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Cytotoxicity and genotoxicity of phenazine in two human cell lines

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ABSTRACT

Phenazine was recently identified as a drinking water disinfection byproduct (DBP), but little is known of its toxic effects. We examined *in vitro* cytotoxicity and genotoxicity of phenazine (1.9–123 μM) in HepG2 and T24 cell lines. Cytotoxicity was determined by an impedance-based real-time cell analysis instrument. The BrdU (5-bromo-2′-deoxyuridine) proliferation and MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assays were used to examine mechanisms of cytotoxicity. Genotoxicity was determined using the alkaline comet assay. Concentration-dependent cytotoxicity was observed in HepG2 cells, primarily due to an antiproliferative effect (BrdU 24 h IC_{50}: 11 μM; 48 h IC_{50}: 7.8 μM) observed as low as 1.9 μM. T24 cells experienced a minor antiproliferative effect (BrdU 24 h IC_{50}: 47 μM; 48 h IC_{50}: 17 μM). IC_{50} values for HepG2 proliferation and viability were 54–77% lower compared to T24 cells. In both cell lines, IC_{50} values for proliferation were 66–90% lower than those for viability. At phenazine concentrations producing equivalent cytotoxicity, HepG2 cells (1.9–30.8 μM) experienced no significant genotoxic effects, while T24 cells (7.7–123 μM) experienced significant genotoxicity at $\geqslant$61.5 μM. While these effects were seen at phenazine concentrations above those found in disinfected water, the persistence of the antiproliferative effect and the differential toxicity in each cell line deserves further study.

1. Introduction

Phenazine (PZ), an N-heterocyclic polyaromatic hydrocarbon (Fig. 1, Table 1), was recently identified as a drinking water disinfection byproduct (DBP) (Zhou et al., 2009). Phenazine and N-chlorinated phenazine were detected by LC/MS/MS after raw water containing diphenylamine was exposed to chloramine. This finding, and previous descriptions of halogen-substituted PZ derivatives (Cross et al., 1969), suggests halogen-containing disinfectants may interact with PZ, creating new halogenated DBPs. Other halogenated PZ DBPs may exist, but have not yet been identified. (*Note: Unsubstituted phenazine* (Fig. 1), *will be referred to as "phenazine" or "PZ"; substituted phenazine compounds will be referred to by their individual names or as "phenazine derivatives".*)

Although PZ was recently identified as a DBP, PZ and its derivatives are well-known secondary metabolites in many bacterial species (Turner and Messenger, 1986). Some bacteria can produce natural precursors to PZ DBPs (Zhou et al., 2009). Bacteria in drinking water distribution system biofilms (Ridgway and Olson, 1981; Wingender and Flemming, 2004), especially *Pseudomonas* and *Streptomyces* spp. (Turner and Messenger, 1986), may be capable of producing PZ or its precursors (Zhou et al., 2009). Therefore, PZ and its derivatives are unique DBPs, as several processes may contribute to their formation: first, as a chemical byproduct from the interaction of naturally occurring precursors in raw water and disinfectants; second, as a biological product from bacteria in distribution system biofilms; and third, as a product of disinfectant residuals reacting with phenazine precursors secreted by distribution system biofilms.

Little is known about the toxicity of PZ; it is mutagenic in *Drosophila* but not *Salmonella* TA98 at low micromolar concentrations (Watanabe et al., 1996). More is known about phenazine derivatives, which show antibiotic, antitumor, anti-malarial, and antiparasitic effects (Laursen and Nielsen, 2004). Pyocyanin, a phenazine derivative, is antiproliferative in human cells (Sorensen et al., 1983), and induces senescence in the A549 cell line (Muller, 2006; Muller et al., 2009). 1-Hydroxyphenazine interferes with cellular respiration by acting as an electron acceptor, preventing ATP generation (Armstrong and Stewart-Tull, 1971; Stewart-Tull and

Abbreviations: $\%DNA_{Tail}$, percent of comet DNA contained in the tail; CI, cell index; DBP, drinking water disinfection byproduct; $Length_{Tail}$, length of comet tail; PZ, unsubstituted phenazine; RTCA, real-time cell analysis; T_M, tail moment.

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Table 1
Properties of phenazine.

	CAS #	Molecular formula	Molecular weight (g/mol)	Source and purity	$\log P_{OW}$
Phenazine (PZ)	92-82-0	$C_{12}H_8N_2$	180.2090	Acros organics, 98%	3.237

Note: $\log P_{OW}$ values were calculated using Molinspiration software (www.molinspiration.com).

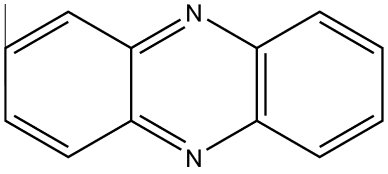

Fig. 1. Structure of phenazine (unsubstituted phenazine, PZ). Structure generated using ChemDraw Pro 13.0 (CambridgeSoft, Waltham, MA).

Armstrong, 1972). Phenazine derivative mixtures can produce reactive oxygen species *in vitro* (Davis and Thornalley, 1983). Other phenazine derivatives may penetrate cellular membranes and intercalate DNA due to their planar structure and hydrophobicity (Laursen and Nielsen, 2004; Sorensen et al., 1983).

Based on these findings, we predicted that PZ may be cytotoxic and genotoxic in mammalian cells. PZ may be present in drinking water, yet its toxicological properties are largely unknown. Therefore, we chose to examine PZ *in vitro* to determine cytotoxicity and genotoxicity, and identify potential need for future investigations (e.g. *in vivo*). (N-chlorophenazine was not examined as no chemical standards exist.) We also examined cell proliferation and viability to examine the mechanism of action of PZ cytotoxicity. HepG2 (human hepatocarcinoma) and T24 (human bladder cancer) cell lines were selected for these assays to represent potential target organs (liver and bladder) for PZ toxicity.

Cytotoxic properties of PZ were investigated using an impedance-based cell sensing technology, called real-time cell analysis (RTCA; ACEA Biosciences). RTCA provides continuous blended measurement of cell number, proliferation, and morphology in real-time, without use of dyes or labels. Adherent cells attached to gold microelectrode-lined multiwell plates are monitored hourly by administering a small electrical current to the microelectrodes. Impedance of this current by the cells is converted to a unitless measurement called "cell index" (CI) (Atienza et al., 2006). Increasing CI indicates cell proliferation, growth, and/or degree of electrode attachment; decreasing or static CI may indicate cell death, detachment, and/or lack of proliferation. Follow-up experiments to determine effects on cell proliferation (5-bromo-2'-deoxyuridine enzyme-linked immunosorbent assay (BrdU ELISA) (Muir et al., 1990; Porstmann et al., 1985)) and viability (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (Mosmann, 1983)) were conducted. (MTT was chosen to examine viability because it does not use phenazine derivatives, unlike other cytotoxicity assays (Neutral Red, XTT, etc.); this avoided confounding with exogenous phenazines.)

As some phenazine derivatives cause DNA damage (Laursen and Nielsen, 2004), PZ was tested for genotoxicity using the alkaline comet assay. To our knowledge, this is the first reported examination of PZ genotoxicity in human cell lines. The alkaline comet assay detects single- and double-stranded breaks, abasic sites, and alkali-labile adducts (Singh et al., 1988; Olive and Banath, 2006; Dhawan et al., 2009). RTCA data were used to determine appropriate PZ concentrations for the comet assay, which requires a high proportion of viable cells (Tice et al., 2000). We hypothesize that PZ will have concentration-dependent cytotoxicity to HepG2 and T24 cells, possibly through an antiproliferative mechanism. We also predict that PZ will evoke DNA damage in these cell lines under these experimental conditions.

2. Materials and methods

2.1. Cell lines and culture conditions

Human hepatocarcinoma (HepG2; HB-8065) and human bladder carcinoma (T24; HTB-4) cell lines were obtained from the American Type Culture Collection (ATCC). Cell lines were maintained in a humidified 37 °C incubator, with 5% CO_2, in Eagle's Minimum Essential Medium (ATCC; #30-2003) supplemented with 10% fetal bovine serum (Sigma; #F1051) and 1% of 10,000 U penicillin/10,000 μg streptomycin solution (Gibco; #15140-122). Cells were subcultured when confluence was 80–95%, and culture medium was refreshed at least twice per week. Cell line passage numbers were restricted (⩽15 passages per individual set of cell cultures) to minimize effects of genetic drift. All manipulations of cell cultures were performed in a biosafety cabinet (Thermo Scientific Forma Class 2 A2; #1284) under aseptic conditions. Manipulation of cell lines was performed in compliance with the University of Alberta "Working with Biohazardous Materials" policy.

2.2. Reagents and toxicant solutions

Phenazine was obtained from Acros Organics (New Jersey, USA; 130150050). DMSO was obtained from Fisher Scientific (Ottawa, Ontario; BP231-1). As phenazine is not soluble in water, a concentrated stock solution in sterile DMSO was prepared fresh for each experiment and diluted to the desired concentration in cell culture medium. A DMSO-spiked sample of culture medium, equivalent to the volume of the most concentrated PZ solution (0.1%), was used as a solvent control treatment. Treatment concentrations ranged from 1.9 to 123 μM; higher concentrations were not used due to the limited solubility of PZ in DMSO and limitations of DMSO concentrations to prevent solvent toxicity.

2.3. RTCA cytotoxicity assay

HepG2 and T24 cells were harvested using a 0.05% trypsin-0.53 mM EDTA solution (Gibco; #25300-05) and plated into 16-well RTCA E-plates at a density of 13,000 and 5000 cells per well, respectively. Both cell lines were used in each replicate, with half of the available wells plated with each cell type. Using the RTCA software (ACEA RT-CES SP v5.3, ACEA Biosciences), diagnostic assays were performed on all plates prior to use; if any well failed quality control tests, results from that well were discarded. If multiple bad wells were found on a plate, the plate was discarded. Cell index (CI) measurements were collected hourly. Cells were allowed to equilibrate in the E-plates without treatment until reaching a CI value of ~1, corresponding to ~40–50% confluence (Xing et al., 2005), within 18–24 h post-plating. Any wells showing abnormal cell growth were not used for treatment or control groups. Plates were then removed from the incubator, and culture medium was removed. Each well was treated with a 200 μL aliquot of PZ solution (1.9–123 μM), DMSO solvent control solution, or culture medium (negative control group). The treatment layout was randomized for each replicate to avoid layout-related artifacts. After treatment, plates were returned to the RTCA unit in the incubator and monitored hourly until a growth plateau had been reached, usually ~72 h post-treatment. All cell index values were "normalized" to a value of 1 at the time of treatment by the RTCA