and 405 nm. The software also converted the 550 nm readings to cell viability percentages. Standard deviations were determined and the *Student's* t-test values were computed to determine if there were significant differences in cell viability and gene expression in treated cells compared to the control cells. Graphs were made to illustrate the dose-response relationship with respect to cytotoxicity and gene expression.

Table 1: Stress gene promoters/response elements-CAT-fusion constructs and their biologic functions.

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Promoter	Biologic Function
CYP1A1	Phase I biotransformation enzyme
GSTYa	Phase II biotransformation enzyme
XRE	Binding site for Ah receptor – hydrocarbon complex
HMTIIA	Sequestration of heavy metals
c-fos	Member of AP-1 transcription factor (TF) complex
HSP70	Cytoplasmic protein chaperone
NF-kBRE	Binding site for NF-kB TF
CRE	Binding site for CREB protein
RARE	Binding site for retinoic acid - receptor
P53RE	Apoptosis / cell cycle arrest
GRP78	ER protein chaperone
GADD45/153	Cell cycle regulation, and genotoxicity

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Results

Cytotoxcity Assay

The cytotoxic effect of iprodione on human liver carcinoma cells (HepG$_2$) is shown in Figure 1. This figure denotes a strong concentration-response relationship with respect to iprodione toxicity to HepG$_2$ cells. The correlation coefficient was computed to be 0.99. The percentages of cell viability were 100.0 ± 0.0%, 12 8 ± 41.4%, 97.5 ± 37.7%, 70.1 ± 35.4%, 33.5 ± 16.1% and 5.1 ± 3.7% at 0, 31.25, 62.5, 125, 250, and 500 µg/mL of iprodione, respectively.

There was therefore a gradual decrease in cell viability at higher iprodione concentrations. Upon 48 hours of exposure the concentration (LC$_{50}$) required to produce 50% reduction in the viability of HepG$_2$ cells was computed to be 208.3 ± 83.3 µg/mL. At lower levels of exposure, iprodione appears to stimulate cell proliferation. In the 31.25 µg/mL concentration the cell viability was 128.0 ± 41.4%.
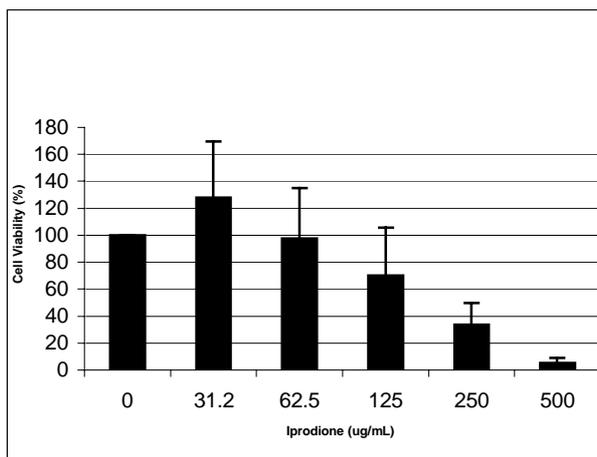
Fig. 1. Cytotoxicity of iprodione to HepG2 cells

Gene Profile Assay

The significant fold induction levels of stress genes in HepG$_2$ cells exposed to iprodione for 48 hours are represented in Figures 2-10 for glutathione S-transferase Ya subunit (GST Ya), xenobiotic response element (XRE), oncogene and transcription factor (*c-fos*), 70-kDa heat shock protein (HSP70), nuclear factor kB response element (NF-kBRE), cyclic AMP response element (CRE), 45-kDa growth arrest and DNA damage protein (GADD45), 153-kDa growth arrest and DNA damage protein (GADD153), and 78-kDa glucose regulated protein (GRP78), respectively.

The constructs that were activated, but did not show a significant induction after 48 hours exposure at concentrations of 0-250 µg/mL were cytochrome P-450 1A1 (CYP1A1), metallothionein (HMTIIA), retinoic acid response element (RARE) and 53-kDa tumor suppressor protein response element (p53RE).

Figures 2 and 3 present the fold inductions of the Ya subunit of the glutathion s-transferase (GSTYa) gene promoter and the xenobiotic response element (XRE) in HepG$_2$ cells exposed to iprodione. Data presented in Figure 2 indicate a strong concentration response relationship with regard to GSTYa induction within the concentration range of 0-250 µg/mL. Figure 3 also shows an activation of the XRE within the concentration range of 0-250 µg/mL; however, the only induction that is statistically significant ($p < 0.05$) is associated with the 250 µg/mL concentration.
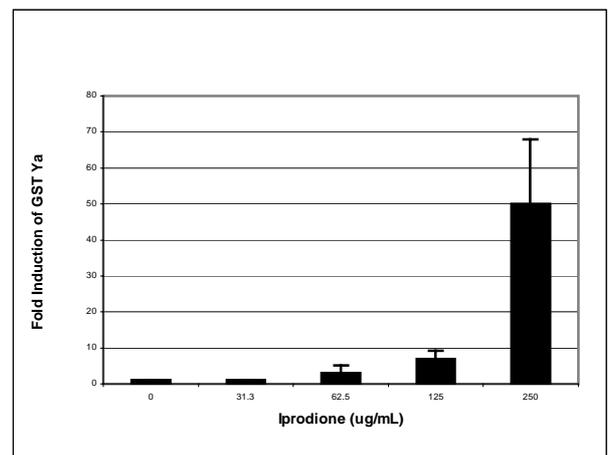

Fig. 2: Fold induction of GST Ya in HepG$_2$ cells
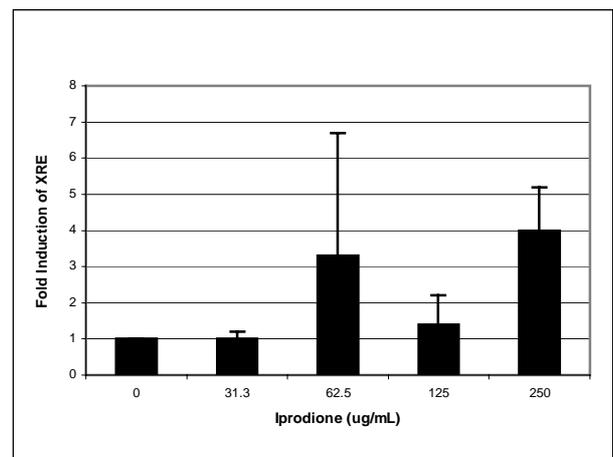
Fig. 3: Fold induction of XRE in HepG$_2$ cells

16

Int. J. Environ. Res. Public Health **2004**, *1*

Figure 4 denotes a strong response of the *c-fos* construct at a concentration of 250 μg/mL. The up-regulation of this construct suggests that iprodione may cause an oncogenic response by binding to the activator protein (AP-1) transcription factor, leading to cell cycle activation of DNA damage, and/or oxidative stress.
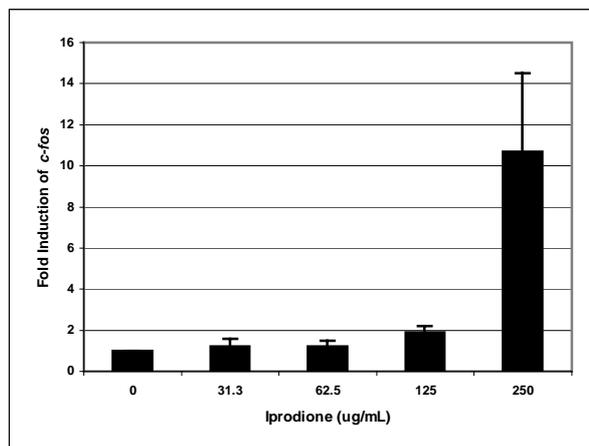

Fig. 4: Fold induction of *c-fos* in HepG$_2$ cells

Figure 5 shows the up-regulation of the 70-kDa heat shock protein (HSP70) at the concentration of 250 μg/mL, indicating that iprodione may cause a range of physiological stress conditions at this level of exposure. Activation of this gene promoter indicates that iprodione is proteotoxic to transformed human liver cells.
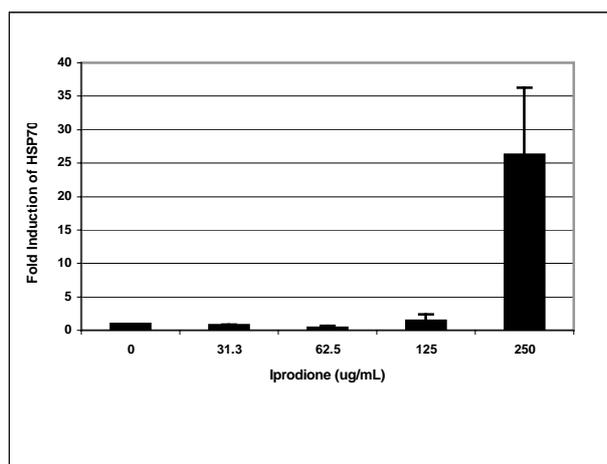

Fig. 5: Fold induction of HSP70 in HepG$_2$ cells

Figure 6 denotes the induction of the nuclear factor k-binding site response element (NF-kBRE) at a concentration of 250 μg/mL, indicating that iprodione causes oxidative damage to HepG$_2$ cells. This may be due to the activation of primary stress factors or secondary stimuli such as inflammatory cytokines.
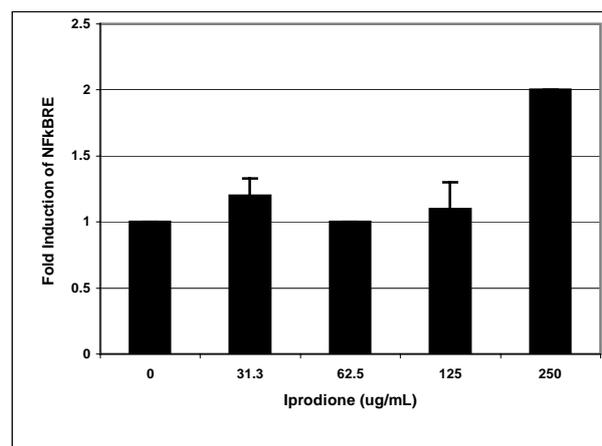

Fig. 6: Fold induction of NF-kBRE in HepG$_2$ cells

Figure 7 denotes a strong concentration response relationship with regard to induction of the cyclic adenosine monophosphate response (CRE) element within the concentration range of 0 to 250 μg/mL. The induction of this construct indicates that this chemical causes intracellular stress in the transformed hepatocytes at 125 and 250 μg/mL.
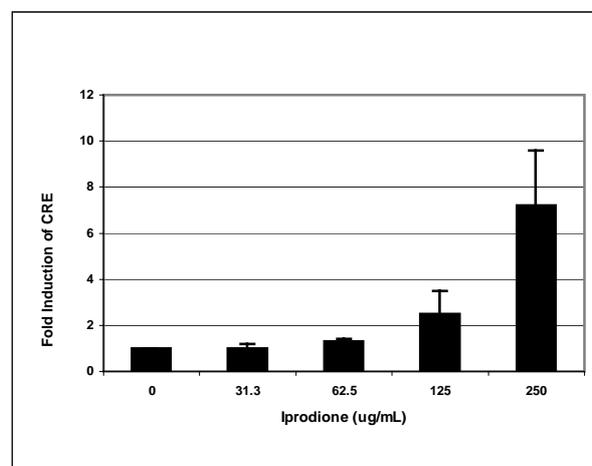

Fig. 7: Fold induction of CRE in HepG$_2$ cells

Figure 8 shows the activation of the 45-kDa growth arrest and DNA damage (GADD45) in HepG$_2$ cells exposed to iprodione. Data presented in this figure indicate a gradual increase in fold induction of this construct as the concentration of iprodione increases. The mean levels of fold inductions were 1.0 ± 0, 1.0 ± 0, 1.0 ± 0, 1.3 ± 0.5, and 2.7 ± 0.5, in 0, 31.3, 62.5, 125, and 250 μg/mL, respectively. A similar trend was observed with regard to the induction of the 153-kDa growth arrest and DNA damage (GADD153) gene promoter (Figure 9). These results indicate the genotoxic potential of iprodione.
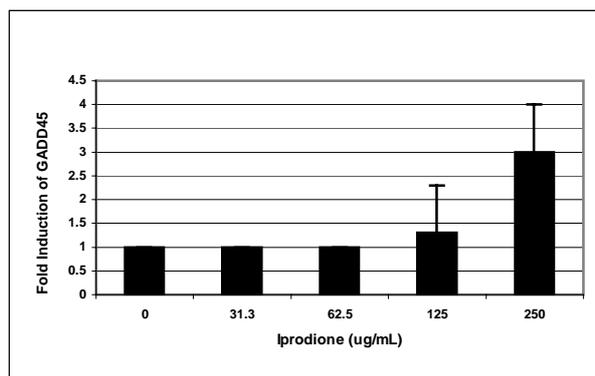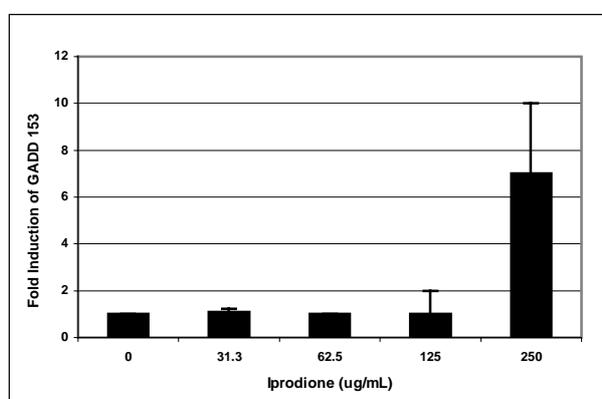
Fig. 8: Fold induction of GADD45 in HepG$_2$ cells

Fig. 9: Fold induction of GADD153 HepG$_2$ cells

The activation of the 78-kDa glucose regulated protein (GRP78) shows a concentration dependent response to iprodione (Figure 10). The activation of this promoter indicates that iprodione has the potential to cause underglycosylation and stress conditions in the endoplasmic reticulum. GRP78 has also been known to respond to DNA damage.
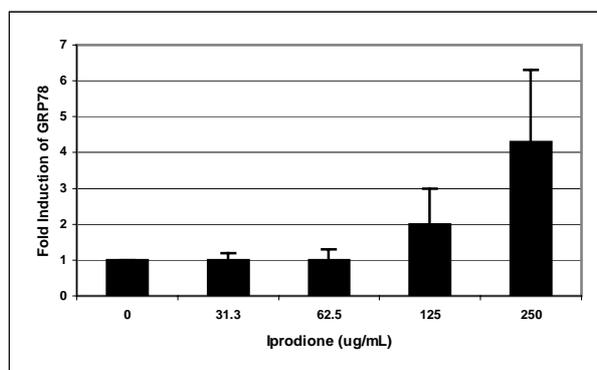

Fig. 10: Fold induction of GRP78 in HepG$_2$ cells

Discussion

Cytotoxicity Assay

Previous studies with experimental animals have demonstrated that iprodione induces liver cell proliferation, increases microsomal enzyme activities, and total cytochrome P-450 content, and causes centrilobular hypertrophy [15,16]. In an *in vitro* cytogenetic study with chinese hamster ovary cells iprodione did not cause chromosomal abberations at adequately high dose levels of 40, 150, and 400 μg/mL, in the presence and absence of metabolic activation [17].

In a study testing the effects of iprodione and vinclozolin on HepG$_2$ cells adaptation to oxidative stress via malonaldehyde and free radical production at concentrations of 125 and 250 μg/mL of iprodione and 50 and 100 μg/mL of vinclozolin, cells treated for 1 hour increased both malonaldehyde and free radical content, and decreased glutathione levels. However the 24-hour treatment at the same concentrations decreased malonaldehyde content and free radical production, and increased glutathione production [10].

In another study where rat lung epithelial L2 cells were exposed to oxidative stress or glutathione depletion for 1 hour in the presence of buthionine sulfoximine (BSO) decreased glutathione (GSH) content at 50 and 100 μg/mL and 125 and 250 μg/mL respectively. The greater increases were observed at the highest concentrations of the two fungicides [10,18].

An increase in intracellular GSH plays a central role in cell defense against oxidative stress by maintaining the intracellular reducing environment and reducing the production of reaction oxygen species. Cells must maintain their intracellular GSH content during oxidative challenges to prevent the loss of cell function and integrity [10,18].

Other organic compounds such as pentachlorophenol (PCP), a biocidal chemical used in agriculture and as a wood preservative, have shown similar but greater cytotoxic effects to HepG$_2$ cells than iprodione. PCP has a LC$_{50}$ of 23.0 ± 5.6 μg/mL compared to 208.3 ± 83.3 μg/mL for iprodione. At lower levels iprodione and PCP increases cell proliferation [19].

A recent investigation on the cytotoxicity of selected munitions compounds reported that 2,4,6-Trinitrotoluene has a relatively higher toxicity to human liver carcinoma cells (showing a LC$_{50}$ of 105 ± 6 μg/mL) compared to its two metabolites, 2,4-Dinitrotoluene and 2,6-Dinitrotoluene with LC$_{50}$'s greater than 300 μg/mL at 48 hours of exposure [12].

Based on the above information, it is obvious that iprodione appears to be less toxic than PCB and TNT, but more toxic than 2,4-DNT and 2,6-DNT. The order of toxicity is PCP > TNT > Iprodione > 2,4-DNT = 2,6-DNT.

18

Int. J. Environ. Res. Public Health **2004**, *1*

Gene Profile Assay

Exposure to iprodione resulted in the activation of a significant number of stress genes in human liver carcinoma cells. These genes included GST Ya, XRE, *c-fos*, HSP70, NF-kBRE, CRE, GADD45, GADD153, and GRP78. Induction of the GST Ya and XRE promoters by iprodione indicates that its metabolism involves Phase II biotransformation in the liver. Upregulation of the GST Ya gene is controlled by the xenobiotic response element [20].

The liver's capacity to detoxify active compounds is correlated with the level of biotransformation enzymes produced. Enzymes such as the gluthathione-S-transferase are transcriptionally induced when cells are exposed to specific chemicals [11]. This family of proteins (GSTs) catalyze the binding of glutathione to a variety of electrophiles such as hydrophobic compounds, heme, and polycyclic aromatic hydrocarbons [21]. Two distinct response elements control the inducible transcription of GST Ya, the xenobiotic response element (XRE-Ah receptor-dependent) and the antioxidant responsive element (ARE-Ah-receptor-independent) [22].

Iprodione significantly induced the *c-fos* gene promoter and the NF-kB response element, indicating its potential to cause inflammatory reactions, cell proliferation, and/or oxidative stress in HepG2 cells within the concentration range of 0-250 µg/mL. The *c-fos* promoter is regulated by a variety of response elements including: 12 O-tetadecanoylphorbol 13-acetate response element (TRE), and serum response element (SRE)[23]. The *c-fos* promoter responds to tumor promoters, DNA damage, and oxidative stresses [24]. The nuclear factor of K enhancer-B site (NF-kB) and the cyclic adenosine monophosphate (cAMP) are two of the nitro aromatic compounds. The NF-kB response element is a multi-subunit transcription factor that can rapidly activate the expression of genes involved in inflammatory, immune, and acute phase responses [25]. Agents that can activate NF-kB include viruses, bacterial lyopolysaccharides, protein synthesis inhibitors, cytokines, lectins and calcium ionophores [26]. The expression of cyclic AMP response element (CRE) indicates the potential of iprodione to cause metabolic disruption in HepG2 cells within the concentration range of 0-250 µg/mL. This response element is involved in protein kinase C and protein kinase A signaling pathways [27].

The significant induction of the 70-kDa heat shock protein (HSP70) and the 78-kDa glucose regulated protein (GRP78) gene promoters indicates iprodione's potential to cause proteotoxic effects at the molecular level. Increased heat shock protein expression may be a result of alterations in protein structure through adducts formation [28]. The GRP78 protein is a major endoplasmic reticulum (ER) protein that functions as a molecular chaperone. Upregulation of this promoter is associated with proteins that are malfolded because of mutagenesis, underglycosylation, or other stress conditions in the ER [29].

Activation of the GADD45 (Figure 8) and GADD153 (Figure 9) promoters from iprodione exposure indicates its potential to cause damage at the genomic level. This damage may be associated with alterations in DNA sequence as well as conformational changes in its helical structure. GADD promoters may respond to a variety of DNA damaging agents such as UV, DNA-crosslinking, and alkylating agents [30-32].

Conclusions

This study shows that iprodione is acutely toxic to HepG$_2$ cells, with an LC$_{50}$ of 208.3 ± 83.3 µg/mL after 48 hours of exposure. With this study, cell proliferation in transformed hepatocytes was observed at a concentration of 31.25 µg/mL and the greatest stress gene response was observed at 250 µg/mL. The gene profile assay provided valuable information on the molecular responses associated with HepG$_2$ cells exposure to iprodione. The study results indicated that iprodione has a high potential to cause protein damage and/or perturbations of protein biosynthesis (HSP70 and GRP78), and to induce growth arrest and DNA damage as a consequence of alterations in DNA sequence or its helical structure.

Induction of the *c-fos* and cAMP gene promoter indicates a potential oncogenic effect. Iprodione involvement in the Phase II biotransformation pathway in the liver is associated with a significant induction of the Ya subunit of the glutathion-S-transferase promoter (GST Ya), as well as the xenobiotic response element (XRE). Induction of nuclear factor kappa (B-site) response element and the cyclic adenosine monophosphate response element (CRE) indicates oxidative stress and metabolic disruption in HepG2 cells.

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