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Assessment of toxicity and genotoxicity of low doses of 5-fluorouracil in zebrafish (*Danio rerio*) two-generation study

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ABSTRACT

Residues of anti-neoplastic drugs represent new and emerging pollutants in aquatic environments. Many of these drugs are genotoxic, and it has been postulated that they can cause adverse effects in aquatic ecosystems. 5-Fluorouracil (5-FU) is one of the most extensively used anti-neoplastic drugs in cancer therapy, and this article describes the results of the first investigation using a two-generation toxicity study design with zebrafish (*Danio rerio*). Exposure of zebrafish to 5-FU (0.01, 1.0 and 100 μg/L) was initiated with adult zebrafish (F0 generation) and continued through the hatchings and adults of the F1 generation, and the hatchings of the F2 generation, to day 33 post-fertilisation. The exposure did not affect survival, growth and reproduction of the zebrafish; however, histopathological changes were observed in the liver and kidney, along with genotoxic effects, at all 5-FU concentrations. Increases in DNA damage determined using the comet assay were significant in the liver and blood cells, but not in the gills and gonads. In erythrocytes, a significant, dose-dependent increase in frequency of micronuclei was observed at all 5-FU concentrations. Whole genome transcriptomic analysis of liver samples of F1 generation zebrafish exposed to 0.01 μg/L and 1 μg/L 5-FU revealed dose-dependent increases in the number of differentially expressed genes, including up-regulation of several DNA-damage-

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responsive genes and oncogenes (i.e., *jun*, *myca*). Although this chronic exposure to environmentally relevant concentrations of 5-FU did not affect the reproduction of the exposed zebrafish, it cannot be excluded that 5-FU can lead to degenerative changes, including cancers, which over long-term exposure of several generations might affect fish populations. The data from this study contribute to a better understanding of the potential consequences of chronic exposure of fish to low concentrations of anti-neoplastic drugs, and they demonstrate that further studies into multi-generation toxicity are needed.

1. Introduction

Potential risks associated with the release of pharmaceuticals into the aquatic environment are an important issue for environmental regulators and for the pharmaceutical industry. Pharmaceuticals predominantly enter the aquatic environment via the effluent from hospital and wastewater treatment plants and as landfill leachates, and to a minor extent in discharge from the pharmaceutical industry. Due to the ubiquitous presence of pharmaceuticals in the environment that has arisen from their continual input into the aquatic compartment, they are considered as 'pseudo'-persistent pollutants (Hernando et al., 2006). The concentrations of the residues of pharmaceuticals in the environment are relatively low compared to other pollutants, which has led to the belief that these compounds have no environmental impact on living organisms. Also, with ecotoxicological data often based on acute toxicity at high concentrations, these have been considered to be far greater than the actual concentrations in the environment. However, based on their therapeutic functions and mechanisms of action, certain groups of pharmaceuticals are suspected to represent risks for non-target organisms, even at concentration of a few nanogrammes per litre, particularly under conditions of chronic exposure (Johnson et al., 2008).

One such group of pharmaceuticals is the cytostatic anticancer drugs. Their main purpose is to prevent uncontrolled proliferation of cancer cells, through inhibition of cell growth or through inducing the death of cancer cells via interactions with DNA function and cell signalling. Due to their mechanisms of action, many cytostatics are classified as mutagenic, carcinogenic and/or toxic to reproductive systems, and it can be assumed that they can elicit these effects in exposed non-target aquatic organisms (Johnson et al., 2008; Lenz et al., 2007).

The fluoropyrimidine 5-fluorouracil (5-FU) is an antimetabolite that exerts its anticancer effects through inhibition of DNA synthesis and replication, by inhibition of thymidylate synthase, and by incorporation of 5-FU metabolites into RNA and DNA (Longley et al., 2003). 5-FU was introduced as a chemotherapeutic agent more than 50 years ago, and is still the cornerstone of most of the currently applied regimens for the treatment of patients with cancers of the gastrointestinal tract, breast, and head and neck (van Kuilenburq and Maring, 2013). Therefore, it is not surprising that 5-FU is one of the most consumed of the cytostatic drugs (Besse et al., 2012). The predicted environmental concentrations have been for 5-FU in different EU countries calculated to be from 2.5 ng/L to 8 ng/L (Straub, 2010; Besse et al., 2012; Johnson et al., 2008).

Ecotoxicity and environmental occurrence of 5-FU have been extensively studied over the last 10 years or so. In reproduction tests with the alga *Pseudokirchneriella subcapitata* and the cyanobacteria *Anabaena flos-aquae*, 5-FU was shown to be highly toxic (half maximal effective concentration [EC_{50}], ~0.12 mg/L and ~0.024 mg/L, respectively) (Brezovsek et al., 2014; Straub, 2010). Much lower toxicity of 5-FU was reported for an acute toxicity assay with a crustacean (EC_{50}, 21 mg/L), while in a chronic reproduction assay in this crustacean, 5-FU was highly toxic with the 'no observed effect concentration' at ~0.002 mg/L (Parrella et al., 2014). In zebrafish (*Danio rerio*), 5-FU was not toxic after acute exposure and showed low toxicity through a chronic 35-day early life-stage toxicity assay (Straub, 2010). Based on these data, and taking into account the predicted environmental concentrations according to the guidelines on the environmental risk assessment of medicinal products for human use (European Medicines Agency, 2006), Straub (2010) concluded that 5-FU does not represent a significant risk to aquatic environments.

However, 5-FU is genotoxic. In mammalian test systems, it has been shown to induce chromosomal damage *in vivo* (Ohuchida et al. 1992) and *in vitro* (Lorge et al., 2006; Oka et al., 2006). It is well known that exposure of aquatic organisms to genotoxic compounds can lead to genetic alterations, even at very low concentrations. In aquatic organisms living in water that is polluted with genotoxic contaminants, numerous studies have shown genotoxic effects (i.e., DNA damage, micronuclei, mutations) (Bolognesi and Hayashi, 2011; Frenzilli et al., 2009), and in fish and mussels, tumours have also been reported (Pinkney et al., 2011; Myers et al., 2003). Chronic exposure to genotoxic contaminants in aquatic organisms might cause gamete loss, decreased reproductive success, embryonic mortality, abnormal development, and changes in genetic diversity (Anderson and Wild, 1994; Fassbender and Braunbeck, 2013).

The present study was conducted to determine whether continuous exposure of zebrafish to 5-FU (0.01, 1.0 and 100 μg/L) through two subsequent generations can induce genotoxic effects, changes in gene expression, histopathological changes, and/or have effects on reproduction and fitness. This exposure to 5-FU was initiated with adult zebrafish, and then continued on through the hatchlings and adults of the F1 generation, and the hatchlings of the F2 generation,