Environmental Pollution 206 (2015) 619–626

Contents lists available at ScienceDirect

Environmental Pollution

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

Cardiomyocyte H9c2 cells present a valuable alternative to fish lethal testing for azoxystrobin

Elsa T. Rodrigues [a, *], Miguel Â. Pardal [a], Vincent Laizé [b], M. Leonor Cancela [b, c], Paulo J. Oliveira [d], Teresa L. Serafim [d]

[a] Centre for Functional Ecology (CFE), Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal
[b] Centre of Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
[c] Department of Biomedical Sciences and Medicine (DCBM), University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
[d] Centre for Neuroscience and Cell Biology (CNC), University of Coimbra Biotech Building, Lot 8A, Biocant Park, 3060-197 Cantanhede, Portugal

ARTICLE INFO

Article history:
Received 25 March 2015
Received in revised form
4 August 2015
Accepted 17 August 2015
Available online xxx

Keywords:
Aquatic ecotoxicology
Azoxystrobin
Alternative assays
Cell-based assays
Cytotoxicity
SRB assay

ABSTRACT

The present study aims at identifying, among six mammalian and fish cell lines, a sensitive cell line whose *in vitro* median inhibitory concentration (IC_{50}) better matches the *in vivo* short-term *Sparus aurata* median lethal concentration (LC_{50}). IC_{50s} and LC_{50} were assessed after exposure to the widely used fungicide azoxystrobin (AZX). Statistical results were relevant for most cell lines after 48 h of AZX exposure, being H9c2 the most sensitive cells, as well as the ones which provided the best prediction of fish toxicity, with a $LC_{50,96h}/IC_{50,48h} = 0.581$. H9c2 cell proliferation upon 72 h of AZX exposure revealed a $LC_{50,96h}/IC_{50,72h} = 0.998$. Therefore, identical absolute sensitivities were attained for both *in vitro* and *in vivo* assays. To conclude, the H9c2 cell-based assay is reliable and represents a suitable ethical alternative to conventional fish assays for AZX, and could be used to get valuable insights into the toxic effects of other pesticides.

1. Introduction

Fish lethal testing is still considered a regulatory requirement to assess the potential hazard of new substances (REACH Regulation, 2006). It is also part of the Whole Effluent Assessment approach (OSPAR Convention, 2000), and is widely used in scientific research. However, concerns about animal welfare and the time and resources necessary to support fish tests highlighted the need for alternative assays (Bradbury et al., 2004). Hence, the development of these assays, such as cell-based assays, has become an important topic of interest. However, this replacement can only be effective if high-level correlations between both assays are found and similar absolute sensitivities are attained. A good correlation between *in vivo* fish results and *in vitro* fish cytotoxicity data, specifically regarding relative sensitivity, has already been confirmed (Kramer et al., 2009). However, when correspondence is considered in absolute terms, fish cells have so far proved to be less sensitive than whole fish (Castaño et al., 2003; Kramer et al., 2009). Thus, it has been stated that fish cell-based assays include a certain risk of false negative results (Castaño et al., 2003). Therefore, scientific knowledge concerning comparative studies, *in vivo*/*in vitro*, with the aim to find sensitive cell models, mammalian or fish derived, are crucial. Also, in the universe of scientific literature, there is a great amount of data concerning cytotoxicity results, even though most are related to drugs for human use and only a few are ecotoxicologically relevant. Thus, in order to accelerate the development of new alternative methods, testing environmentally relevant hazardous substances is also essential.

Pesticides are considered environmentally relevant substances,

List of acronyms and abbreviations: ΔΨm, inner mitochondrial membrane potential; AZX, azoxystrobin; CV, coefficient of variation; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; FCCP, p-trifluoromethoxy carbonyl cyanide phenyl hydrazine; FBS, foetal bovine serum; IC_{50}, median inhibition concentration; K_{OW}, octanol-water partition coefficient; LC_{50}, median lethal concentration; PBS, phosphate buffered saline; PMT, photomultiplier tube; SRB, sulforhodamine B; TMRE, tetramethylrhodamine ethyl ester perchlorate; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; ww, wet weight.

* Corresponding author.
E-mail addresses: etrodrig@zoo.uc.pt (E.T. Rodrigues), mpardal@zoo.uc.pt (M.Â. Pardal), vlaize@ualg.pt (V. Laizé), lcancela@ualg.pt (M.L. Cancela), pauloliv@cnc.uc.pt (P.J. Oliveira), tserafim@ci.uc.pt (T.L. Serafim).

http://dx.doi.org/10.1016/j.envpol.2015.08.026
0269-7491/

and strobilurin related-pesticides were developed after the discovery of a group of active natural compounds which display a potent activity against yeasts and filamentous fungi (Sauter et al., 1999). Azoxystrobin (AZX) was the first strobilurin to be patented and is currently the world's leading agricultural fungicide (Pesticide Action Network UK, 2015). The molecular target of AZX is the mitochondrial respiratory complex III, thus affecting the translocation of protons across the mitochondrial membrane by inhibiting the quinone outside center (EFSA, 2010). Some of the environmental concentrations of AZX found in aquatic systems and reported by Rodrigues et al. (2013) are above the Regulatory Acceptable Concentration (RAC, 3.3 μg L^{-1}) in what concerns risk to aquatic invertebrates and the No Observed Ecologically Adverse Effect Concentration (NOEAEC, 10 μg L^{-1}) reported for freshwater communities. Even though this fungicide is considered to have low acute and chronic toxicity to mammals, birds, and bees (Bartlett et al., 2002; EFSA, 2010; EPA, 1997), it is toxic to freshwater and estuarine/marine fish and aquatic invertebrates (EPA, 1997). However, data on AZX aquatic toxicity are scarce (Rodrigues et al., 2013), with only four fish AZX LC_{50} values available: *Oncorhynchus mykiss*: $LC_{50,96h}$ = 470 (400–5800) μg L^{-1} (EPA, 1997), *Ctenopharyngodon idella* larvae (<10 days): $LC_{50,48h}$ = 549 (419–771) μg L^{-1} (Liu et al., 2013), *Lepomis macrochirus*: $LC_{50,96h}$ = 1100 (900–1700) μg L^{-1} (EPA, 1997), and the euryhaline species *Cyprinodon variegatus*: $LC_{50,96h}$ = 670 (560–800) μg L^{-1} (EPA, 2012).

AZX toxicity has been tested using adrenocortical H295r and hepatocellular HepG2 carcinoma cell lines (Prutner et al., 2013; Rudzoka et al., 2009). The chosen endpoints were oestrone production by H295r and cytochrome P450 1A induction by HepG2. Nevertheless, none of these studies determined IC_{50s}, i.e., the concentration of AZX required to achieve 50% *in vitro* cell inhibition.

The present study proposes to compare the growth inhibition potential of AZX on mammalian (A549, HepG2, BJ and H9c2) and fish (VSa16 and ABSa15) cell lines, with the juvenile *Sparus aurata* short-term lethal test. The sulforhodamine B (SRB) colorimetric assay was used as a measure of cell proliferation after 24, 48 and 72 h of AZX incubation. The LC_{50}/IC_{50} ratio was determined for all cell lines in order to find which cell line better matches fish lethality test results. Several endpoints were then tested using the selected cell line: (1) metabolic function and cellular fitness by the resazurin reduction assay, (2) mitochondrial membrane electric potential ($\Delta\Psi m$) using the mitochondrial marker tetramethylrhodamine ethyl ester perchlorate (TMRE), and (3) mitochondrial superoxide anion radical production by mitochondria using the MitoSOX fluorescent assay. In parallel, fluorescence microscopy was performed to image $\Delta\Psi m$ and superoxide anion production in mitochondria after 48 and 72 h of AZX exposure. Outcomes of the present study are expected to promote the development of alternative testing methods, as well as achieve regulatory acceptance and implementation of *in vitro* assays.

2. Material and methods

2.1. Ethical statement

All animal experiments were conducted in accordance with the ethical guidelines of the European Union Council (Directive 2010/63/EU) and the Portuguese Agricultural Ministry (Decreto-Lei 113/2013) for the protection of animals used for experimental and other scientific purposes. The person in charge of experimental procedures with live animals has accreditation for the use of live animals for scientific purposes (category C) according to the Federation of European Laboratory Animal Science Associations (FELASA) education and training guidelines, granted by the Portuguese General Directorate of Veterinary.

2.2. Analytical standard solutions

Azoxystrobin PESTANAL analytical standard (purity 99.9%) was purchased from Sigma–Aldrich (31697). For the fish lethal test, an AZX stock solution (1 g L^{-1}) was prepared in acetone p.a. (Sigma–Aldrich 32201) and stored at −18 °C. Exposure solutions were prepared in filtered (11 μm, Whatman 1001-047) natural seawater on the day of use according to the ASTM E729 96 (2002) guideline. No analytical measurements were performed to the exposure solutions. All concentrations are, therefore, presented as nominal values. Solvent control was also prepared on the day of use with acetone (1.14 ml L^{-1}) in filtered natural seawater. For cell-based assays, AZX stock solutions were prepared in DMSO (Sigma–Aldrich D2650) and stored at 4 °C. Exposure solutions were prepared on the day of use in filtered (0.2 μm sterilized cellulose nitrate filter, Sartorius 11407-47-ACN) Dulbecco's modified Eagle's medium (DMEM)-high glucose (Sigma–Aldrich D5648) adjusted to contain 1.8 g L^{-1} of sodium bicarbonate and supplemented with 10% foetal bovine serum (FBS, Gibco 10270-106) and 1% antibiotic-antimycotic (Gibco 15240-062), at pH 7.3. Solvent control medium was prepared on the day of use with DMSO in DMEM-high glucose prepared as previously described.

2.3. Fish lethal test

Fish median lethal concentration ($LC_{50,96h}$) was determined according to ASTM E729 96 (2002) guideline using juveniles (≈5.0 g) of gilthead seabream *S. aurata* from a commercial fish farm (MARESA, Spain). Fish were maintained in a temperature-controlled room (19 ± 1 °C) under a natural light regime, and in aerated recirculating aquatic systems composed of round 200-L tanks supplied with natural seawater at 18 °C and an appropriate life support system. All fish were from the same batch and were kept under laboratory acclimation conditions for 20 days with an initial density of 3.6 kg m^{-3}. Physico-chemical acclimation conditions such as salinity, pH and dissolved oxygen were measured daily in the water using WTW probes (Weilheim, Germany). Fish were fed daily with Aqualgold 3 (Sorgal, Portugal), and were starved for the 24 h prior to the test to ensure that all animals were at a similar starting point. After the acclimation period, seven active and externally undamaged fish were selected for each of the six AZX exposure treatments performed in a geometric series with a factor of 1.5, as well as for the negative (fish in filtered natural seawater alone) and solvent controls. The selected fish, weighing from 3.6 to 10.7 g ww, were placed individually into 3-L glass flasks (15 cm ∅). Each flask was aerated using PTFE tubing (Bola S1810-18) and air filtration was carried out by using a syringe filter (0.2 μm, Sartorius 17761-Q). The test room was maintained at the same environmental conditions as the acclimation room. Since AZX is considered stable to hydrolysis (EPA, 1997), a 96-h static non-renewal test was performed. Physico-chemical experimental conditions such as salinity, pH and dissolved oxygen were measured at 0, 48 and 96 h using WTW probes. Fish behaviour and mortality were monitored daily. At the end of the test, total ammonia nitrogen ($N–NH_4$) was determined in water samples following the Limnologisk metodik (1992) methodology. The amount of toxic (un-ionized) ammonia nitrogen ($N–NH_3$), which is a function of temperature (18 °C), pH (mean value of 7.7) and salinity (34), was determined according to Spotte and Adams (1983). At the end of the test, surviving fish were over-anesthetized with tricaine methane sulfonate (MS-222, Pharmaq Vm 11003/4013) using a solution of 200 mg L^{-1} (buffered at pH 7.0–7.5 with sodium