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The characterization of γH2AX and p53 as biomarkers of genotoxic stress in a rainbow trout (*Oncorhynchus mykiss*) brain cell line

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HIGHLIGHTS

- Three genotoxicants were studied on rainbow trout cells *in vitro*.
- Methyl methanesulfonate induced γH2AX but not p53.
- 4-nitroquinoline *N*-oxide induced both γH2AX and p53.
- Bleomycin induced γH2AX and p53, but γH2AX was more sensitive.

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ABSTRACT

Rainbow trout cell cultures were exposed to three genotoxicants and examined for effects on γH2AX and p53 levels by western blotting and on cell viability using the indicator dyes Alamar Blue (AB) for energy metabolism and 5'-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) for plasma membrane integrity. Bleomycin induced γH2AX and p53 in a dose- and time-dependent manner and had little cytotoxic effect. However, induction was first seen at 0.3 μM for γH2AX but not until 16.5 μM for p53. Methyl methanesulfonate (MMS) increased H2AX phosphorylation but diminished p53 levels as the dose was increased from 908 μM up to 2724 μM. Over this dose range cell viability was progressively lost. 4-nitroquinoline N-oxide (NQO) induced both γH2AX and p53, beginning at 62.5 nM, which was also the concentration at which cell viability began to decline. As the NQO concentration increased further, elevated γH2AX was detected at up to 2.0 μM, while p53 was elevated up to 1.0 μM. Therefore, H2AX phosphorylation was superior to p53 levels as a marker of DNA damage caused by genotoxicants that act by introducing double-stranded DNA breaks (bleomycin), alkyl groups (MMS), and quinoline adducts (NQO).

1. Introduction

Rainbow trout (RT), *Oncorhynchus mykiss* (Walbum), and RT cell lines are increasingly being used to study and detect the genotoxicity of environmental contaminants and of commercial products that might reach the aquatic environment (Antunes et al., 2016; Capkin et al., 2017; Rodrigues et al., 2016; Vacchi et al., 2016; Zeng et al., 2016a). *O. mykiss* is used because the species is widely distributed, easily maintained in the laboratory, and has long been used in research, especially in toxicology (Wolf and Rumsey, 1985). RT cell lines have been developed from most tissues and organs of this species and are collectively referred to as the RT invitrome (Bols et al., 2017). Their experimental advantages include savings in cost and time, simplified design and dosing, and reducing the use of animals (Bols, 1991). In environmental toxicology, they can be used to detect and rank toxicants and to identify and evaluate biomarkers (Bols et al., 2005; Schirmer, 2006). Biomarkers can be used *in vivo* to determine whether fish have been exposed to and impacted by a particular toxicant or toxicant class. For genotoxicants, biomarkers might be sought among the proteins involved in the DNA damage response (DDR), which is the collection of mechanisms for ameliorating DNA damage and involves proteins that detect DNA lesions, signal their presence, and promote their repair (Jackson and Bartek, 2009). However, relatively little is known about the DDR in RT cell lines. Two DDR proteins that might be used as biomarkers of genotoxicants are H2AX and p53.

H2AX is the X variant of histone 2A and undergoes phosphorylation in response to DNA damage (Fernandez-Capetillo et al.,

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Abbreviations

AB	Alamar Blue
CFDA-AM	5'-carboxyfluorescein diacetate acetoxymethyl ester
DDR	DNA damage response
DSB	Double-stranded break
MMS	Methyl methanesulfonate
NQO	4-nitroquinoline N-oxide
RT	Rainbow trout

2004), while p53 is a central regulatory protein of many fundamental activities including DNA repair (Vogelstein et al., 2000a,b) and can accumulate in response to DNA-damaging agents (Fritsche et al., 1993). Upon the formation of double-stranded DNA breaks (DSB) via oxidative stress, replicative stress, enzyme activity, chemical exposure, or ionizing radiation, the Mre11-Rad50-Nbs1 (MRN) end-processing and sensor complex interacts with DNA proximal to the break through Rad50 (van den Bosch et al., 2003). Nbs1 is required in order to recruit Ataxia telangiectasia mutated (ATM) to lesions, while Mre11 possesses endonuclease activity (Ackermann and El-Deiry, 2008; Furuta et al., 2003; Stracker and Petrini, 2011; van den Bosch et al., 2003). ATM, a sensor of the phosphoinositide-3-kinase (PIKK) family, along with ataxia telangiectasia and Rad3-related (ATR) and the DNA protein kinase catalytic subunit (DNA-PK), phosphorylate Ser139 of mammalian H2AX in the C'-terminal SQ motif, and the result is commonly referred to as γH2AX (Rogakou et al., 1998; Shiloh, 2003). The regulation of p53 activity and expression is complex. Some post-translational modifications include ubiquitylation, methylation, ADP-ribosylation, glycosylation, SUMOylation (small ubiquitin-like modifiers), NEDDylation (neural precursor cell expressed, developmentally down-regulated 8), acetylation, and phosphorylation at multiple sites (Vogelstein et al., 2000b; Zhou et al., 2017). Although mammalian cell lines have been used intensively to study H2AX and p53 regulation, the focus has most commonly been to understand the development and treatment of tumors (Mantovani et al., 2017; Tran et al., 2017) rather than for an environmental purpose. However, several human cell lines have shown to be useful for assessing the genotoxicity of polycylic aromatic hydrocarbons (PAHs) (Audebert et al., 2010).

Relatively few studies have been done on H2AX and p53 in RT and RT cell lines. H2AX was phosphorylated in fish after exposure to tritiated water (Festarini et al., 2016) and in the cell lines, RTgill-W1 and RTH-149, upon exposure to cadmium (Krumschnabel et al., 2010). Thus, γH2AX would appear to be a biomarker for DNA damage in RT but studies on more classical DNA damaging agents would help to confirm a relationship. Studies on p53 in RT cell lines hint at p53 behaving slightly differently in RT cells than in mammalian cells. Several DNA damaging agents that induced p53 in mammalian cells failed to increase p53 levels in RTL-W1 (Embry et al., 2006) and RTbrain-W1 (Liu et al., 2011). Also, two p53 inhibitors, 2-phenylethynesulfonamide and pifithrin-α, had off-target actions on RTgill-W1 (Zeng et al., 2014, 2016b). Thus, more studies are needed on H2AX and p53 in RT cell lines.

In the current study, changes in H2AX phosphorylation and p53 levels were investigated in RTbrain-W1 in response to three model DNA-damaging compounds: bleomycin (BLEO), methyl methanesulfonate (MMS), and 4-nitroquinoline N-oxide (NQO). Although the capacity of this cell line for xenobiotic metabolism has yet to be explored, RTbrain-W1 was used because it has been characterized previously for expression of several genes in the DDR (Liu et al., 2011; Steinmoeller et al., 2009) and because recent mammalian research has shown surprising roles for the DDR in the brain. DNA repair is now being viewed as an essential part of brain physiology (Suberbielle et al., 2013) and alterations in DNA repair are associated with neurodegenerative diseases (Merlo et al., 2016) and stress (Hare et al., 2018). The compounds were chosen because their mode of genotoxicity is reasonably well known in yeast and in mammals. Bleomycin generates double-stranded breaks (DSBs) (Povirk and Finley Austin, 1991) which are repaired by homologous recombination (HR) or non-homologous end joining depending on cell phase (Thompson, 2012). MMS monoalkylates DNA (Beranek, 1990; Ma et al., 2011), causing single-stranded breaks repaired by base excision (Pascucci et al., 2005), or HR upon replication-dependent double-stranded breaks (Nikolova et al., 2010). NQO metabolites form quinoline adducts (Galiègue-Zouitina et al., 1985; Kohda et al., 1991) that can be repaired by nucleotide excision repair (Snyderwine and Bohr, 1992), or when left unrepaired, lead to DSBs and chromosomal rearrangements (Brüsehafer et al., 2016). They were also chosen because their actions on mammalian cell lines have been intensively studied (Arima et al., 2006; Banáth and Olive, 2003; Khoury et al., 2016; Liu et al., 2014; Schroeder et al., 2014; Valentin-Severin et al., 2003; Wang et al., 2013). Our results showed that when RTbrain-W1 cells were exposed to these genotoxicants, γH2AX levels were elevated in a dose- and time-dependent fashion, suggesting that H2AX phosphorylation might be a good biomarker of genotoxicant exposure in rainbow trout.

2. Materials and methods

2.1. Chemicals

Biomarker testing was performed using methyl methanesulfonate (MMS; CAS 66-27- 3, #129925), 4-Nitroquinoline *N*-oxide (NQO; CAS 56-57-5, #N8141), and bleocin (proprietary name for bleomycin, referred to as BLEO; CAS 55658-47-4, #203408-M) that were all purchased from Sigma-Aldrich. 4-NQO was dissolved in dimethyl sulfoxide (DMSO; CAS 67-68-5, #472301) from Sigma-Aldrich for dosing. The final concentration of DMSO in media was 0.5% v/v, which is not cytotoxic on its own to cells (Schnell et al., 2009). BLEO and MMS were dissolved in ultrapure deionized water for dosing, where the solvent never exceeded 1% of total exposure volume. These solvents were used as mock-treated negative controls for exposure of the respective chemicals they were used to dissolve.

2.2. Cell cultures and exposure

Testing was performed in an adherent glial cell line cultured from the brain of rainbow trout (*Onchorhynchus mykiss*) named RTbrain-W1, which has seen limited use in the literature (Fischer et al., 2011; Liu et al., 2011; Lončar et al., 2010; Steinmoeller et al., 2009; Vo et al., 2015). Cells were routinely grown in 75 cm^2 (T75) polystyrene tissue culture flasks (BioLite, Thermo Fisher Scientific) at room temperature (RT; 20 ± 2 °C) in L-15 basal medium (HyClone, GE Healthcare) supplemented with 15% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific) and a 1% penicillin-streptomycin cocktail (P/S; HyClone, GE Healthcare). Routine passaging and seeding was performed using 0.25% v/v trypsin (HyClone, GE Healthcare) diluted in Dulbecco's phosphate-buffered saline (DPBS; from HyClone, GE Healthcare), and cells were used between their 5th and 25th passages.

Prior to exposing cells to genotoxicants or solvent only for controls, cells were removed from T75s by trypsinization and centrifuged (3000 RPM for 5 min at 18 °C). The trypsin was removed, and cells were seeded on either a 96-well plate or a 6-