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Investigation of nuclear enzyme topoisomerase as a putative molecular target of monohaloacetonitrile disinfection by-products

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ABSTRACT

Disinfection by-products occur widely as the unintended effect of water disinfection and are associated with toxicity and adverse human health effects. Yet the molecular mechanisms of their toxicity are not well understood. To investigate the molecular basis of hyperploidy induction by monohaloacetonitriles, the interaction of monohaloacetonitriles with topoisomerase II in Chinese hamster ovary cells was examined. We showed a concentration-dependent inhibition of DNA decatenation activity of topoisomerase under acellular conditions while *in vitro* monohaloacetonitrile treatment expressed mixed results. The working hypothesis, that topoisomerase II is a molecular target of monohaloacetonitriles, was only partially supported. Nevertheless, this research serves as a starting point toward molecular mechanisms of toxic action of monohaloacetonitriles.

Introduction

For the past century the disinfection of municipal drinking water reduced morbidity and mortality rates in society. Formation of disinfection by-products (DBPs) is a side effect of the disinfection process, and these toxic agents affect human health due to exposure to disinfected water (Cantor, 2010; Hrudey, 2009; Richardson et al., 2007; Villanueva et al., 2004). Risk trade-offs between the inactivation of microbial pathogens and DBP generation exist but simultaneous compliance of both microbial inactivation and DBP levels is required (U.S. Environmental Protection Agency, 2006a, 2006b, 2007). Compliance involves only the regulated species of DBPs, yet, only 11 agents are currently regulated by the U.S. EPA out of the hundreds of possibly toxic DBPs (Plewa and Wagner, 2015). It is established that consumption of chlorinated water is associated with bladder cancer, but the causality of human urinary bladder cancer is not yet identified (Hrudey et al., 2015). Better toxicological characterization of DBPs may advance the health risk analyses of exposure to DBPs.

Currently few molecular mechanisms for specific DBP-induced toxicity have been reported (Dad et al., 2013; Du et al., 2013; Pals et al., 2011). We recently demonstrated that the monohaloacetonitriles induced hyperploidy in mammalian cells, which was a consequence of mitosis inhibition (Komaki

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et al., 2014). Haloacetonitriles (HANs) and other nitrogen-containing DBPs (N-DBPs) are gaining more attention recently since these classes of DBPs are far more cytotoxic and genotoxic than the regulated DBPs such as trihalomethanes and haloacetic acids (Muellner et al., 2007; Plewa et al., 2008). N-DBP formation in drinking waters will increase due to the use of compromised source waters with algal or wastewater influence. Such source waters are linked to increased dissolved organic nitrogen levels, as well as the transition to disinfection with chloramines which generates higher levels of N-DBPs (Bond et al., 2011; Shah and Mitch, 2011). HANs are associated with mutagenicity in *Salmonella typhimurium* (Bull et al., 1985; Muller-Pillet et al., 2000; Simmon et al., 1977), genotoxicity in mammalian cells (Bull et al., 1985; Muellner et al., 2007; Muller-Pillet et al., 2000), clastogenicity (Le Curieux et al., 1995), developmental toxicity (Smith et al., 1987; Smith et al., 1989) and carcinogenicity (National Toxicity Program, 2010). HANs were also identified as inducers of aneuploidy (Osgood and Sterling, 1991). In a recent fingerprinting study, 4 HANs were demonstrated to be highly potent in AREc32, ARE-bla, p53-bla, microtox, umuC±S9 assays (Stalter et al., 2016). Pals et al. demonstrated that bromoacetonitrile (BAN) was thiol-reactive and depleted glutathione or cellular thiols as a molecular initiating event as compared to bromoacetic acid (Pals et al., 2016). However, these brominated DBPs with different molecular initiating events still converged to the point where intracellular Ca^{2+} homeostasis was disrupted, and generated reactive oxygen species-mediated genotoxicity. Pals et al. showed a potentiating effect in genotoxicity when the mixed compounds shared the same molecular initiating event. Although there are several publications surrounding toxicity of DBPs, downstream events such as oxidative stress or DNA damage induction are not sufficient descriptors of toxicological properties.

Despite of the severity of the cytogenetic endpoint there are no publications to date that explain the molecular basis of the massive cell cycle alternations induced by monohaloacetonitrile DBPs (Komaki et al., 2014). Hyperploidy is known to occur in p53-deficient cells due to errors in mitosis, including spindle assembly, chromosome segregation, and cytokinesis (Andreassen et al., 1996; Margolis et al., 2003). Conserved nuclear enzyme topoisomerase II plays a major role in chromosome segregation during mitosis by generating a transient double-stranded break and letting a separate intact double helix pass through the break. Vertebrates contain two isoforms, topoisomerase IIα and topoisomerase IIβ. Topoisomerase IIα is regulated over both cell and growth cycles, while the concentration of topoisomerase IIβ is independent of the cell cycle (Gentry and Osheroff, 2013). Only topoisomerase IIα is required for DNA decatenation and chromatid separation during anaphase (Gardner et al., 2011). Several research reports that failure to properly segregate daughter chromosomes due to catalytic inhibition of topoisomerase II leads endoreduplication (Andreassen et al., 1996; Cortés and Pastor, 2003). Although there are several other molecular mechanisms that induce endoreduplication (for example, spindle inhibitor-induced endoreduplication (Motwani et al., 2000)), as the first step, we developed a working hypothesis that topoisomerase II is a molecular target of monohaloacetonitriles that may disrupt the mitosis checkpoint.

1. Experimental

1.1. Chemicals and reagents

General reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma-Aldrich Co. (St. Louis, MO). Iodoacetonitrile (IAN; 98%, CAS 624-75-9), BAN (97%, CAS 590-17-0) and chloroacetonitrile (CAN; 99%, CAS 107-14-2) were purchased from Sigma-Aldrich. The DBPs were diluted in dimethyl sulfoxide (DMSO) at a concentration of 1 M each time, and diluted to the treatment concentration in serum-free F12.

1.2. Cell culture

Chinese hamster ovary (CHO) cell line AS52 clone 11–4–84, was maintained in modified Ham's F12 medium (Mediatech, Inc., Manassas, VA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO_2. The cells exhibit normal morphology, express cell contact inhibition, and grow as a monolayer without expressing neoplastic foci. The doubling time was approximately 14 hr.

1.3. Topoisomerase II decatenation assay

Topoisomerase II catalyzes the decatenation of intact double-stranded DNA by allowing the enzyme to separate replicated DNA molecules at mitosis. The Human Topoisomerase II Assay Kit (TopoGEN Inc., Buena Vista, CO) utilizes the kinetoplast DNA (kDNA) from *Crithidia fasciculata* (Nitiss et al., 2001). Topoisomerase II decatenates the interlocked (catenated) circles from the network. Catenated and liberated minicircles upon decatenation can be separated and detected as discrete bands on an agarose electrophoresis gel. Viable CHO cells or nuclear protein extracts were treated with monoHANs to determine if they could inhibit the decatenation activity of topoisomerase II under cellular and acellular conditions.

Unsynchronized CHO cells were plated in sterile flat bottom 6-well tissue culture plates at 2×10^5 cells/2 mL F12 + 5% FBS/well. After 16–18 hr of incubation to allow cells to attach onto the surface of the plate, cells were rinsed twice with 1 mL of divalent cation-free Hank's balanced salt solution (HBSS), and treated with each HAN in 1 mL of serum-free F12 for 4 hr at 37°C, 5% CO_2. Multiple wells were used per treatment group as needed. A sheet of sterile AlumnaSeal™ (RPI Corporation, Mt. Prospect, IL) was pressed over the wells before covering the plate with a lid to prevent volatilization. After the 4-hr exposure, the treatment solution was aspirated, and the cells were washed twice with 1 mL HBSS. Two millimeters F12 + 5% FBS were added and incubated for 14 hr. After the 14-hr post-treatment incubation, the cells were washed twice with 1 mL HBSS, and harvested with 0.025% trypsin + 0.1 g/L EDTA (Hyclone Laboratories, South Logan, UT). An aliquot of the harvested cells was used for cell number count with Beckman Coulter Z1 Particle Counter (Beckman Coulter, Inc., Brea, CA) and for determination of acute cytotoxicity using the trypan blue dye exclusion assay (Phillips, 1973; Plewa and Wagner, 2009). The rest of the cells were pelleted and used for nuclear

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