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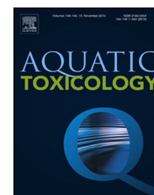

Infrared spectroscopy detects changes in an amphibian cell line induced by fungicides: Comparison of single and mixture effects

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ABSTRACT

Amphibians are regarded as sensitive sentinels of environmental pollution due to their permeable skin and complex life cycle, which usually involves reproduction and development in the aquatic environment. Fungicides are widely applied agrochemicals and have been associated with developmental defects in amphibians; thus, it is important to determine chronic effects of environmentally-relevant concentrations of such contaminants in target cells. Infrared (IR) spectroscopy has been employed to signature the biological effects of environmental contaminants through extracting key features in IR spectra with chemometric methods. Herein, the *Xenopus laevis* (A6) cell line was exposed to low concentrations of carbendazim (a benzimidazole fungicide) or flusilazole (a triazole fungicide) either singly or as a binary mixture. Cells were then examined using attenuated total reflection Fourier-transform IR (ATR-FTIR) spectroscopy coupled with multivariate analysis. Results indicate significant changes in the IR spectra of cells induced by both agents at all concentrations following single exposures, primarily in regions associated with protein and phospholipids. Distinct differences were apparent in the IR spectra of cells exposed to carbendazim and those exposed to flusilazole, suggesting different mechanisms of action. Exposure to binary mixtures of carbendazim and flusilazole also induced significant spectral alterations, again in regions associated with phospholipids and proteins, but also in regions associated with DNA and carbohydrates. Overall these findings demonstrate that IR spectroscopy is a sensitive technique for examining the effects of environmentally-relevant levels of fungicides at the cellular level. The combination of IR spectroscopy with the A6 cell line could serve as a useful model to identify agents that might threaten amphibian health in a rapid and high throughput manner.

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1. Introduction

Large declines in amphibian populations have been reported since the 1990s (Houlahan et al., 2000; Stuart et al., 2004), with environmental pollution reported as a significant factor in these declines (Sparling et al., 2001). The life cycle of amphibians usually encompasses reproduction and early development in the aquatic environment, meaning that this group of organisms may be susceptible to run-off from agricultural sources, such as pesticide application, which is often coincident with this sensitive period of development (Hanlon and Parris, 2014; Hayes et al., 2006; Mann et al., 2009). Such factors, in addition to the permeable skin of amphibians (Quaranta et al., 2009), mean that this group is a be a sentinel organism, indicative of early deterioration in environmental quality (Sparling et al., 2010).

Fungicides are widely used in agriculture in order to prevent and treat diseases in commercial crops such as wheat and soybean (Belden et al., 2010; McMullen et al., 2012). Two classes of fungicide frequently used in agricultural practice, either singly or in combination are the benzimidazole and triazole fungicides. Benzimidazole fungicides exert their toxic effect on fungal spores through inhibition of microtubule assembly, by binding to tubulin, the major component of microtubules (Berg et al., 1986; Davidse, 1986; Wolff, 2009). Triazole fungicides, in contrast, interfere with steroid biosynthesis and therefore formation of fungal cell walls through inhibition of sterol-14α-demethylase (CYP51), an enzyme

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present in all eukaryotes (Bossche et al., 1995; Zarn et al., 2003). As a consequence, the structure of the plasma membrane is disrupted, making it prone to further damage (Georgopapadakou, 1998; Lorito et al., 1996). Both benzimidazole and triazole fungicides have been associated with negative effects in non-target organisms, including amphibians. Such effects include endocrine disruption in adult amphibians (Poulsen et al., 2015), and developmental defects such as craniofacial abnormalities in the case of triazole fungicides (Di Renzo et al., 2011; Groppelli et al., 2005; Papis et al., 2006), or inhibition of the differentiation of neural tissues and organ dysplasia following exposure to benzimidazole fungicides (Yoon et al., 2003, 2008).

Investigating the effects of environmental pollutants at the cellular level is of importance in ecotoxicological research because the key interaction between chemical contaminants and organisms initially occurs within cells (Fent, 2001). In addition, the use of an *in vitro* cell culture model reduces the number of vertebrates used in environmental risk assessment, thus reducing ethical concerns (Scholz et al., 2013). Infrared (IR) spectroscopy is being increasingly applied in cell-based assays in order to determine molecular modifications caused by chemical stressors, based on changes in the IR absorbance spectra (Holman et al., 2000a). Exposure of a sample to IR radiation will cause the functional groups within the sample to absorb the IR radiation and vibrate in several ways, including stretching, bending and deformation. These absorptions and vibrations can then be directly correlated to biochemical molecules, with peaks in the spectrum corresponding to the chemical structure of a particular entity, *e.g.*, lipid ~1740 cm^{-1}, DNA ~1080 cm^{-1}, Amide I and II ~1650 and 1550 cm^{-1} respectively, thus providing a 'biomolecular fingerprint' in the form of an IR spectrum (Ellis and Goodacre, 2006; Kelly et al., 2011; Martin et al., 2010). Previous studies have found a high concordance between traditional toxicological endpoints and those measured by FTIR spectroscopy. For example, in HEPG2 cells exposed to TCDD, there was a positive correlation between CYP1A1 expression and IR absorption of the phosphate band (Holman et al., 2000b). MCF-7 cells exposed to 17β-estradiol showed comparable EC-50 values when assessed with either the E-screen assay or FTIR spectroscopy, with the results from FTIR spectroscopy obtained in a much shorter time, a key advantage of this technique (Johnson et al., 2014). As IR spectroscopy is able to analyse lipids, carbohydrates, proteins and nucleic acids concurrently, it is valuable technique for metabolic fingerprinting (Ellis and Goodacre, 2006).

The resulting fingerprint is highly complex and information rich, comprising hundreds of features (wavenumbers), therefore multivariate techniques such as principal component analysis (PCA) or linear discriminant analysis (LDA) are often applied in order to reduce the complexity of the data sets into a small number of factors (scores). The application of chemometric methods like PCA and LDA allows the extraction of key features from the IR spectrum in the form of loadings and cluster vectors, which denote which regions of the IR spectrum are responsible for segregation between control and treated cells when viewed alongside the scores plots (Baker et al., 2014; Martin et al., 2010; Trevisan et al., 2012). The combination of IR spectroscopy and multivariate techniques for feature extraction has previously been applied in human, algal and bacterial cell types in order to distinguish between treated and control cells and generate potential biomarkers based upon the loadings and cluster vectors generated (Heys et al., 2014; Johnson et al., 2014; Llabjani et al., 2010, 2011; Mecozzi et al., 2007; Riding et al., 2012a; Ukpebor et al., 2011).

In this study, ATR-FTIR spectroscopy coupled with multivariate feature-extraction techniques was employed in order to detect the effects of two commonly used fungicides: carbendazim, a benzimidazole fungicide, and flusilazole, a triazole-derived fungicide at low, environmentally relevant concentrations (Chatupote and Panapitukkul, 2005; Palma et al., 2004) ranging from 0.05–5 nM in A6 cells, a continuous epithelial cell line derived from the kidney of the African clawed frog, *Xenopus laevis*. A6 cells are a well characterised cell line, having previously been used in toxicity studies (Gorrochategui et al., 2016) measuring responses such as expression of heat shock proteins (HSPs), intracellular calcium and cell cycle progression after exposure to a variety of environmental contaminants (Bjerregaard, 2007; Bjerregaard et al., 2001; Darasch et al., 1988; Faurskov and Bjerregaard, 1997, 2000, 2002; Heikkila et al., 1987; Khamis and Heikkila, 2013; Music et al., 2014; Thit et al., 2013; Woolfson and Heikkila, 2009; Yu et al., 2007). Additionally, as amphibians are exposed to multiple chemical stressors in the environment (Hua and Relyea, 2014; Relyea, 2009), cells were also exposed to mixtures of carbendazim and flusilazole. The aims of the study were as follows: (1) To determine if ATR-FTIR spectroscopy coupled with multivariate feature-extraction techniques could detect changes induced to cellular biomolecules by carbendazim and flusilazole across a concentration range in the A6 cell line; (2) To determine differences in the mechanism of action of each agent through direct comparison of the features extracted from their IR spectra; and, (3) To determine the combined effects of carbendazim and flusilazole on cells in binary mixtures in comparison to single agent effects through comparison of the features extracted from their IR spectra.

2. Materials and methods

2.1. Cell culture

Xenopus laevis A6 kidney epithelial cells were purchased from Sigma Aldrich (Dorset, UK) and grown at 22 °C in T75 tissue culture flasks in 70% (diluted with distilled water to adjust to amphibian osmolarity) Leibovitz's (L15) media supplemented with 10% v/v fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 U/mL/100 μg/mL). Cells were routinely subcultured every 7 days by partial digestion in 0.25% trypsin-EDTA and prior to incorporation into experiments. Media was replaced every 72 h. Flasks that had reached 80–90% confluency were used for experiments. All cell culture consumables were purchased from Gibco Life Technologies (Paisley, UK) unless otherwise stated.

2.2. Test agents

Flusilazole (product no. 45753) and carbendazim (product no. 45368) were purchased as PESTANAL® analytical standards from Sigma Aldrich (Poole, Dorset) and made up to 10 μM stocks solutions in dimethylsulfoxide (DMSO) (also from Sigma Aldrich). Serial dilutions of the stock solutions were made to give the appropriate concentrations in the treatment flasks. Test agent/vehicle control solutions did not exceed 1% v/v in the treatment flasks.

2.3. Cell treatments

Routinely cultured A6 cells were trypsin-disaggregated, resuspended in complete media and seeded in T25 flasks at a density of 5×10^4 cells/ml. Cells attached for 24 h before treatment with the test agents as either single concentrations or binary mixtures for a further 24 h. This treatment time is optimal for IR spectroscopy studies as it allows the recording of distinct spectral variations, whilst avoiding large amounts of damage from apoptosis and necrosis to the cells (Derenne et al., 2012). For the single concentrations, cells were treated with 5 nM, 1 nM, 0.5 nM, 0.1 nM and 0.05 nM of carbendazim or flusilazole, plus a vehicle control (DMSO). For the binary mixtures, cells were treated with 5 nM and 0.05 nM of flusilazole and carbendazim in the following

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