

## Toxicological assessment of industrial solvents using human cell bioassays: assessment of short-term cytotoxicity and long-term genotoxicity potential

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There is an increasing demand for simple toxicological screening methods to assess the human health risk associated with exposure to environmental toxicants. Such screening tools should allow for risk evaluation in terms of both short-term/acute toxicity and longer-term genetic damage, which may lead to mutagenicity and carcinogenicity. We employed a battery of human cell bioassays using the human hepatoma cell-line, HepG2, to assess the cytotoxic and genotoxic potential of environmental pollutants. Here, we demonstrate direct application of these human cell bioassays to the toxicological assessment of a number of industrial solvents that are in common use worldwide. HepG2 cells were exposed to various solvents at concentrations ranging from 25 to 500 ppm. The cells were then analysed using specific protocols for four different adverse effects: cell death/acute cytotoxicity using a neutral red uptake assay, altered enzyme function (often an indicator of cell stress) using the ethoxyresorufin *O*-deethylase (EROD) bioassay, DNA single strand breaks (SSB), and DNA repair induction, which evaluates mutagenic activity. Using the positive controls, linear dose–response curves were achieved for all four bioassays. The high sensitivity of the tests allowed for environmentally meaningful assessments, and precision studies showed excellent reproducibility for all four bioassays. Comparing the results of the four bioassays on each of 16 industrial solvents allowed for ranking of the anticipated relative human toxicity of these solvents, which were comparable with data from standard toxicity tests and human occupational data. Overall, the study clearly supports the application of the HepG2 cell bioassay system for rapid toxicological screening of many candidate toxicants in both short- and long-term toxicity potential. *Toxicology and Industrial Health* 2006; 22: 1–15.

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## Introduction

There is an urgent need to develop and establish new toxicological approaches to assess the potential cytotoxic and genotoxic effects of toxicants found in the environment (Hasspieler *et al.*, 1996; Bakand *et al.*, 2005; Bhogal *et al.*, 2005). In response to such growing demand, the development of rapid, simple, and sensitive toxicological screening methods for the biomonitoring of environmental pollutants that affect human health, is a universal goal. In previous years, numerous *in vitro* and *in vivo* assays have been utilized to assess the effects of environmental pollutants on their cellular targets. Increasing public interest in these issues has created a demand for alternatives to using animals in such testing. Bacterial assays are used for fundamental studies of mutagenesis and for screening of environmental samples as potential genotoxins. Mammalian cell culture systems have also been used in risk evaluation in order to investigate the mechanisms of chemical carcinogenesis and as bioassay systems for monitoring environmental genotoxins. Isolated cells have been extensively used in toxicological studies *in vitro* (Andreoli *et al.*, 2003; Bakand *et al.*, 2005; Hayashi, 2005; Dambach *et al.*, 2005).

The types of organ and cell systems – including the liver, kidney, neural tissue, the hematopoietic system, the immune system, the reproductive organs and the endocrine system – currently available to perform *in vitro* tests for toxicity testing have been extensively reviewed (Tahti *et al.*, 2003; O'Brien *et al.*, 2004; Farkas and Tannenbaum, 2005). Perfused organs, such as the liver and kidney, brain, lung etc., are examples of one model used for *in vitro* testing (Speilman *et al.*, 1998). The prime advantage of using entire organs is that the general morphology and cell–cell interactions are preserved. Primary cell cultures from organs of interest (liver, kidney, etc.) may also be prepared. Their use permits longer-term studies encompassing a few days and/or a few passages. Some differentiated functions may be retained, and co-culture is possible with other cellular types (Speilman *et al.*, 1998; Pfaller and Gstraunthaler, 1998; Costa, 1998). On the contrary, primary cell cultures have unstable phenotypes, and may quickly lose many differentiated functions. The use of

immortalized and continuous cell lines offers ease of propagation, and the ability to generate unlimited numbers of cells for testing (Speilman *et al.*, 1998; Pfaller and Gstraunthaler, 1998; Costa, 1998). Many different cell types have been used in toxicity studies, including continuous renal epithelial cell lines to assess nephrotoxicity (Pfaller and Gstraunthaler, 1998; Prieto, 2002), neuroblastoma or glioma cell lines to assess neurotoxicity (Costa, 1998; Lindahl *et al.*, 1999; Tahti *et al.*, 2003; Madia *et al.*, 2004), embryonic stem cells and ovarian and testicular cell types to assess reproductive and developmental toxicity (Spielmann, 1998; Riecke and Stahlmann, 2000; Bishop, 2003; Bremer *et al.*, 2005). Such lines are useful for specific mechanistic studies and may be co-cultured. They may also be genetically manipulated to express proteins of interest, and can be cryopreserved. As with all primary cell cultures, their disadvantage is they may have lost a variety of specific cell functions and have an unstable genotype.

One organ of particular importance to toxicological research is the liver. The use of *in vitro* hepatic systems for toxicity studies has received increasing attention in recent years (Hasspieler *et al.*, 1996; Andreoli *et al.*, 2003; Tahti *et al.*, 2003; O'Brien *et al.*, 2004; Bakand *et al.*, 2005; Bhogal *et al.*, 2005; Dambach *et al.*, 2005; Farkas and Tannenbaum, 2005; Hayashi, 2005). These have been used advantageously in hepatocyte-based cytotoxicity and genotoxicity assays *in vitro*. One human hepatic cell line, HepG2, provides a convenient and sensitive tool for the rapid screening of environmental samples for potential genotoxic and cytotoxic effects (Hasspieler *et al.*, 1996). The human hepatocyte, HepG2, retains many functions of the normal hepatocyte (liver cell), including the synthesis and secretion of hepatic-specific proteins (Adeli and Sinkevitch, 1990), and expression of xenobiotic-metabolizing enzymes (Dufresne *et al.*, 1993), and has been used extensively for many biological studies. Our laboratory previously used the HepG2 cell culture system to develop a number of toxicity bioassays, in order to assess both acute cytotoxicity as well as genotoxic potential, as assessed by measurement of DNA strand breaks (Ali *et al.*, 1994), and DNA repair activity (Hasspieler *et al.*, 1995).

Genetic approaches to measuring toxicological effects are becoming increasingly popular, as our expertise in this area of technology quickly advances. Over the past decades, many *in vitro* assays have been used to assess the genotoxic effects of xenobiotics, such as heavy metals, on target organisms. For example, bacterial assays, such as the *Salmonella* mutagenicity assay (Ames *et al.*, 1975), have been used, not only for fundamental studies of mutagenesis, but also for the screening of environmental samples for potential genotoxicity. The methods used in this test system have been extensively reviewed elsewhere (Claxton *et al.*, 2004; Chung *et al.*, 2006). Several mammalian cell lines have also been used to investigate the mechanisms of chemical carcinogenesis, and as bioassay systems for monitoring environmental genotoxins (Maurici *et al.*, 2005; Ohno *et al.*, 2005; Fernandes *et al.*, 2005; Lambert *et al.*, 2005).

Of the various endpoints that have been used as indices of genotoxic insult, the formation of DNA single-strand breaks (SSB) has experienced increasing use. This trend may be attributed to the relatively high sensitivity of the SSB response to xenobiotic exposure, as well as to the toxicological sequelae that are associated with the SSB response, including clastogenesis, heritable mutations, and cancer. The methods of quantifying SSBs are generally based on exposing the DNA strand to alkaline conditions (pH > 11.5), so that unwinding of the helix occurs at the SSB sites. If an appropriate, fixed period of unwinding is used, the formation of single-strand (SS) DNA will be proportional to the number of 'alkali-labile' break sites present. At least four procedures have been developed to assess SSBs including: (1) alkaline unwinding and DNA quantification using a fluorescent DNA-binding stain (Hoechst 33258) (Shugart, 1998); (2) alkaline elution of SS DNA using a porous membrane (Kohn *et al.*, 1980), where the DNA is quantified radiometrically using cells that are prelabeled in culture with [<sup>3</sup>H]thymidine; (3) the single-cell gel electrophoresis assay (Singh *et al.*, 1988); and (4) hydroxylapatite DNA chromatography (Daniel *et al.*, 1985). We have previously improved upon this latter method for use with human cells in culture (Hasspieler *et al.*, 1995).

Another approach to assessing genotoxicity is monitoring DNA repair activity in cells after genotoxic insult. The most widely used approach involves quantifying unscheduled DNA synthesis (UDS), which indicates the repair of DNA lesions. UDS assays are based on the incorporation of radiolabeled nucleotides (commonly [<sup>3</sup>H]thymidine) into the DNA of cells that are not undergoing replicative (scheduled) DNA synthesis. The two general methods for quantifying [<sup>3</sup>H]thymidine incorporation are (1) autoradiography (Dusenbery and Lee-Chen, 1988), and (2) liquid scintillation counting (LSC) (Martin *et al.*, 1978). In the interest of developing methods to assess genotoxicity in ways that are sensitive and rapid, our laboratory has optimized the LSC-based UDS assay for use in human cultured cell lines, following the procedures described by Martin *et al.* (1978). This technique differs from the autoradiography assay in that the cellular DNA is assessed in a batchwise manner rather than by the visual examination of individual cells (Martin *et al.*, 1978).

This study focuses on the application of a panel of *in vitro* human cell bioassays to provide a toxicological assessment of a number of industrial solvents that are currently in common use worldwide. A combination of assays that measure acute cytotoxicity and enzyme induction, together with genotoxic assays that assess DNA damage and repair induction, should allow for a comprehensive assessment of both short- and long-term toxicity potential. Data presented from the screening of 16 industrial solvents supports the potential use of such a test battery for mass screening of suspected environmental toxicants, providing useful information for human health risk assessment.

## Materials and methods

### Materials

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cell culture media were obtained from Life Technologies (Burlington, Canada). [<sup>3</sup>H]Thymidine was obtained from ICN Biomedicals (Mississauga, Canada). Tissue culture equipment was obtained from Corning Inc. (New York, NY, USA). NTB-2 emulsion was purchased from Eastman Kodak

(Rochester, NY, USA). Hydroxylapatite gel (DNA grade) was obtained from Bio Rad Laboratories (Richmond, CA, USA). All other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Table 1 lists the chemicals tested, their CAS numbers, and structural formulas.

### Cell culture

HepG2 monolayer cell cultures were maintained in  $\alpha$ -Minimal Essential Medium (MEM) (supplemented with 5% fetal bovine serum (FBS), 0.1 U/L penicillin (base), 100 mg/L streptomycin (base), and 0.25 mg/L amphotericin B) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. The cell lines were subcultured weekly by digesting the cells with 0.25% Trypsin/1 mM sodium EDTA (Gibco) at 37°C for 7 min. The cells were then separated by four passages through a 20-gauge needle and dilution in complete medium. Culture plates were inoculated with the appropriate number of cells to reach confluence within a given time period. The culture medium was replaced with fresh medium every two to three days.

### Neutral red uptake assay for cytotoxicity

The cytotoxicity of a test compound was quantified using the neutral red dye uptake (NRU) assay, as described previously (Hasspieler *et al.*, 1996). Approximately  $3 \times 10^4$  cells in 0.2 mL complete medium were added to each well of a 96-well tissue-culture microtiter plate. The cell cultures were incubated at 37°C for 24 h and then treated with fresh medium containing various concentrations of a test compound delivered in solvent (eg, acetone, DMSO). Solvent alone (to a final concentration of 1% (v/v)) was used as a negative control. The cells were then incubated for a given time period (eg, 0–24 h), and then the treatment medium (pre-incubated overnight at 37°C) containing 50 mg/L NR was introduced. After incubation for another 3 h to allow uptake of the dye into viable cells, the cells were immediately washed with a fixative, and the NR dye was extracted. After a brief agitation on a microtiter plate shaker, the absorbance of the extracted dye was measured using a Dynatech MRX spectrophotometric plate reader (Dynatech Laboratories Inc., Chantilly, VA, USA) with a 540-nm filter.

**Table 1.** Chemicals quantitatively evaluated

Chemical name	Trade name	CAS No(s)	Structural formula
Isopropylbromide	Several	75-26-3	C3H7Br
<i>n</i> -Propylbromide	Several	106-94-5	C3H7Br
<i>n</i> -Propylbromide (stabilized)	EnSolv	106-94-5	C3H7Br
Perchloroethylene	Several	127-18-4	C2Cl4
trans-1,2-Dichloroethylene	Several	540-59-0	C2H2Cl2
Trichloroethylene	Several	79-01-6	C2HCl3
1,1,1,2,3,4,4,5,5,5-Decafluoropentane/ trans-1,2-dichloroethylene	VERTREL MCA	NA/540-59-0	C5F10H2/C2H2Cl2
1,1,1,2,3,4,4,5,5,5-Decafluoropentane/ trans-1,2-dichloroethylene/methanol	VERTREL SMT	NA/540-59-0/67-56-1	C5F10H2/C2H2Cl2/CH2OH
1,1,1-Trichloroethane	Several	71-55-6	C2HCl3
3,3-Dichloro-1,1,1,2,2-pentafluoropropane/ 1,3-dichloro-1,1,1,2,2-pentafluoropropane	DPF	422-56-0/NA	C3Cl2HF5 (two isomers)
3,3-Dichloro-1,1,1,2,2-pentafluoropropane/ 1,3-dichloro-1,1,1,2,3-pentafluoropropane	AK225ca/cb	422-56-0/507-55-1	C3Cl2HF5 (two isomers)
Methoxy-nonafluorobutane	HFE-7100	163702-08-7/163702-07-6	Two isomers: (CF3)2CF2CF2OCH3/ CF3CF2CF2CF2OCH3
Ethoxynonafluorobutane	HFE-7200	163702-06-5/163702-05-4	Two isomers: (CF3)2CF2CF2OC2H5/ CF3CF2CF2CF2OC2H5
Methylnonafluorobutylether/ethylnonofluorobutylether/ trans-1,2-dichloroethylene	HFE-72DE	NA/NA/540-59-0	C4F9OCH3/C4F9OCH3/C2H2Cl2
Methylnonafluorobutylether/trans-1,2-dichloroethylene	HFE-71DE	NA/540-59-0	C4F9OCH3/C2H2Cl2
Methylnonafluorobutylether/ trans-1,2-dichloroethylene/ethanol	HFE-71DA	NA/540-59-0/64-17-5	C4F9OCH3/C2H2Cl2/CH2OH

NA, not available.

### Ethoxyresorufin *O*-deethylase (EROD) bioassay

The EROD bioassay presented here is a modification of the method described by Pohl and Fouts (1980). HepG2 cells were grown to confluence in 96-well tissue culture microtiter plates and then treated with test compounds for 24 h under standard culture conditions. After a 24-h exposure period, the cells were washed and preincubated for 5 min in medium containing 40  $\mu$ M digitonin (to permeabilize cells), glucose 6-phosphate dehydrogenase, and ethoxyresorufin (8  $\mu$ M). The reaction was started by the addition of glucose 6-phosphate (5 mM) and NADPH (0.5 mM). EROD, measured as an increase in fluorescence, was monitored at 37°C in a Labsystems Fluoroskan II plate reader, at the respective excitation and emission wavelength optima of 538 and 591 nm. The rates of resorufin production were determined using resorufin standards, and the protein content for the same microtiter plates was measured using a modification of the method described by Lowry *et al.* (1951), using bovine serum albumin in incubation medium as standard. To each microtiter well containing cells and medium, sodium dodecyl sulfate was added to solubilize protein, and the plates were shaken on an orbital shaker. The solubilized protein from each microtiter well was transferred to corresponding wells in a new plate containing 200  $\mu$ L of premixed copper/tartrate reagent. The plates were shaken, diluted Folin reagent was added to each well, and the plates were shaken at room temperature. The absorbance at 650 nm was read in a spectrophotometric plate reader (MRX, Dynatech Laboratories Inc.). The standard protein assay was simultaneously used to calculate protein content. The specific enzyme activity was expressed as pmol resorufin produced/mg protein/min.

### DNA SSB assay

The procedure used for analysing DNA SSB is a modification of the procedure described by Hasspieler *et al.* (1995). HepG2 cells were grown to confluence in 24-well (16 mm diameter) tissue culture plates containing complete medium supplemented with 50 nCi/mL [<sup>3</sup>H]thymidine. The cells were later treated for a given period of time with test compounds, as described above.

After the treatment period, the treatment medium was replaced, and immediately followed by alkalization with sodium hydroxide. After this alkaline unwinding period, the samples were neutralized with hydrochloric acid, followed by the immediate addition of 2% sodium lauryl sarcosinate/20 mM EDTA. The plates were then sonicated and the cell lysates stored at 4°C until hydroxylapatite chromatography. Hydroxylapatite chromatography was performed using a circulating, heated water bath that was fitted with 24 disposable columns, with the thermostat set at 60°C. The hydroxylapatite gel was prepared as a slurry containing 2.5 g gel suspended in 25 mL potassium phosphate buffer (PB). The slurry was mixed, heated to boiling, allowed to cool, and then the gel slurry (1 mL) was combined with each cell lysate. The mixtures were incubated at 60°C, poured into individual columns, and the columns were washed. The SS DNA fraction and the DS DNA fraction were eluted, and each DNA fraction was combined with concentrated hydrochloric acid and the samples were digested. Each digested sample was combined with scintillation cocktail and the radioactivity was measured in a  $\beta$ -liquid scintillation counter. Data are expressed as *F*-values, corresponding to the radioactivity (disintegrations/min) in the DS fraction divided by the sum of the radioactivity in the SS and DS fractions.

### UDS-DNA repair assay

To minimize scheduled (replicative) DNA synthesis, HepG2 cells were grown to confluence in 35-mm Petri dishes, maintained at this stage for four days in 0.5% FBS, and then incubated for 1 h with hydroxyurea, to inhibit residual scheduled DNA synthesis. Test compounds were added to the cells in solvent (to a final concentration of 1%) together with [<sup>3</sup>H]thymidine. After incubating for a given time period, the cells were collected onto 0.6  $\mu$ m Sartorius filters, and washed successively with PBS, 5% trichloroacetic acid, and 95% ethanol. The filters were air-dried, combined with scintillation cocktail, and the radioactivity was measured in a  $\beta$ -liquid scintillation counter. In this assay, an increase in the incorporation of [<sup>3</sup>H]thymidine over background levels indicated the induction of UDS.

## Results

In the present study, we screened a number of industrial solvents for potential short-term cytotoxicity and long-term genotoxic potential using a battery of four human cell bioassays. Two bioassays were developed to assess the carcinogenic and mutagenic potential of suspected toxicants at the cellular and DNA levels. These assays were combined with two other bioassays that measure cytotoxicity as well as enzyme induction, and this four-test battery was used to screen a number of industrial solvents for acute and chronic toxicity potential.

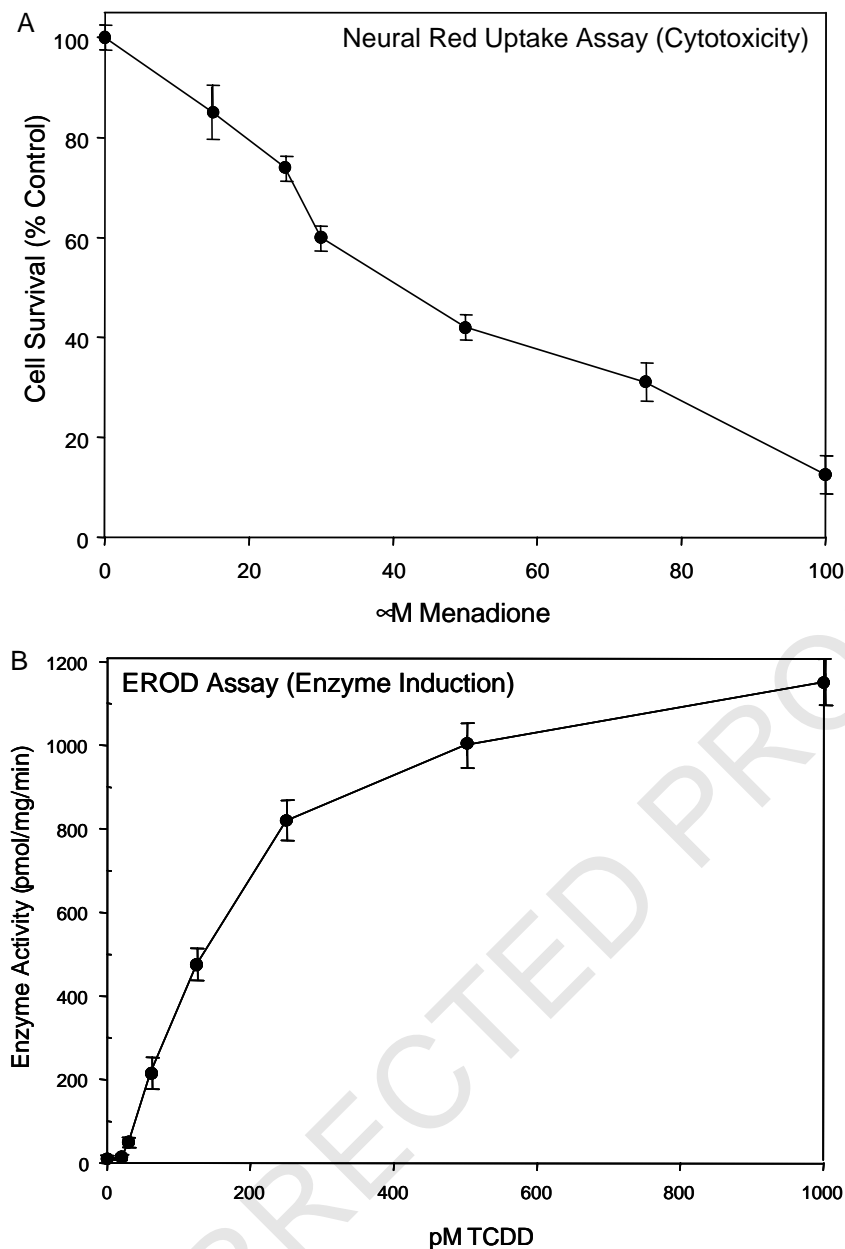
The cell death/cytotoxicity bioassay is based on the ability of normal, healthy cells to take up nutrients. A toxic response lowers this rate of uptake, indicating loss of cell viability. The positive control used for this test is menadione, which is known to be cytotoxic and affects, among other things, calcium transport across the cell membrane. The test introduces a non-toxic dye into the culture medium, which is then taken into living cells, but not into damaged or dead cells. Toxicity is quantified by extracting the dye from the cells, measuring the absorbance with a spectrophotometer, and comparing the results to the positive control. Figure 1(A) shows a typical standard curve generated using different concentrations of menadione to induce cytotoxicity assessed by decreasing uptake rate of NRU. This is a highly reproducible assay with intra-assay (within-run) and inter-assay (between-day) imprecision of 8.6 and 8.5%, respectively, at 18.8  $\mu\text{M}$  menadione. The lowest concentration of menadione that showed a toxic effect on cells was 15.6  $\mu\text{M}$ .

The altered enzyme function bioassay is based on the increased enzyme activity, primarily mixed function oxidases (MFOs), that often results from exposure to toxic chemicals. The positive control for this test is 2,3,7,8-TCDD (dioxin), which is known to induce MFO activity at very low levels. The specific MFO activity measured in the test focuses on the CYP1A1/2 P450 enzymes. The test measures enzyme activity with a fluorometer after 24-h exposure to the chemical of interest. Toxicity is quantified by comparison with the positive control. Figure 1(B) shows a typical standard curve generated using different

concentrations of TCDD to induce EROD. The assay is very reproducible with intra-assay (within-run) and inter-assay (between-day) imprecision of 5.6 and 13.6%, respectively, at 60 and 115.0 pM TCDD ( $N=24$  for both studies). The lowest concentration of TCDD to consistently show a statistically significant increase in EROD activity over the negative control was 15.6 pM.

The DNA damage bioassay evaluates the ability of a chemical to cause damage to DNA by monitoring strand breakage. The positive control for this test is 4-nitroquinoline *N*-oxide, a known mutagen that induces SSBs. For this test, the cells are labeled with radioactive DNA, exposed to the chemical, and then analysed. Analysis involves separating SS DNA (ie, broken strands) from double-stranded (DS) DNA (ie, intact DNA), and then counting the radioactivity of both types of DNA in a liquid scintillator. Toxicity is quantified by comparing the ratio of SS to DS DNA to the positive control. Figure 2(A) shows a typical standard curve generated using different concentrations of 4-NQO. This assay was optimized to ensure precision and typically has intra-assay (within-run) and inter-assay (between-day) imprecision of 4.8 and 7.1%, respectively ( $N=25$  for each study). The lowest concentration of 4-NQO that showed a statistically significant genotoxic effect on cells was 125 nM. This sample concentration yielded an average *F* value of 89.9%, compared to 96.8% for the control.

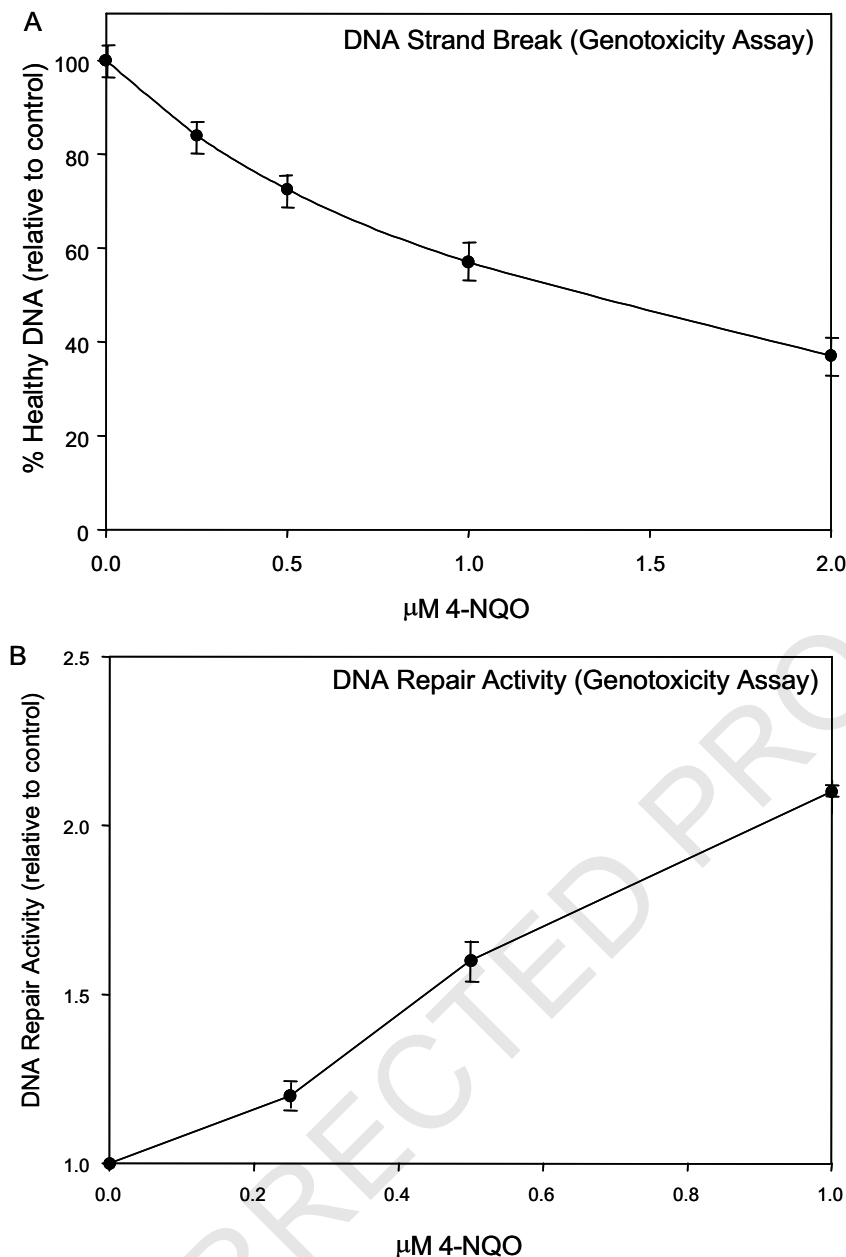
The DNA repair bioassay evaluates the ability of a chemical to both damage and repair DNA. This activity is often an indicator of mutagenicity. Repair of damaged DNA is measured by the test. The positive control for this test is the same as for the DNA damage bioassay. In this test, the incorporation of healthy nucleotides at a previously damaged DNA site is measured. When combined with the DNA strand break assay above, this test provides an indicator of the genotoxic potential of a chemical. Figure 2(A) shows a typical standard curve generated using different concentrations of 4-NQO. This assay was optimized to ensure precision and typically has intra-assay (within-run) and inter-assay (between-day) imprecision of 5.5 and 8.3%, respectively.



**Figure 1.** Dose–response curves for the neural red dye uptake and EROD assays in HepG2 cells. (A) Effect of menadione on HepG2 cells in the neural red uptake assay for cytotoxicity. Vertical axis represents absorbance at 540 nm expressed as percent of solvent-treated controls. (B) Effect of TCDD on EROD activity in HepG2 cells. A typical profile of percent maximum EROD activity versus TCDD concentration is shown. The dose–response relationship is readily evident from these plots. Vertical bars represent standard error of the mean of four replicates.

Once all four human cell bioassays were fully optimized, this battery of assays was used to screen a number of industrial solvents. A series of comparative human cell bioassay tests was carried out on 16 solvents, and the results of these tests were interpreted in terms of relative toxicity. The comparative human cell bioassay tests were

carried out for two groups of compounds. The first group included *n*-propyl bromide (nPB), stabilized nPB, 1,1,1-trichloroethane (TCA), trichloroethylene (TCE), perchloroethylene (PCE), and isopropyl bromide (iPB). The second group of solvents included a mixture of 3,3-dichloro-1,1,1,2,2-pentafluoropropane and 1,3-dichloro-1,1,



**Figure 2.** Dose–response curves for the DNA break and DNA repair assays. (A) Effect of 4-NQO on HepG2 cells in the DNA single stranded break assay for genotoxicity. Vertical axis represents percent of healthy DNA calculated based on the *F* value (ratio of single stranded to double stranded DNA) at each concentration of 4-NQO. (B) Effect of 4-NQO on DNA repair activity in HepG2 cells. Liquid scintillation assay for unscheduled DNA synthesis was performed in the presence of different concentrations of 4-NQO. Vertical axis represents [<sup>3</sup>H]thymidine uptake relative to solvent-treated controls. Vertical bars represent standard error of the mean of four replicates.

1,2,2-pentafluoropropane (AK225ca/cb), 3,3-dichloro-1,1,1,2,2-pentafluoropropane/1,3-dichloro-1,1,1,2,2-pentafluoropropane (DPF), a mixture of methyl nonafluorobutyl ether, ethyl nonafluorobutyl ether and trans-1,2-dichloroethylene (HFE-72DE); methoxy-nonafluorobutane (HFE-7100), ethoxy-nonafluorobutane (HFE-7200), a hydro-

fluoroether, methylnonafluorobutyl ether in an azeotrope formulation with trans-1,2-dichloroethylene (HFE-71DE), a hydrofluoroether, methylnonafluorobutyl ether in an azeotrope formulation with trans-1,2-dichloroethylene and ethanol (HFE-71DA), 1,1,1,2,3,4,4,5,5,5-decafluoropentane, trans-1,2-dichloroethylene and stabilizer

(VERTEL SMT), 1,1,1,2,3,4,4,5,5,5-decafluoropentane, trans-1,2-dichloroethylene (VERTEL MCA), and trans 1,2-dichloroethylene (TDE).<sup>1</sup>

Both nPB and a stabilized nPB product (EnSolv<sup>®2</sup> stabilized nPB) were tested to evaluate the comparative toxicity of the compound and a stabilized formulation. Other stabilizing formulations for nPB exist, but were not tested. Since the components of these other stabilizing formulations vary widely from those used in the EnSolv formulation, extrapolation from the results of the EnSolv stabilized nPB to any product with a different stabilizing formulation is not warranted or advised.

Four tests for all compounds tested were run using a range of tissue concentrations from 25 to 500 ppm (Table 2). Figures 3–5 show the results of human cell bioassays for three of the 16 compounds (nPB, iPB, and TCE). Each chemical was tested using all four bioassays. The results for the 16 chemicals are summarized in Table 2. Graphs are not shown for the remaining chemicals to limit the number of figures shown to 5. The tissue concentrations tested reflect higher actual workplace air concentrations because only a portion of chemicals in inhaled air are actually absorbed, and then only a fraction of the chemical mass actually absorbed reaches target tissues and cells. Therefore, these tissue concentrations provide a good biomarker of the potential toxicity of the chemical product in humans exposed to them in the workplace. The sensitivity of these tests was sufficient to identify impacts due to nPB at low concentrations that are environmentally meaningful. The sensitivity of these tests ranged from 0.02 ng/m<sup>3</sup> (0.00004 parts per million (ppm)) for the EROD enzyme induction test, to 0.02 mg/m<sup>3</sup> (0.004 ppm) for the cytotoxicity assay.

## Discussion

There are a number of important general considerations to take into account when choosing a

system and method to measure *in vitro* toxic effects (Tiffany-Castiglioni *et al.*, 1999). If permanent cell lines are used (which have both technical and economic advantages), the observations and conclusions made may differ greatly from what actually occurs *in vivo* after toxicant exposure. Many continuous cell lines are hardy, and may not show realistic exposure effects unless they are subjected to unusually high toxicant concentrations. Continuous lines do not exhibit the usual cellular stages of development. When primary cell cultures are used, batch-to-batch cellular variability may influence observed toxicant responses. If tissue slices are used, it is important to consider the method by which they are prepared. Cell–cell interactions may also be crucial to toxic effects, and should be taken into consideration when a test system is selected. Cell–cell interactions between different cell types may be implicated in toxic effects. Concentrations of toxicants that are effective *in vivo* may be very different than those relevant *in vitro*. If the results obtained from *in vitro* studies are to be meaningful, they must mimic as closely as possible those conditions present *in vivo*. It is important to generate both time and dose response curves to cover a variety of scenarios and gain meaningful information. It is also important to note that *in vitro* systems only allow monitoring of short-term effects and that a clear understanding of the advantages and limitations of such *in vitro* systems will need to be considered when interpreting data generated from *in vitro* toxicological assessments.

We believe this study is the first time these solvents have been subjected to an identical set of *in vitro* cell bioassay tests on human cells. This is one of the important functions of this testing, as it affords a basis for comparing the anticipated relative human toxicity of these solvents. What follows is a critical analysis of relative toxicity, based strictly on the outcome of the human bioassay tests.

Six chemicals were positive in three of the four tests (isopropyl bromide, AK225ca/cb, DPF, HFE-72DE, Vertrel SMT, and Vertrel MCA). Four chemicals were positive in only one of the four tests (TCA, *n*-propylbromide, EnSolv, and HFE-7100). The other six were positive in two of the four tests. For the purposes of discussion, it was

<sup>1</sup>AK225ca/cb is a registered trademark/tradename of Asahi Glass Company. HFE-71DA, HFE-71DE, HFE-72DE, HFE-7100 and HFE-7200 are tradenames and/or trademarks of 3M Corp. Vertrel, VERTEL MCA and VERTEL SMT are trademarks and tradenames of DuPont, Inc.

<sup>2</sup>EnSolv<sup>®</sup> is a registered trademark of Enviro Tech International, Inc.

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B Hasspieler *et al.*

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**Table 2.** Toxicological assessment of organic solvents using human cell bioassays

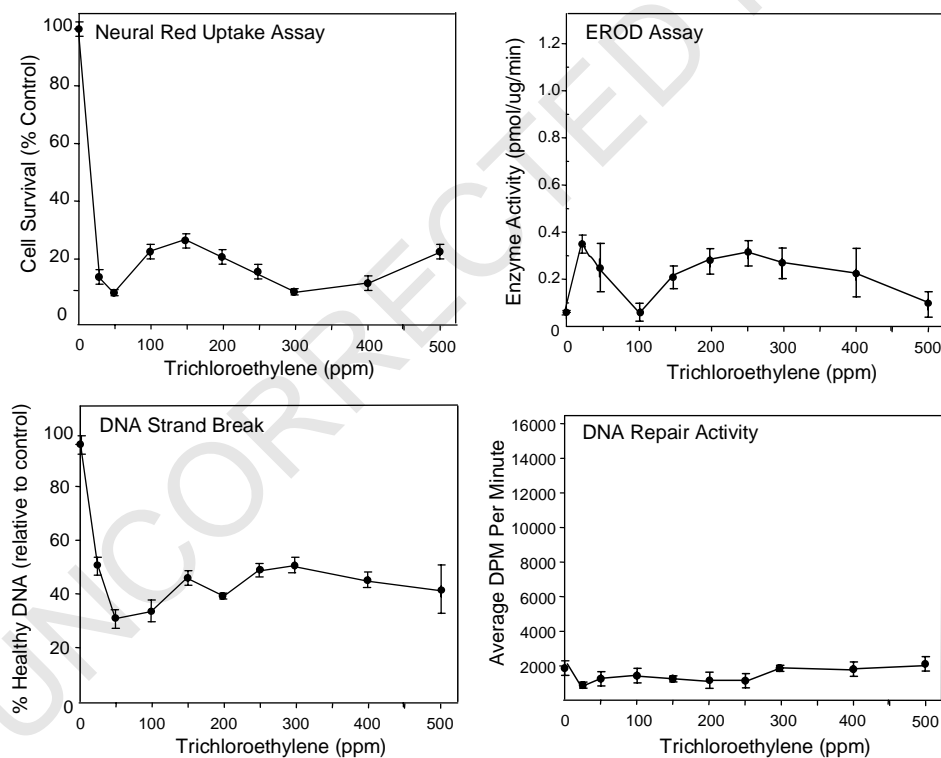
	Cytotoxicity	EROD	DNA strand break	DNA repair activity
TCE	Positive (>25 ppm)	Negative (at 25–500 ppm)	Positive (>25 ppm)	Negative (at 25–500 ppm)
PCE	Positive (>25 ppm)	Negative (at 25–500 ppm)	Positive (>25 ppm)	Negative (at 25–500 ppm)
TCA	Negative (at 25–500 ppm)	Negative (at 25–500 ppm)	Negative (at 25–500 ppm)	Positive (>25 ppm)
<i>n</i> -Propyl bromide (nPB)	Positive (at 500 ppm)	Negative (at 25–500 ppm)	Negative (at 25–500 ppm)	Negative (at 25–500 ppm)
EnSolv® (stabilized nPB)	Positive (at 500 ppm)	Negative (at 25–500 ppm)	Negative (at 25–500 ppm)	Negative (at 25–500 ppm)
Isopropyl bromide	Positive (at >150 ppm)	Negative (at 25–500 ppm)	Positive (at >150 ppm)	Positive (at >150 ppm)
AK225ca/cb	Positive (at >100 ppm)	Positive (at >200 ppm)	Negative (at 25–500 ppm)	Positive (at 500 ppm)
DPF	Positive (at >200 ppm)	Positive (at 500 ppm)	Negative (at 25–500 ppm)	Positive (at 500 ppm)
HFE-72DE	Positive (>25 ppm)	Negative (at 25–500 ppm)	Positive (>25 ppm)	Positive (at >200 ppm)
HFE-7100	Positive (>25 ppm)	Negative (at 25–500 ppm)	Negative (at 25–500 ppm)	Negative (at 25–500 ppm)
HFE-7200	Positive (>100 ppm)	Negative (at 25–500 ppm)	Positive (>200 ppm)	Negative (at 25–500 ppm)
HFE-71DE	Positive (>25 ppm)	Negative (at 25–500 ppm)	Positive (>100 ppm)	Negative (at 25–500 ppm)
HFE-71DA	Positive (>25 ppm)	Negative (at 25–500 ppm)	Positive (>25 ppm)	Negative (at 25–500 ppm)
VERTEL SMT	Positive (>25 ppm)	Negative (at 25–500 ppm)	Positive (>25 ppm)	Positive (>200 ppm)
VERTEL MCA	Positive (>25 ppm)	Negative (at 25–500 ppm)	Positive (>25 ppm)	Positive (>100 ppm)
TDE	Positive (>25 ppm)	Negative (at 25–500 ppm)	Positive (>25 ppm)	Negative (at 25–500 ppm)

Each solvent was tested at various doses of 25–500 ppm using each of four cell bioassays in HepG2 cells to assess cytotoxicity, EROD activity, DNA strand breakage, and DNA repair activity. A test was scored as positive when percent change in activity was statistically different from the negative control ( $P < 0.05$ ).

assumed that relative toxicity could be established on the combined basis of the number of positive tests and the concentrations at which significant results were obtained. On this basis, the six

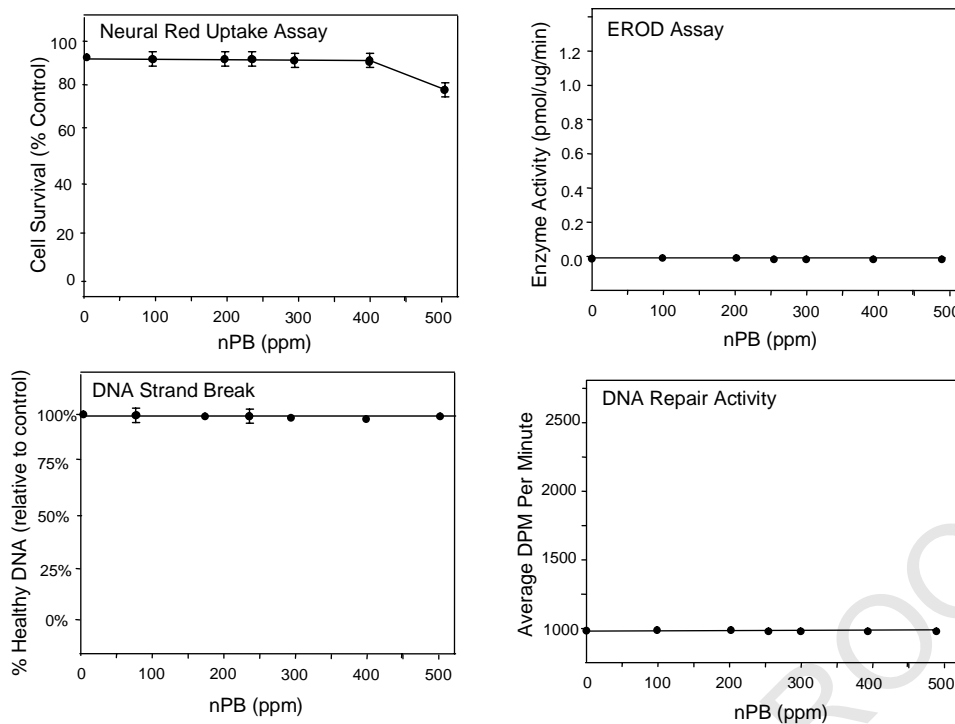
chemicals that were positive in three of the four tests would be considered most toxic. Based on the relative concentrations in the three positive tests across these chemicals, the comparative relative

### Trichloroethylene (TCE) Screen



**Figure 3.** Dose–response curves for trichloroethylene (TCE) in the neural red dye uptake, EROD, and DNA break and repair activity assays. The effect of TCE at concentrations of 25–500 ppm was tested on HepG2 cells using the four cell bioassays. (A) Neutral red dye uptake assay, (B) EROD assay, (C) DNA stand break assay, and (D) DNA repair activity assay. Positive controls (4-NQO and TCDD) were run in parallel with each chemical to validate each of the four assays. Vertical bars represent standard error of the mean of four replicates.

## 1-Bromopropane (nPB) Screen



**Figure 4.** Dose–response curves for 1-bromopropane (nPB) in the neural red dye uptake, EROD, and DNA break and repair activity assays. The effect of nPB at concentrations of 25–500 ppm was tested on HepG2 cells using the four cell bioassays. (A) Neutral red dye uptake assay, (B) EROD assay, (C) DNA strand break assay, and (D) DNA repair activity assay. Positive controls (4-NQO and TCDD) were run in parallel with each chemical to validate each of the four assays. Vertical bars represent standard error of the mean of four replicates.

toxicity across these six tested compounds is as follows: Vertrel MCA (most toxic) > Vertrel SMT and HFE-72DE > iPB > AK225ca/cb > DPF.

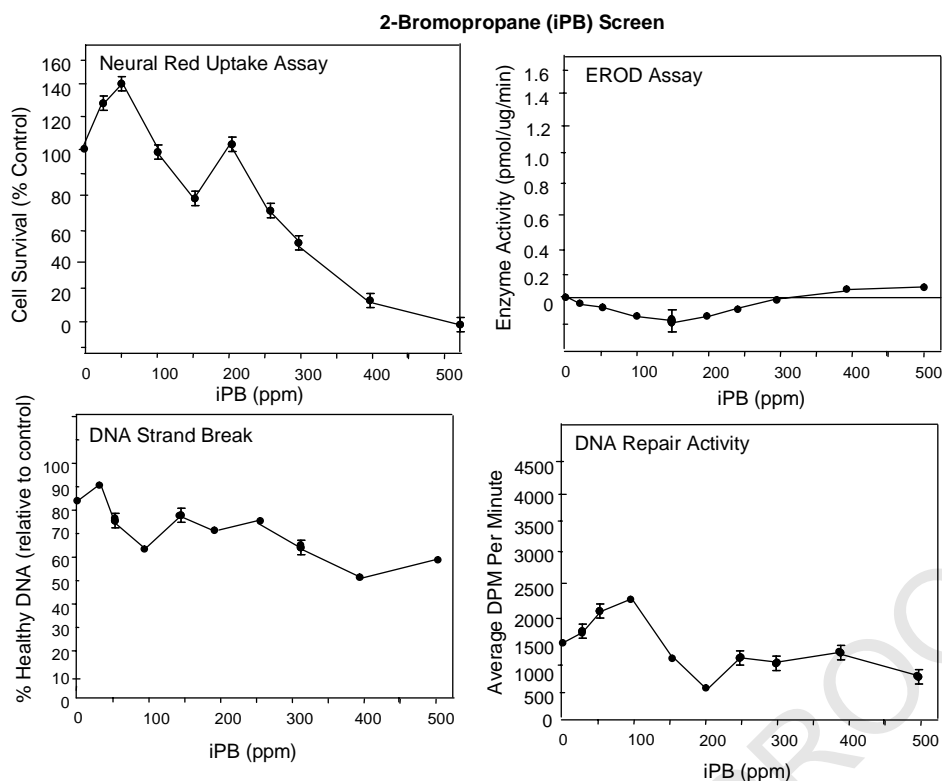
For the group of chemicals that tested positive in two of the four assays, the comparative relative toxicity, using the same concentration scale, is as follows: TCE = PCE = TDE = HFE-71DA > HFE-71DE > HFE-7200. Finally, for the group of chemicals that tested positive in only one of the four assays, the comparative relative toxicity is estimated as follows: HFE7100 > TCA > nPB and stabilized nPB (least toxic). nPB, stabilized nPB, and HFE-7100 were positive only for cytotoxicity, and TCA was positive only in the DNA repair assay. While nPB and stabilized nPB were positive at only the highest tested dose (500 ppm), HFE-7100 and TCA were positive at all dose levels except 25 ppm. TCA was ranked lower in toxicity than HFE-7100 because cytotoxicity was considered a more direct toxic effect than DNA repair activity, considering the effects occurred at the same dose levels. nPB and stabilized nPB were considered least toxic

because the only positive result was at the highest tested dose level. This was not the case for the other 14 solvents. The tests showed that a particular set of compounds, comprising the stabilizing formulation (about 7% of the total mixture for EnSolv<sup>®</sup>) added to nPB, had no effect on overall toxicity. Other formulations of nPB were not tested. nPB was also negative in standard genotoxicity tests conducted elsewhere (Elf Atochem, 1994). In addition, the human bioassay testing clearly indicates that nPB, although a structural analog of iPB, has neither the mutagenic nor inherent toxicity indicated by the test for iPB. The chlorinated solvents were tested only in their neat form due to the wide variety of stabilizing compounds used with these materials.

Of those solvents comprised of a single active chemical constituent, iPB appears to have the most potential for genotoxicity, inducing DNA strand breaks and repair activity at concentrations > 150 ppm. TDE was positive for strand breakage at a lower concentration, but was negative for repair activity. The genotoxic potential of TDE

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**Figure 5.** Dose–response curves for 2-bromopropane (iPB) in the neural red dye uptake, EROD, and DNA break and repair activity assays. The effect of iPB at concentrations of 25–500 ppm was tested on HepG2 cells using the four cell bioassays. (A) Neutral red dye uptake assay, (B) EROD assay, (C) DNA strand break assay, and (D) DNA repair activity assay. Positive controls (4-NQO and TCDD) were run in parallel with each chemical to validate each of the four assays. Vertical bars represent standard error of the mean of four replicates.

appears to be enhanced when combined with nonafluorobutyl ether and ethyl nonafluorobutyl ether (ie, HFE-72DE), resulting in both DNA strand breaks as well as DNA repair induction. TDE tested negative in other standard genotoxic tests, but the *cis*-isomer of 1,2-dichloroethylene tested positive (Agency for Toxic Substances and Disease Registry, 1996). HFE-7100 and HFE-7200 exhibited significant potential for cytotoxicity, but only ethoxy-nonafluorobutane showed activity in the DNA break assay. Of the products containing mixtures of active chemicals, AK225ca/cb induced cytotoxicity, DNA strand breaks, and DNA repair induction, and DPF induced cytotoxicity, EROD activity, and DNA repair induction, but generally at higher concentrations than other mixtures (500 ppm).

Use of human *in vitro* bioassays provides an indication of potential chemical toxicity. Such tests evaluate cellular biomarkers for potential toxicity. Chemical activity *in vitro* cannot be assumed to completely reflect *in vivo* effects, but provides

critical information when attempting to relate the results of whole body animal toxicity tests to humans. The US Environmental Protection Agency (USEPA) has incorporated *in vitro* data into their revised approach to evaluate potential carcinogens (United States Environmental Protection Agency, 2005). They state that ‘[t]he application of techniques for measuring cellular and molecular alterations due to exposure to specific environmental agents may allow conclusions to be drawn about the mechanisms of carcinogenesis.’

Although specific in this case to carcinogenic analysis, this indicates that the USEPA has realized that human cellular studies can provide important information when evaluating the potential human toxicity of a chemical. USEPA further states that ‘... cells and tissues [can] serve as biomarkers of exposure in both animals and humans.’ Such tests can ‘... aid in interspecies extrapolations when data are available from both experimental animal and human cells’. Clearly, use of *in vitro* bioassays alone does not provide conclusive evidence regarding

potential whole body toxicity in humans. As the USEPA also says '... although important information can be gained from *in vitro* test systems, a higher level of confidence is generally given to data that are derived from *in vivo* systems. However, such *in vitro* tests can provide useful information when included as part of an overall weight-of-evidence analysis for a chemical'.

Such a weight-of-evidence analysis is provided below for all tested solvents. The solvents containing only a single active ingredient included two chemicals with no toxicity-based standards (nPB and iPB), and four other solvents with toxicity-based standards (TCA, TDE, TCE, PCE). The latter two chemicals are suspected human carcinogens and all four have known non-cancer toxicity, especially on the liver. Based on the USEPA human health-based criteria (eg, reference doses), the non-cancer potency of these four chemicals can be ranked from high to low as follows: TCE > PCE > TDE > TCA.

Results obtained for all six solvents tested at seven dose levels ranging from 25 to 500 ppm are summarized in Table 2. Based on these results, it may be concluded there is no significant toxicity posed by nPB or stabilized nPB at concentrations in the range 100–400 ppm. These results indicate that TCE, TDE, and PCE are similarly toxic of the four tested chlorinated chemicals, and TCA is less toxic, which is consistent with the USEPA's ranking of these chemicals. Although TCA is not a suspected human carcinogen, it was positive in the DNA repair bioassay. This result suggests that TCA has an inherent induction effect on DNA repair activity. As this activity does not involve DNA strand breaks (negative response on DNA break test), the positive result in DNA repair assay may not be an indicator of significant DNA damage. These data indicate that the *in vitro* results obtained from human liver cells are consistent with the relative toxicity of chemicals based on whole animal toxicity testing. Compared with these *in vitro* results, nPB has less inherent toxicity to humans than TCA, TDE, TCE, and PCE. In addition, it clearly lacks the genotoxic potential of TCE and PCE. Results for iPB, a structural analog of nPB, but with *in vivo* evidence of greater inherent toxicity than nPB, indicated that only the EROD test was negative at all dose levels. The cytotoxicity

test was positive at concentrations of 150 ppm and higher, while the two DNA-based tests were positive at concentrations of 100 ppm and higher. These results support the contention, based on standard toxicity tests and human occupational data, that iPB is mutagenic and more inherently toxic than nPB. Such results further support the likelihood that the metabolism of nPB is different to iPB. Specifically, it seems likely that the formation of radicals (ie, charged, highly reactive oxygen atoms) that can interact with DNA or impact cellular transport, either does not occur with nPB, or occurs at such a low rate that no cellular toxicity results from target tissue concentrations < 500 ppm. Again, we believe this study marks the first time these solvents have been subjected to an identical series of human cell bioassay tests.

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#### Conflict of interest statement

The bioassays discussed in this article were performed by EnviroMed Laboratories of Windsor, Canada, at the request of Enviro Tech International, Inc., which also funded the studies. Enviro Tech International, Inc., is the manufacturer of the EnSolv stabilized nPB-based solvent discussed in the article. Enviro Med Labs and Enviro-Tech International, Inc., are unrelated entities with no affiliation of any kind. Three of the authors (Bruce Hasspieler, Douglas Haffner and Khosrow Adeli) were employed by EnviroMed at the time the bioassays were conducted. In addition, SLR International Corp., an environmental consulting firm, received fees from Enviro-Tech International, Inc., to provide critical review of the bioassay results and to prepare text evaluating the relative toxicity of the first group of tested chemicals. There is no corporate relationship between Enviro-Tech and SLR. One author, Mark Stelljes, is employed by SLR International Corp.

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