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Cytotoxicity of atorvastatin and simvastatin on primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes

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ABSTRACT

Statins are cholesterol-lowering pharmaceuticals and commonly prescribed drugs in European countries. Their discharge into the aquatic environment has increased in the last few years and they are present at detectable levels in most sewage effluents. The aim of the present study was to quantify the cytotoxic effects of acid and lactone forms of two statins, atorvastatin and simvastatin, as well as selected metabolites (*ortho*- and *para*-hydroxy atorvastatin acid, *ortho*-hydroxy atorvastatin lactone, simvastatin hydroxyl carboxylic acid, and 3″hydroxy simvastatin lactone) to hepatocytes from rainbow trout (*Oncorhynchus mykiss*). Hepatocytes were exposed for 24, 48, and 72 h to different concentrations of each test substance (0.4–400 μM). Cytotoxicity was measured as metabolic inhibition and loss of membrane integrity with the fluorescent probes alamar blue (AB) and 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA–AM), respectively. Atorvastatin, simvastatin, and *ortho*-hydroxy atorvastatin lactone had dose-dependent cytotoxic effects on hepatocytes. Simvastatin was more toxic than atorvastatin and the lactone form more toxic than the acid form. Exposure time affected atorvastatin and *ortho*-hydroxy atorvastatin lactone but not simvastatin toxicity.

1. Introduction

Pharmaceuticals have received increasing attention as emerging aquatic pollutants in the last decade (Besse and Garric, 2008; Sanderson et al., 2003). Although most pharmaceuticals are designed to act in the human body, it is likely that non-target organisms that possess similar biochemical pathways will also be affected (Fent et al., 2006).

Statins, or HMGR (3-hydroxy-3-methylglutaryl coenzyme A reductase) inhibitors, are among the most prescribed human pharmaceuticals in western European countries and reach the aquatic environment in increasing concentrations (Walley et al., 2005). They pass waste-water treatment plants as the parent compounds or as metabolites (Metcalfe et al., 2003). Predicted environmental concentrations (PECs) for atorvastatin and simvastatin in Norwegian aquatic environments have been estimated to 0.25 and 0.63 μg/L, respectively (Grung et al., 2007).

Statins are human pharmaceuticals that block the mevalonic acid (MVA) pathway and consequently cholesterol biosynthesis (Endo et al., 2004). The rate limiting step in this pathway is the competitive inhibition of the enzyme HMGR. Statins are used to lower plasma cholesterol levels and are prescribed against cardiovascular disease (Davignon, 2004). Negative effects have been reported in combination with statin use (Evans and Rees, 2002; Kiortsis et al., 2007) with the most common unwanted effects being myotoxicity and hepatotoxicity in humans, an effect that occurs in 1–7% of statin-treated patients (Sirvent et al., 2005).

Most statins are administered in the biologically active acid form except for simvastatin and lovastatin that are administered as the lactone prodrug (Fujino et al., 2004). *In vivo*, the acid and the lactone forms interconvert in plasma until equilibrium is reached (Kearney et al., 1993). The lactone prodrug generally has higher toxicity than the biologically active acid form of statins, presumably because of its higher potential for passive diffusion across cell membranes (Skottheim et al., 2008). However, the full mechanism of statin toxicity is not yet understood (Evans and Rees, 2002).

Intracellularly, statins are metabolized by cytochrome P-450 (CYP) enzymes, mainly CYP3A4 in humans (Fujino et al., 2004; Kantola et al., 1998; Prueksaritanont et al., 1997). The proposed

Abbreviations: HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; PEC, predicted environmental concentration; MVA, mevalonic acid; CYP, cytochrome P-450; AB, alamar blue; CFDA–AM, 5-carboxyfluorescein diacetate, acetoxymethyl ester; GLM, general linear model; OATP1B1, organic anion transporting polypeptide 1B1.

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main route of metabolism for atorvastatin and simvastatin occurs via the lactone form followed by an interconversion of the hydroxylated statin lactone metabolites to their acid forms (Jacobsen et al., 2000; Vickers et al., 1990). Most acid metabolites have been shown to contribute to HMGR inhibition (Christians et al., 1998; Hermann et al., 2005; Lins et al., 2003; Mauro, 1993).

Few studies have investigated the effect of statins on non-target organisms. Growth inhibition in the duckweed *Lemna gibba* was observed after atorvastatin and lovastatin exposure (Brain et al., 2006). Fluvastatin was shown to inhibit insect juvenile-hormone synthesis (Debernard et al., 1994) and abnormal gut formation in frog larvae was observed after exposure to atorvastatin and another statin, lovastatin (Richards and Cole, 2006). To our knowledge this is the first study investigating the toxic effect of statin metabolites on a non-target organism.

The aim of the present study was to quantify the cytotoxicity of acid and lactone forms as well as selected metabolites of atorvastatin and simvastatin to rainbow trout (*Oncorhynchus mykiss*) hepatocytes. The statin concentrations used in the present study were similar to concentrations used with human skeletal muscle cells *in vitro* (Skottheim et al., 2008). The cytotoxic effect of the statins was measured after 24, 48, and 72 h of exposure. The toxic impact of atorvastatin and simvastatin in both forms and their metabolites were determined for primary hepatocytes from juvenile and/or adult fish *in vitro*.

2. Material and methods

2.1. Chemicals

Atorvastatin acid (CAS 134523-03-8, purity 98%) and lactone (CAS 125995-03-1, purity 98%), simvastatin acid (CAS 139893-43-9, purity 98%) and lactone (CAS 79902-63-9, purity 98%), *ortho*-hydroxy atorvastatin lactone (CAS 163217-74-1, purity 98%), and 3″hydroxy simvastatin lactone (CAS 126313-98-2, purity 98%) were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). *Para*-hydroxy atorvastatin acid (CAS 214217-88-6, purity 98%), *ortho*-hydroxy atorvastatin acid (CAS 214217-86-6, purity 98%), and simvastatin hydroxyl carboxylic acid (CAS 170729-80-3, purity 98%) were purchased from Varda Biotech (Mumbai, India). The parent compounds of statins were dissolved in 99.9% dimethyl sulfoxide (DMSO, CAS 67-68-5) from Sigma–Aldrich Norway AS (Oslo, Norway) and the metabolites in waterfree DMSO. The stock solutions were stored in the dark at −20 °C until use. Alamar blue (AB) and 5-carboxyfluorescein diacetate, actetoxymethyl ester (CFDA–AM) were obtained from Molecular Probes (Paisley, United Kingdom). CFDA–AM was dissolved in DMSO (4 mM).

2.2. Fish

Juvenile and adult rainbow trout (*O. mykiss*, body lengths 15–20 cm and 30–35 cm, respectively) were obtained from Valdres Oppdrettsanlegg (Leira, Norway) and kept in tanks at the Department of Biology, University of Oslo, Norway. Water temperature in the tanks was 8–10 °C, oxygen saturation approximately 100%, pH 6.6 and an artificial photoperiod (100 lux) of 12:12 h light:dark was used. Fish were fed daily with commercial fish pellets (Skretting Averøy, Averøy, Norway) in amounts corresponding to 0.5% body mass.

2.3. Liver perfusion and hepatocyte culturing

Isolation of rainbow trout hepatocytes were done as described in Tollefsen et al. (2008a,b). Fish were sacrificed with a blow on the head and the liver was perfused (10 mL/min, 10–15 min) with a calcium-free buffer (perfusion buffer) containing NaCl (122 mM), KCl (4.8 mM), $MgSO_4$ (1.2 mM), Na_2HPO_4 (3.3 mM), $NaHCO_3$ (3.7 mM), and *ethylene glycol tetraacetic acid*, EGTA (26 μM, CAS 67-42-5), pH 7.5, until all blood was removed. The liver was then perfused (10 mL/min, 10–15 min) with the same buffer containing 0.3 mg/mL collagenase (type IV, Sigma–Aldrich Norway AS, Oslo, Norway, CAS 9001-12-1) and $CaCl_2$ (1.5 mM) instead of EGTA and heated to 37 °C, the optimum for collagenase activity. Following the perfusion, the liver was removed and the cells were dispersed in an ice-cold EGTA free perfusion buffer containing 0.1% (w/v) bovine serum albumin, BSA (Sigma–Aldrich Norway AS, Oslo, Norway, CAS 9048-46-8). The cell solution was then filtered through sterile 250 μm and 100 μm nylon meshes before centrifuged three times at 50*g* for 3–4 min at 4 °C. The cells were then resuspended in serum-free L-15 medium containing L-glutamine (0.29 mg/mL, CAS 56-85-9), $NaHCO_3$ (4.5 mM, CAS 144-55-8), penicillin (100 Units/mL, CAS 69-57-8), streptomycin (100 μg/L, CAS 3810-74-0), and amphotericin (0.25 μg/mL, 1397-89-3), all supplied by Cambrex (East Rutherford, NJ, USA), and filtered once more through a sterile 100 μm nylon mesh. The cells were tested for viability with the trypan blue exclusion test (trypan blue, CAS 72-57-1) and only utilized for experiments if the viability was larger than 90%. The cells were diluted to 0.5 million cells/mL. The cells were then seeded out into 96-well Primaria® plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) at a density of 0.1 million cells/well and kept at 15 °C in an ambient atmosphere. Retention of cells in wells through the experiment was checked by microscopy. All glassware and instruments were autoclaved before use and solutions were sterilized by filtration (0.22 μm).

2.4. Exposure conditions

Cells were maintained for 24 h before half of the culture medium was replaced with new medium containing the statins. Isolated hepatocytes were treated in two series of experiments: cells from adult fish were exposed to the parent compounds, whereas hepatocytes from juvenile rainbow trout were exposed to either the parent compounds of statins or their metabolites. Final statin concentrations in the exposed wells were 3.13, 6.25, 12.5, 25, 50, 100, 200, and 400 μM from the lowest to the highest concentration (corresponding to approximately 1–200 mg/L) for the parent compounds and 0.39, 0.78, 1.56, 3.13, 12.5, 25, and 50 μM (corresponding to approximately 0.1–30 mg/L) for the statin metabolites. Cells were exposed to each concentration in triplicate wells. Statin stock solutions were made in DMSO followed by serial dilution of each compound. The final vehicle concentrations for the different treatment groups were not equal and constituted <1% for the parent compounds, and <2.7% for the metabolites, with no apparent toxic effects. The vehicle was used as control and a range of concentrations of copper ($CuSO_4 \times 5H_2O$, CAS 7758-99-8, highest concentration 0.64 mg/mL) as a reference for toxicity. The cells were exposed for 24, 48, and 72 h prior to assessment of cytotoxicity.

2.5. Cytotoxicity measurement

The cytotoxic effects of statins on trout hepatocytes were determined by the fluorescent probes AB and CFDA–AM to measure metabolic activity and membrane stability in the cells, respectively. This method was previously developed for primary rainbow trout hepatocytes (Schreer et al., 2005) and modified for the current experiment as described in Tollefsen et al. (2008a).

All culture medium was removed from the wells and 100 μL Tris buffer (50 mM, pH 7.5) containing 5% AB and 4 μM CFDA–AM was added. The use of cell-binding culture plates (96-well