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# Chronic environmentally relevant levels of simvastatin disrupt embryonic development, biochemical and molecular responses in zebrafish (*Danio rerio*)

Susana Barros[a], Rosa Montes[b], José Benito Quintana[b], Rosario Rodil[b], Ana André[a], Ana Capitão[a], Joana Soares[a], Miguel M. Santos[a,c,*], Teresa Neuparth[a,*]

[a] CIMAR/CIIMAR—Interdisciplinary Centre of Marine and Environmental Research, Endocrine Disruptors and Emerging Contaminants Group, University of Porto, Avenida General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal
[b] Department of Analytical Chemistry, Nutrition and Food Sciences, IIAA—Institute for Food Analysis and Research, University of Santiago de Compostela, Constantino Candeira S/N, 15782 Santiago de Compostela, Spain
[c] FCUP - Department of Biology, Faculty of Sciences, University of Porto (U. Porto), Porto, Portugal

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ABSTRACT

Simvastatin (SIM), a hypocholesterolaemic compound, is among the most prescribed pharmaceuticals for cardiovascular disease prevention worldwide. Several studies have shown that acute exposure to SIM causes multiple adverse effects in aquatic organisms. However, uncertainties still remain regarding the chronic effects of SIM in aquatic ecosystems. Therefore, the present study aimed to investigate the effects of SIM in the model freshwater teleost zebrafish (*Danio rerio*) following a chronic exposure (90 days) to environmentally relevant concentrations ranging from 8 ng/L to 1000 ng/L. This study used a multi-parameter approach integrating distinct ecologically-relevant endpoints, i.e. survival, growth, reproduction and embryonic development, with biochemical markers (cholesterol and triglycerides). Real Time PCR was used to analyse the transcription levels of key genes involved in the mevalonate pathway (*hmgcra*, *cyp51*, and *dhcr7*). Globally, SIM induced several effects that did not follow a dose-response relationship; embryonic development, biochemical and molecular markers, were significantly impacted in the lower concentrations, 8 ng/L, 40 ng/L and/or 200 ng/L, whereas no effects were recorded for the highest tested SIM levels (1000 ng/L). Taken together, these findings expand our understanding of statin effects in teleosts, demonstrating significant impacts at environmentally relevant concentrations and highlight the importance of addressing the effects of chemicals under chronic low-level concentrations.

## 1. Introduction

In the past, pharmaceuticals were overlooked as aquatic pollutants because exposure levels were considered to be too low to induce significant effects in non-target organisms (Arnold et al., 2014; Daughton, 2016; EEA, 1999). However, from the mid-90 s, a growing attention has been devoted to this class of compounds. Indeed, the detection of pharmaceuticals in the aquatic environments has increased in the last years, not only because of the pharmaceutical industry growth, but also due to improvements of analytical methods (LaLone et al., 2014). Most pharmaceuticals are detected in surface waters at trace levels, generally at concentrations ranging between ng/L and low μg/L levels (Arnold et al., 2014; Azzouz and Ballesteros, 2012; BIO Intelligence Service, 2013; Daughton, 2016; Fent et al., 2006). However, since they are bioactive substances, designed to produce biological effects at rather low concentrations, the scientific community is now in broad agreement that pharmaceuticals may pose a considerable environmental risk (BIO Intelligence Service, 2013; Ferreira et al., 2009; Rodrigues et al., 2006). In fact, several pharmaceuticals have been demonstrated to induce effects at environmentally relevant concentrations in non-target organisms (Arnold et al., 2014; Fent et al., 2006; Neuparth et al., 2014). Nevertheless, the ecological risk assessment of pharmaceuticals is still in its infancy with studies dealing with this class of compounds reporting mostly acute or sub-lethal toxicity effects, with concentrations above environmental relevance (Dahl et al., 2006; Fent et al., 2006; Neuparth et al., 2014; Santos et al., 2016; Sária et al., 2011). Hence,

* Corresponding authors at: CIMAR/CIIMAR—Interdisciplinary Centre of Marine and Environmental Research, Endocrine Disruptors and Emerging Contaminants Group, University of Porto, Avenida General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal.
*E-mail addresses:* santos@ciimar.up.pt (M.M. Santos), tneuparth@ciimar.up.pt (T. Neuparth).

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given that non-target aquatic organisms may be continuously exposed to low levels of this class of compounds for several generations, there is an urging need to assess the chronic effects of environmentally relevant concentrations of pharmaceuticals (Arnold et al., 2014; BIO Intelligence Service, 2013; Fent et al., 2006).

Simvastatin (SIM) is a hypolipidemic drug of the statin class used in humans as the primary treatment of hypercholesterolemia to decrease serum LDL-cholesterol levels (Burg and Espenshade, 2011; Igel et al., 2001; Neuparth et al., 2014; Nicolás Vázquez et al., 2017). Simvastatin, as well as other statins, is known to specifically inhibit the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), which is essential for the *de novo* synthesis of cholesterol in mammalian cells through the mevalonate pathway (Al-Habsi et al., 2016; Blumenthal, 2000; Endo et al., 1976a, 1976b; Fent et al., 2006; Sehayek et al., 1994). Simvastatin competes with 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) for the active binding site in the enzyme HMGCR, having an affinity of about three orders of magnitude greater than the natural substrate. Once bound to the enzyme, statins alter its conformation, inhibiting its function and thereby decreasing the cholesterol synthesis (Al-Habsi et al., 2016; Istvan, 2003; Moghadasian, 1999).

Statins, including SIM, have been reported to be among the most prescribed human pharmaceuticals in the western countries (Ellesat et al., 2010; Kaufman et al., 2002; Miao and Metcalfe, 2003; Neuparth et al., 2014; Walley et al., 2005). Due to the lack of mechanisms that ensure complete removal of statins in wastewater treatment plants (WWTP), significant amounts of these pharmaceuticals are discharged in the aquatic environments (Fent et al., 2006; Lapworth et al., 2012). Several authors have reported the presence of SIM in WWTPs worldwide (Kasprzyk-Hordern et al., 2009; Miao and Metcalfe, 2003; Ottmar et al., 2012; Pereira et al., 2015, 2016; de Sousa, 2013; Verlicchi et al., 2012). Concentrations up to 8.9 µg/L and 1.23 µg/L (influents) and 1.5 µg/L and 90 ng/L (effluents), were reported in WWTPs in Portugal and USA, respectively (Ottmar et al., 2012; Pereira et al., 2016). The predicted environmental concentrations for SIM in Norwegian and Portuguese surface waters have been estimated at 630 ng/L and 369.8 ng/L, respectively (Grung et al., 2007; Pereira et al., 2015). Considering that SIM is a common prescribed drug in western countries, and its discharge into aquatic environment has increased in the last years, many aquatic taxa might be at risk. This is particularly true given that statins were predicted to inhibit HMGR in a broad range of animal *taxa* (Santos et al., 2016). In fact, the main concern regarding the presence of SIM in the aquatic ecosystems is its environmental persistence, toxicity, and bioactivity, at rather very low concentrations. Furthermore, SIM has a high log $K_{ow}$ of 4.68, which might indicate high bioaccumulation potential in aquatic organisms (Santos et al., 2016). Previous studies have reported multiple detrimental effects of SIM in aquatic organisms at several levels of biologic organization, such as impairment of embryo development (*Danio rerio* and *Parachentrotus lividus* – Ribeiro et al., 2015), decreased metabolic activity and membrane stability (*Oncorhynchus mykiss* hepatocyte – Ellesat et al., 2010), alteration of acetylcholinesterase and lipid peroxidation levels (*Fundulus heteroclitus* – Key et al., 2009; *Palaemonetes pugio* – Key et al., 2008), disturbances in growth (*Dunaliella tertiolecta, Nitocra spinipes* and *Gammarus locusta* – Dahl et al., 2006; DeLorenzo and Fleming, 2008; Neuparth et al., 2014), severe reproductive impairments (*Gammarus locusta* – Neuparth et al., 2014), and even mortality (*Plaemonetes pugio* – Key et al., 2008). With the exception of the Neuparth et al (2014) study that reported chronic effects of SIM in the ng/L range, all the aforementioned studies have been based on acute toxicity tests, with SIM concentrations above environmental relevance.

Despite the diversity of studies regarding SIM toxicity, the underlying mechanisms of action in aquatic organisms are still not fully understood (Gee et al., 2015). Acknowledging the mode of action (MOA) of SIM in non-target aquatic organisms is important not only to establish its ecotoxicological response upon SIM exposure, but also to anticipate the effects of other compounds acting through homologous pathways. This is particularly relevant for bioactive molecules designed to produce biological effects at very low concentrations, such as pharmaceuticals. Hence, there is an urgent need to perform long-term, low level exposure assays, that integrate effects of ecologically-relevant endpoints with molecular and biochemical responses.

Therefore, the main aim of the present study was to evaluate SIM effects on zebrafish, following a chronic partial life-cycle exposure to environmentally relevant concentrations (ng/L) and early offspring embryonic development effects of parental SIM exposure. We integrate multiple key ecological endpoints (survival, growth, reproduction, and embryonic development), with biochemical markers of lipid homeostasis (cholesterol and triglycerides) and molecular analysis (expression of key genes coding for modules of the mevalonate pathway: *hmgcra*, *cyp51*, and *dhcr7*), to gain insights into the long-term adverse effects of SIM on ecologically-relevant endpoints and to address the underlying mechanism of toxicity in fish.

## 2. Material and methods

### 2.1. Species selection

Zebrafish *(Danio rerio)* is recommended as a test species in a wide range of ecotoxicological test protocols (Oberemm, 2000). Its small size, robustness, multiple progeny from a single mating, embryo transparency and easy maintenance under laboratory conditions are advantages for its use as test organism (Fang and Miller, 2012; Lawrence, 2007; Soares et al., 2009). In addition, the close phylogeny of zebrafish and mammals, with highly conserved mevalonate pathway genes, makes this species ideal for performing the present study.

### 2.2. Zebrafish maintenance

Wild-type zebrafish, 50-day old, were obtained from Orniex, Portugal (purchased from local suppliers in Singapore). Animals were acclimated to controlled laboratory conditions, in 250 L aquarium with dechlorinated filtered and aerated water. During this period, fish were kept at 28 ± 1 °C, under a photoperiod of 14:10 h (light:dark) and fed, *ad libitum*, three times per day with commercial fish diet Tetramin (Tetra, Melle, Germany). These conditions were maintained for 15 days until the beginning of the chronic bioassay.

### 2.3. Chronic toxicity bioassay

The chronic bioassay was carried out at "Biotério de organismos aquáticos" (BOGA) located at Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Matosinhos, Portugal. The experiment was subject to a previous ethical review process carried out by CIIMAR animal welfare body (ORBEA, 2010/63/EU Directive). The bioassay was performed in compliance with the European Directive 2010/63/EU, on the protection of animals used for scientific purposes, and the Portuguese "Decreto Lei" 113/2013.

A partial life-cycle bioassay was performed for 90 days (Fig. 1). The experiment was started by randomly allocating 25 sub-adult zebrafish in 30 L aquaria (two per treatment), under a flow-through system. The water flow was maintained at 1.08 L per hour by means of a peristaltic pump (ISM 444, ISMATEC) supplied with dechlorinated, heated and charcoal filtered tap water. Each aquarium was maintained with a water temperature of 28 ± 1 °C, 14:10 h (light:dark) photoperiod, pH 7.5 ± 0.2 and a mean ammonia concentration of 0.08 ± 0.04 mg/L. Fish were fed twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany), supplemented with 48-h-old live brine shrimp (*Artemia* spp.) from one week before the onset of reproduction until the end of the experiment. During the bioassay, the amount of food delivered was adjusted according to fish development and size. The amount of food provided was equal for all aquaria.

The experiment consisted of six treatments in duplicate: a control