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Effect of acid blue 80, an anthracenedione dye, on rainbow trout liver, gill and gut cells *in vitro*

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ABSTRACT

Acid Blue 80 (AB80) is a dark blue colorant that like other synthetic dyes can get into the environment. Cultures of rainbow trout cell lines were dosed with AB80 either directly, which involved mixing AB80 stock solution into the medium over cells, or indirectly, which involved replacing the medium in cultures with medium that had AB80. A dose-dependent decline in cell viability was found in cultures with or without fetal bovine serum (FBS) after direct dosing. However, for FBS cultures, indirect dosing caused no loss of viability over 24 h and in the long term was detrimental to RTgill-W1 but not RTL-W1 cultures. After 6 days at 50 mg/L cytotoxicity was evident and by 9 days RTgill-W1 cell number had declined. Yet AB at 1 mg/L elicited no changes over 9 days in any cell line. AB80 appears to have the potential to be toxic at only very high concentrations.

1. Introduction

Synthetic dyes are an important class of environmental contaminants (Martinez-Huitle and Brillas, 2009) and an example of one is Acid Blue 80 or Blue Nylosan F.L. (Prevot et al., 2001). Acid Blue 80 is benzenesulfonic acid, 3,3′-[(9,10-dihydro-9,10-dioxo-1,4-anthracenediyl) diimino] bis [2,4,6-trimethyl-, disodium salt, which is an anthracenedione dye and a dark blue colorant. The dye is used in several industries in a wide variety of products including printing inks, paints and cleansers. The Canadian Ministers of the Environment and of Health have identified Acid Blue 80 as a priority substance because of its potential for environmental persistence, bioaccumulation and toxicity to non-human organisms (The Chemical Substances Portal, chemical substances.gc.ca). Being an anionic dye with two sulfonic acid groups and no reactive functional group, Acid Blue 80 is expected to have a low to moderate toxicity to aquatic organisms. To date the median or nominal Lethal Concentration (LC_{50}) to rainbow trout and guppies has been found to be above 10 mg/L, but other than this little is known about the toxicity of this dye. This lack of toxicological data is a concern because of the huge volume of the dye that could potentially be released into environment through its daily commercial use especially in cleaning products.

One approach to investigate potential ecotoxicants is to use fish cells in culture (Bols et al., 2005; Schirmer, 2006). As well fulfilling a societal desire to reduce the use of animals in toxicity testing, cell cultures offer experimental advantages. They allow mechanisms of toxicity to be explored definitively at the cellular level in a controlled setting independent of the complexities and variableness of systemic or physiological regulation. Control extends to the physical environment, with parameters such as temperature and light can be easily modulated. Other advantages include dosing of cell cultures, which is done more reproducibly and with less toxic waste, and the more rapid and less expensive acquisition of results. Once cellular responses to a toxicant or toxicant class are known, the responses of cells in culture can be used to develop routine assays for addressing other ecotoxicological issues such as the toxicants presence and persistence in environmental samples to compare the relative toxicity of members of a similar group of ecotoxicants and to evaluate the sensitivity of potential target tissues or organs and even the sensitivity of different aquatic species.

Although little or nothing is known about the effects of Acid Blue 80 on animal cells in culture, studies on anthracenedione derivatives suggest possible cellular responses that might be examined. Anthracenedione or anthraquinone can refer to a specific isomer, 9,10-anthraquinone, or to a group of compounds that can be considered as derivatives of anthraquinone. Exposing a human cell line (JEG-3) to 9,10 anthraquinone had no effect on cell viability as evaluated with Alamar Blue (AB) for metabolism and with 5-carboxyfluorescein diacetate acetoxymethyl (CFDA–AM) for cell membrane

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integrity (Peters et al., 2007). By contrast many derivatives of anthraquinone have been developed as chemotherapeutic agents and screened for their ability to kill animal cells in culture with viability being measured by a variety of techniques, including trypan blue exclusion for membrane integrity and neutral red (NR) for lysosomal activity (Andersson et al., 1999; Barnard et al., 1995). Cytotoxicity could be elicited through the generation of reactive oxygen species (ROS) or by binding to DNA (Barasch et al., 1999; Zagotto et al., 2004; Zhang et al., 2007). Other more chronic mechanisms have been studied less frequently. However a few anthraquinone derivatives weakly induced 7 ethoxyresorufin o-deethylase (EROD) activity in the rainbow trout liver cell line, RTL-W1, suggesting that they might modulate xenobiotic metabolism and be metabolized themselves (Schirmer et al., 2001).

Therefore, in this study, two rainbow trout cell lines, RTgill-W1 and RTL-W1, and to a lesser extent a third cell line, RTgutGC, have been used to examine possible toxic actions of Acid Blue 80.

2. Materials and methods

2.1. Cell lines and their growth

Three rainbow trout epithelial cell lines, RTL-W1, RTgill-W1 and RTgutGC, were used between passages 11 and 40. RTL-W1 was developed from liver (Lee et al., 1993) RTgill-W1 from gills (Bols et al., 1994) and RTgutGC from the intestine (Kawano et al., 2011). The cell lines were cultured routinely in 75 cm^2 culture flasks at room temperature in Leibovitz's L-15 culture medium (Sigma-Aldrich, Ltd., Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin–streptomycin solution (10,000 units/mL penicillin, 10 mg/mL streptomycin, Sigma-Aldrich).

2.2. Exposure of fish cells to acid blue (AB80)

AB80 (CAS Number: 4474-24-2) was obtained as a dark blue powder from Sigma-Aldrich Canada Ltd. (Cat. No. 210323; Oakville, Ontario), which contained 40% dye and 60% proprietary filler. HPLC separation of AB80 followed by UV and MS analyses failed to detect either colored or ionizable impurities (Balakrishnan, V., 2010 personal communication). A stock solution of 100 mg/ml was prepared and serially diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Usually exposures were done in 96-well tissue culture plates (Becton-Dickinson, Mississauga, ON, Canada) in which cells had been seeded at a cell density of 40,000 cells per well in 200 μL of L-15 with or without FBS. Cells were allowed to attach for 24 h prior to exposure. The cultures were dosed to a final concentration 1, 5, 10, 25 and 50 mg/L of AB80. Additions were done in sextuplicates and the cells were incubated for 24 h at room temperature.

Application of AB80 to cell cultures was done by two different methods: direct dosing and indirect dosing. Direct dosing involved adding a small volume (1 μL) of stock solutions directly to the culture wells. The final concentration of DMSO in the directly dosed culture wells was 0.5% (v/v). Indirect dosing involved a complete replacement of the culture medium of wells with medium containing different concentrations of AB80. This medium was prepared from the same AB80 stock solution by mixing 8 μL of stock solution with 1.6 mL of medium in centrifuge tubes. The final concentration of DMSO in the indirectly dosed culture wells was also 0.5% (v/v). For each assay a control was performed by adding only the solvent (DMSO) to the cells.

2.3. Cell viability assays to evaluate cytotoxic action

Three cellular parameters were monitored with 3 fluorescent dyes to determine a change in cell viability. Metabolic activity was measured with Alamar Blue (AB, reazurin) (Biosource). Cell membrane integrity was evaluated with 5-carboxyfluorescein diacetate (CFDA–AM; Molecular Probes, Eugene, OR, USA). Lysosomal function was assessed with neutral red (NR; 3-amino-7-dimethylamino-2-methylphenazine hydrochloride; Sigma-Aldrich). The assays were performed as described previously in detailed step-by-step protocols on the use of these dyes and on the analysis and interpretation of the results (Dayeh et al., 2005). Results were recorded as relative fluorescent units (RFUs) with a fluorescence plate reader (Spectramax Gemini XS microplate spectrofluorometer; Molecular Devices, Sunnyvale, CA). The excitation and emission wavelengths used were,respectively, 530 and 590 nm, for AB, 485 and 530 nm for CFDA–AM, and 530 and 645 nm for NR.

2.4. Measuring the induction of 7-ethoxyresorufin o-deethylase (EROD) activity

Microwell cultures were dosed with 23,7,8-tetrachlorodibenzo-p-dioxin (TCDD; Cambridge Isotopes Laboratory, And over, MA) at up to 96.7 nM as a positive control and with AB80 at up to 25 mg/L and assayed for EROD activity 24 h later as described previously in step-by-step format (Ganassin et al., 2000). The assay ran for 60 min within the wells and was read at an excitation wavelength of 530 nm and emission of 595 nm with the fluorescence plate reader. The RFU data from each experiment over an exposure range, which usually consisted of 8–10 concentrations, was examined by a one-way analysis of variance (ANOVA). If the p value was greater than 0.05, the compound was concluded not to be an EROD inducer over this concentration range. If the p value was less than 0.05, the test agent was concluded to be an EROD inducer. In this case EROD activity was expressed as p moles resorufin/mgP/min.

2.5. Monitoring cell proliferation to evaluate cytostatic actions

The proliferation of RTL-W1 and RTgill-W1 was studied in wells of 12-well plates (Becton-Dickinson) in L-15 with 10% FBS after indirect dosing with AB80. Approximately 30,000 cells were added per well and allowed to attach for 24 h prior to the treatments. Cells were dosed in triplicates with AB80. Control wells received only DMSO. Plates were incubated at room temperature and at up to 12 days later, cell number was enumerated with a Colter counter (Colter Electronics of Canada, Burlington, ON). At least two independent experiments were done for each condition.

2.6. Data analysis

All graphs, calculations and statistical analyses were done using GraphPad InStat (version 3.00 for Windows 95, GraphPad Software, San Diego, CA USA, www.graphpad.com). From plots of RFUs vs. AB80 concentrations, the effective concentrations causing a 50% (EC50s) decline in viability were determined. EC50s between two groups and within multiple groups were compared, respectively, by unpaired t-test and one-way analysis of variances (ANOVA). If the ANOVA was significant, the Tukey–Kramer multiple comparisons test was performed to find the pairs of means that were significantly different. In all cases a p-level < 0.05 was considered as significantly different.

3. Results

3.1. Cytotoxicity of Acid Blue 80 (AB80) dosed into cultures without serum

When rainbow trout cell lines were exposed for 24 h to acid Blue 80 (AB80) in medium with no serum, cell viability declined in cultures dosed directly (Fig. 1A) or indirectly (Fig. 1B) at concentrations beyond 1 mg/L (Table 1). The loss of viability was seen as a dose-dependent decline in AB reduction, the conversion of CFDA–AM to CF, and the uptake of NR (Fig. 1). From these curves the effective concentrations (EC50s) were calculated allowing the results with different dosing methods, viability endpoints and cell lines to be compared (Table 1). For each endpoint with each cell line, AB80 was more toxic (lower EC50) after direct than after indirect dosing (unpaired t-test, $p < 0.05$). The exception was RTgutGC evaluated with Alamar Blue ($p = 0.06$). Among the three endpoints, the EC50s for AB and NR were not significantly different from each other in RTgill-W1 and RTL-W1 but were significantly lower than the EC50s for CFDA–AM (ANOVA, $p < 0.05$; Tukey–Kramer Multiple Comparisons Test, $p < 0.005$). For RTgutGC cultures that were directly dosed, the EC50s were the same for the three viability endpoints (ANOVA, $p > 0.05$). Among the three cell lines, the EC50s were not significantly different between RTgill-W1 and RTgutGC but were significantly lower for RTL-W1 (ANOVA, $p < 0.05$; Tukey–Kramer Multiple Comparisons Test, $p < 0.005$). This was true for both directly and indirectly dosed cultures.