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Cytotoxicity of binary mixtures of human pharmaceuticals in a fish cell line: Approaches for non-monotonic concentration–response relationships

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HIGHLIGHTS

- The *in vitro* cytotoxicity of ten pharmaceuticals was investigated using RTG-2 cells.
- Biphasic concentration–response relationships were observed for some compounds.
- Cytotoxicity of binary mixtures could be predicted using concentration addition.
- The *in vitro* mode of toxic action for the pharmaceuticals tested was non-specific.

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ABSTRACT

Predicting the effects of mixtures of environmental micropollutants is a priority research area. In this study, the cytotoxicity of ten pharmaceuticals to the rainbow trout cell line RTG-2 was determined using the neutral red uptake assay. Fluoxetine (FL), propranolol (PPN), and diclofenac (DCF) were selected for further study as binary mixtures. Biphasic concentration–response relationships were observed in cells exposed to FL and PPN. In the case of PPN, microscopic examination revealed lysosomal swelling indicative of direct uptake and accumulation of the compound. Three equations describing non-monotonic concentration–response relationships were evaluated and one was found to consistently provide more accurate estimates of the median and 10% effect concentrations compared with a sigmoidal concentration–response model. Predictive modeling of the effects of binary mixtures of FL, PPN, and DCF was undertaken using an implementation of the concentration addition (CA) conceptual model incorporating non-monotonic concentration–response relationships. The cytotoxicity of the all three binary combinations could be adequately predicted using CA, suggesting that the toxic mode of action in RTG-2 cells is unrelated to the therapeutic mode of action of these compounds. The approach presented here is widely applicable to the study of mixture toxicity in cases where non-monotonic concentration–response relationships are observed.

1. Introduction

A large number of studies have reported the occurrence of pharmaceuticals in treated wastewater destined for release to the environment (Kasprzyk-Hordern et al., 2009; Miège et al., 2009; Behera et al., 2011; Jelic et al., 2011; Martín et al., 2012; Ratola et al., 2012; Yu et al., 2013). Active pharmaceutical ingredients designed to modulate a specific molecular target in the human body also have the potential to modulate structurally related targets in aquatic animals, particularly vertebrates such as fish (Fent et al., 2006). Reported concentrations of most pharmaceuticals found in municipal wastewater treatment plant (WWTP) effluents and receiving waters are generally below the lowest concentrations known to cause acute toxicity in fish when applied singly (Corcoran et al., 2010), although bioaccumulation of some pharmaceuticals has been observed in fish sampled from effluent-dominated water courses (Brooks et al., 2005; Ramirez et al., 2009) and during laboratory exposures (Schwaiger et al., 2004), indicating that tissue concentrations can reach higher levels than concentrations measured in environmental samples. Furthermore, some compounds such as synthetic steroid contraceptives are known to affect the reproductive success of fish at environmentally relevant concentrations (Kidd et al., 2007). Investigating the possible ecological

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effects of pharmaceutical mixtures has been identified as a priority research area (EEA, 2010; Boxall et al., 2012). However, predicting the effects of complex mixtures of low concentrations of pharmaceuticals remains a challenge, particularly when possible adverse interactions have not been characterized in susceptible aquatic organisms.

Fish cell lines have long been used for toxicity screening of individual chemicals and complex mixtures, for ranking relative toxicity, and for establishing structure–toxicity relationships (Segner, 1998). Cultured cells represent a convenient model system for examining mixture toxicity prior to undertaking whole-organism studies as they allow large numbers of compounds and combinations to be tested rapidly and cost-effectively.

In this study, we investigated the *in vitro* toxicity of selected pharmaceuticals in RTG-2 cells (Wolf and Quimby, 1962), a fibroblast-like cell line derived from mixed male and female gonad tissue of rainbow trout (*Oncorhynchus mykiss*). Cytotoxicity was determined using the neutral red uptake (NRU) assay (Repetto et al., 2008) a cell viability assay widely utilized for *in vitro* toxicity studies conducted using cultured animal cells. Compounds were selected on the basis of their reported occurrence in WWTP effluents and surface waters, and included four β-adrenergic receptor blockers (β-blockers; atenolol [ATL], metoprolol [MP], pindolol [PD] and propranolol [PPN]), two selective serotonin reuptake inhibitors (fluoxetine [FL] and venlafaxine [VFX]), a non-steroidal anti-inflammatory drug (diclofenac [DCF]), an anti-convulsive (carbamazepine [CBZ]), an anti-rheumatic chemotherapeutic (methotrexate [MTX]), and a synthetic steroidal contraceptive (17α-ethinylestradiol [EE2]). For compounds exhibiting cytotoxicity, cells were exposed to binary mixtures and the resulting concentration–response profiles were compared to responses predicted using mixture modeling approaches.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents were purchased from Sigma–Aldrich Pty. Ltd., Australia, unless otherwise specified.

2.2. Cell culture and treatments

RTG-2 cells (ECACC 90102529) were obtained from Sigma–Aldrich Pty. Ltd., Australia. Cells were maintained in Liebovitz' L-15 (Gibco) supplemented with 50 μg mL^{-1} streptomycin (Gibco), 50 U mL^{-1} penicillin (Gibco), 1 × MEM non-essential amino acids (Gibco), 15 mM HEPES buffer (pH 7.4; Gibco), and 10% fetal bovine serum (FBS; Gibco). This medium is referred to hereafter as 'standard growth medium'. Cells were maintained at 22 °C under a standard atmosphere. All cell culture reagents were purchased from Life Technologies Pty. Ltd., Australia.

RTG-2 cells were seeded into 96 well plates at a density of 1×10^4 cells per well in 100 μL standard growth medium and incubated overnight at 22 °C. The outer wells of the plate were filled with 100 μL sterile water to prevent edge effects. On the following day, dilutions of the compounds were prepared in a suitable solvent (DMSO or methanol), added to standard growth medium under sterile conditions and mixed well. Cells were treated with test compounds by replacing the overnight growth media with media containing dilutions of the test compound. Initial cytotoxicity screens were performed using a 72 h exposure period, while binary mixture studies were conducted using 24 h exposures. All wells including solvent controls contained an equivalent volume of solvent in growth medium (0.5%).

After initial screening to prioritize compounds for further analysis, treatment with binary combinations of selected pharmaceuticals was undertaken. Cells were treated in fixed-ratio regimes for 24 h, with concentration ranges chosen based the toxicity of individual compounds such that concentrations used for binary mixture exposures were equitoxic and covered a range from 4 × EC_{50} to 1/64th × EC_{50} in twofold dilution series. Controls for the mixture studies contained an equivalent volume of solvent. Exposures were repeated on three separate occasions ($n = 3$).

2.3. Cytotoxicity assays

Cell viability was estimated using the NRU assay according to Repetto et al. (2008), with minor modifications. In brief, media containing test compounds were removed by aspiration and replaced with 100 μL per well standard growth medium containing 10 μg mL^{-1} NR (Sigma–Aldrich). After 2 h at 22 °C, NRU assay media were removed by aspiration and the cells washed briefly with 100 μL PBS. Cell-associated NR was solubilized by adding 100 μL of 50% ethanol, 5% acetic acid to each well and mixing well. Absorbance was determined at 540 nm using a plate spectrophotometer (Thermo Multiskan Ascent).

For microscopy, RTG-2 cells were seeded into the wells of 24-well plates and incubated overnight. After exposure to test compounds, growth media were removed by aspiration and replaced with 0.5 mL per well standard growth medium containing 10 μg mL^{-1} NR. After 2–3 h at 22 °C, cells were washed briefly with 0.5 mL PBS and a further 0.5 mL PBS added to each well before imaging with an inverted microscope fitted with a color CCD camera (Olympus). Experiments were repeated on three separate occasions.

2.4. Concentration–response modeling

2.4.1. Non-linear regression

NRU data were background-corrected and normalized to solvent controls. Concentration–response models were fit to experimental data using least-squares non-linear regression in Prism® ver. 6.01 (GraphPad Software Inc., La Jolla, CA, USA).

Because biphasic responses (often referred to as hormesis) were observed for some pharmaceuticals and combinations, we selected three non-monotonic concentration–response models and compared the goodness-of-fit of each model with that of a sigmoidal four-parameter log–logistic equation (Eq. (1); Hill (1913)), where y is the response; x is the concentration of the effector; ω represents the response at maximum concentration, which for survival data is usually the minimum asymptote of the response curve; α represents the response as concentration approaches zero; the ε parameter is the inflection point corresponding to the median effect level (EC_{50}); and β describes the maximum slope of the curve. One of the first models describing biphasic concentration–response relationships was the Brain–Cousens equation (Eq. (2); Brain and Cousens (1989)), which incorporates a linear stimulatory phase (represented by the function φx) into the log–logistic equation. In this equation, the ε parameter can no longer be interpreted as the EC_{50} but does provide a lower bound for the EC_{50}; the other parameters retain their previous interpretations. The two other biphasic models implemented here were the Cedergreen equation (Eq. (3); Cedergreen et al. (2005)), which incorporates a non-linear stimulatory phase, replacing the φx term in the Brain–Cousens equation with $\varphi \times \exp(-1/x^a)$, and the Beckon equation, which is a combination of two log–logistic models, one of which describes the stimulatory phase of the concentration–response relationship and the other the inhibitory phase (Eq. (4); Beckon et al. (2008)). Parameter nomenclature for the equations presented here is the same that used by Beckon et al. (2008), with the exception of the