Aquatic Toxicology 128–129 (2013) 113–123

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Mode of action of human pharmaceuticals in fish: The effects of the 5-alpha-reductase inhibitor, dutasteride, on reproduction as a case study

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ARTICLE INFO

Article history:
Received 17 September 2012
Received in revised form
27 November 2012
Accepted 2 December 2012

Keywords:
Pharmaceutical
Anti-androgen
5-Alpha reductase
Fish
Read-across
Reproductive toxicology

ABSTRACT

In recent years, a growing number of human pharmaceuticals have been detected in the aquatic environment, generally at low concentrations (sub-ng/L–low μg/L). In most cases, these compounds are characterised by highly specific modes of action, and the evolutionary conservation of drug targets in wildlife species suggests the possibility that pharmaceuticals present in the environment may cause toxicological effects by acting through the same targets as they do in humans. Our research addressed the question of whether or not dutasteride, a pharmaceutical used to treat benign prostatic hyperplasia, may cause adverse effects in a teleost fish, the fathead minnow (*Pimephales promelas*), by inhibiting the activity of both isoforms of 5α-reductase (5αR), the enzyme that converts testosterone into dihydrotestosterone (DHT). Mammalian pharmacological and toxicological information were used to guide the experimental design and the selection of relevant endpoints, according to the so-called "read-across approach", suggesting that dutasteride may affect male fertility and steroid hormone dynamics. Therefore, a 21-day reproduction study was conducted to determine the effects of dutasteride (10, 32 and 100 μg/L) on fish reproduction. Exposure to dutasteride significantly reduced fecundity of fish and affected several aspects of reproductive endocrine functions in both males and females. However, none of the observed adverse effects occurred at concentrations of exposure lower than 32 μg/L; this, together with the low volume of drug prescribed every year (10.34 kg in the UK in 2011), and the extremely low predicted environmental concentration (0.03 ng/L), suggest that, at present, the potential presence of dutasteride in the environment does not represent a threat to wild fish populations.

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1. Introduction

In recent years, a growing number of human pharmaceuticals have been detected in the aquatic environment, generally at low concentrations (sub-ng/L–low μg/L). In most cases, these compounds are characterised by highly specific modes of action (MoA), high potency, persistence and prolonged activity in order to minimise dosing requirements and potential toxicity in patients (Winter et al., 2010).

Generally, it is possible to hypothesise that these compounds will be pharmacologically active in organisms in which the drug targets are expressed and functional; therefore, the presence of conserved targets in a given species could potentially increase the risk of eco-toxicological effects (Gunnarsson et al., 2008; Seiler, 2002). Gunnarsson et al. (2008) predicted the presence of evolutionary conserved human drug targets in 16 species from different taxonomic groups, showing that the majority of the drug targets are conserved in aquatic vertebrates (i.e. up to 86% in fish), and to a lesser extent in invertebrates and plants (i.e. 61% in Daphnia and 35% in green algae). As a consequence, one of the first steps of the environmental risk assessment (ERA) process should be the translation of the knowledge of a pharmaceutical's MoA and toxicity in mammals into predictions on its MoA and toxicity in the non-target species showing a conserved drug target.

In order to perform this step and to enhance the reliability of the ERA, all relevant data should be taken into account, including the information generated during pre-clinical and clinical studies in mammals during the drug development phase (Berninger and Brooks, 2010; Winter et al., 2010). These data should include physical–chemical properties, primary and secondary pharmacodynamics, toxicology, metabolism, excretion, degradability, and persistence of the active pharmaceutical ingredient (API) and/or of the relevant metabolites (e.g. pharmacologically active metabolites) (EMEA, 2006).

These pieces of information can then be integrated with the knowledge of the physiology of the species used as the experimental model. A key role in this process is played by bioinformatics databases, available for a growing number of environmentally

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http://dx.doi.org/10.1016/j.aquatox.2012.12.003

relevant species. These databases can provide valuable information not only on the degree of evolutionary and functional conservation of drug targets, but also on the physiological, biochemical, or molecular pathways that could potentially be affected by the interaction with a specific target (Gunnarsson et al., 2008; Kostich and Lazorchak, 2008; Seiler, 2002; Winter et al., 2010).

Finally, this background knowledge can be used to make predictions and to tailor the experimental design of the eco-toxicity tests to the species used as the experimental model; for example, by selecting specific endpoints that are relevant to the MoA, or by choosing an appropriate duration of exposure to the drug (Ankley et al., 2007; Christen et al., 2010; Owen et al., 2007; Panter et al., 2012).

The conceptual approach described above is generally defined as "read-across" or "intelligent testing strategy", and at other times, more specifically, "mode-of-action toxicology". In our study, we used this approach to address the question of whether or not dutasteride, a human pharmaceutical mainly used to treat benign prostatic hyperplasia, may cause adverse effects in a teleost fish, the fathead minnow (*Pimephales promelas*), by inhibiting the activity of both isoforms of 5α-reductase (5αR), the enzyme that converts testosterone (T) into dihydrotestosterone (DHT). This drug was considered as a case study since a general assumption in fish endocrinology is that DHT is not synthesised in teleosts, or if it is, it has modest or no physiological relevance (Borg, 1994); therefore, the question arising was: "why study the effects of dutasteride, a 5αR inhibitor, in fathead minnow, if this species seems to lack the biochemical apparatus targeted by the drug?"

However, the screening of bioinformatics databases combined with a critical review of the literature led us to hypothesise that the genes coding for both isoforms of 5αR were expressed in the fathead minnow. In fact, in our previous work, we confirmed that the genes coding for the targets of dutasteride (*srd5a1* and *srd5a2*) are expressed in fish tissues (i.e. testis), both genes show a medium to high degree of similarity to the human isoforms (respectively 78% for *srd5a1* and 65% for *srd5a2*), and that 5αR is also functionally conserved, as demonstrated by the detection of DHT in fathead minnow plasma by LC–MS/MS (Margiotta-Casaluci et al., submitted for publication). Furthermore, DHT had an in vivo androgenic potency comparable to that of the putative fish androgen, 11-ketotestosterone (KT), in juvenile fathead minnow males, and higher than the potency of KT in females (e.g. it induced severe disruption of ovarian physiology and morphology, including the development of spermatogenic tissue) (Margiotta-Casaluci and Sumpter, 2011).

This set of evidence constituted the rationale for testing the effects of dutasteride, a dual 5αR inhibitor, in the fathead minnow.

2. Materials and methods

2.1. Test species

Fathead minnows were supplied from breeding stocks maintained at Brunel University, London, UK. Two weeks before the beginning of the study, sexually mature males and females were separated to prevent any spawning activity and acclimated to the test conditions. Fish were fed three times per day, once with adult brine shrimp (Tropical Marine Centre, Gamma irradiated) and twice with flake food (King British Tropical flake food, Lillico, Surrey). Fish were not fed on the sampling day. The test was run at a nominal water temperature of 25 ± 1 °C, with a photoperiod of 16 h light:8 h of dark, and with 20 min dawn/dusk transition periods. The light intensity at the surface of the test vessels was measured by a lux metre (Lutron, LX-101) once a week during the study, and was 740 ± 120 lux (n = 6).

2.2. Test substances and dilution water

The test substance, dutasteride (CAS number 164656-23-9), was obtained from GlaxoSmithkline (GSK) as 99.9% pure. Concentrated stock test solutions were prepared in N,N-dimethylformamide (DMF; CAS number 68-12-2; ≥99%) (Sigma, Poole, UK). One master concentrated stock solution (10.0 g/L) was made in DMF, stored at +4 °C, and used to make up fresh dosing stock solutions at 0.4, 1.28, and 4 g/L. Fresh stock solutions were made up weekly, in order to avoid potential degradation in the stock bottle. Dechlorinated tap water (5 and 10 μm carbon filtered) was used as dilution water, and general parameters (pH, temperature and dissolved oxygen) were monitored daily throughout the study. Water pH ranged from 7.4 to 8.1, temperature from 24.79 to 26.12 °C, and dissolved oxygen from 7.06 to 7.95 mg/L.

2.3. 21-Day reproduction study

The 21-day reproduction study was performed according to the method proposed by Harries et al. (2000) and Ankley et al. (2001), and subsequently adapted by Winter et al. (2008). The experiment was carried out using a continuous flow-through system comprised of 10.5 L glass fish tanks containing a spawning substrate. The latter consisted of a U-shaped PVC tile fitted above a glass collection tray covered with a 0.5 cm^2 stainless steel mesh. The tile was used as the nest-site for spawning, while any eggs that fell off the tile were collected in the tray underneath; the mesh allowed eggs to be trapped in the tray, without them being affected by the fish. Screens were placed between all test vessels to prevent fish visually interacting with those in neighbouring tanks and to minimise disturbance due to operator movements.

Thermostatically heated (25 ± 1 °C) dechlorinated tap water from a header tank flowed through six flow-metres into six glass mixing chambers at a rate of 600 mL/min. The same chambers also received the stock solution of the test chemical in DMF at a rate of 0.015 mL/min, in order to achieve the desired nominal concentrations. Separate lines from each mixing chamber supplied about 12 tank volume changes per day to each of eight replicate test tanks, each containing one male and one female fish (8 males and 8 females per treatment). The final experimental setup included five groups: one dilution water control group (DWC), one solvent control group (SC), and three groups exposed to dutasteride (10, 32 and 100 μg/L). The SC vessel received the same rate of addition of DMF, such that the water in all test vessels contained DMF at 0.0025% (OECD suggested limit: 0.0095%).

The reproductive performance of the fish was assessed during a 21-day pre-exposure period, a 3-day transition (when dosing of dutasteride was started to allow the system to equilibrate), and a further 21 days of exposure to dutasteride. Each day the spawning tiles and the collection trays were removed from each tank, and if eggs had been laid, the apparatus was replaced with a clean set. Under microscope observation, the numbers of fertilised, non-fertilised, and dead eggs were counted. Furthermore, fish behaviour was monitored daily (e.g. feeding behaviour and male aggressiveness), and any abnormal situations recorded.

After 21 days of exposure to dutasteride, fish were terminally anaesthetised using ethyl 3-aminobenzoate methanesulfonate salt (MS-222, 0.5 g/L; adjusted to pH 7.5 with 1 M NaOH) (Sigma, Poole, UK; CAS No: 144-55-8). Sperm samples were collected using a 5 μL graduated capillary tube (Sigma Aldrich) using the protocol described by Kime et al. (2001) and Hala et al. (2009). The collected milt was expelled into a microfuge tube and diluted 1:50 in cold Catfish extender (94 mM sodium chloride, 27 mM potassium chloride, 15 mM Trizma hydrochloric acid and 50 mM glycine pH to 7.5) and then stored at +4 °C until density and motility analyses were conducted (22 h later). Blood samples were collected from each fish

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