Chemosphere 108 (2014) 314–319

Contents lists available at ScienceDirect

Chemosphere

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

Acute toxicity of 8 antidepressants: What are their modes of action?

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HIGHLIGHTS

- Different acute toxicities of the 8 antidepressants on *Daphnia magna.*
- Correlation between *in vivo* and *in vitro* toxicity data.
- Manifold modes of action for the antidepressants.
- A narcotic effect associated to a more specific effect on lysosomal membranes.

ARTICLE INFO

Article history:
Received 31 July 2013
Received in revised form 8 December 2013
Accepted 5 January 2014
Available online 14 February 2014

Keywords:
Pharmaceutical
Daphnia magna
Haliotis tuberculata
Multi-approach
Narcosis
Lysosomotropism

ABSTRACT

Currently, the hazard posed by pharmaceutical residues is a major concern of ecotoxicology. Most of the antidepressants belong to a family named the Cationic Amphipathic Drugs known to have specific interactions with cell membranes. The present study assessed the impact of eight antidepressants belonging to selective serotonin reuptake inhibitors or serotonin norepinephrine reuptake inhibitors by the combination of multi-approaches (*in vivo*, *in vitro*, *in silico*) and gives some insights on the mode of action for these molecules. Antidepressants were from the most to the least toxic compound for *Daphnia magna*: Sertraline (EC_{50} = 1.15 mg L^{-1}) > Clomipramine (2.74 mg L^{-1}) > Amitriptyline (4.82 mg L^{-1}) > Fluoxetine (5.91 mg L^{-1}) > Paroxetine (6.24 mg L^{-1}) > Mianserine (7.81 mg L^{-1}) > Citalopram (30.14 mg L^{-1}) and Venlafaxine (141.28 mg L^{-1}). These acute toxicities were found correlated to Log K_{ow} coefficients ($R = 0.93$, $p < 0.001$) and to cytotoxicity assessed on abalone hemocytes through the neutral red uptake assay ($R = 0.96$, $p < 0.001$). If narcosis as mode of action is typically expected during acute ecotoxicity bioassays, we showed by molecular modeling that particular interactions can exist between antidepressants and phosphatidylcholine, a major component of cell membranes, leading to a more specific mode of action corresponding to a potential acidic hydrolysis of ester functions.

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1. Introduction

The pollution by pharmaceutical residues in surface waters represents a recent preoccupation of the scientific community. Over 3000 different substances used in human and veterinary medicines can be found in aquatic environments (Richardson and Ternes, 2005; Fent et al., 2006). The main pathway for environmental contamination is *via* the sewage network that focuses wastewaters through sewage treatment plants (STP). Compounds not well-degraded in the STP are being discharged in treated effluents and thus end up in rivers, streams and marine ecosystems (Fent et al., 2006; Santos et al., 2010). Contrary to pollutants commonly monitored like metals, pesticides or hydrocarbons, pharmaceutical residues are continuously discharged into superficial waters involving for aquatic organisms an exposure during their entire life cycle. Since they are designed to highly interact with biological systems, the examination on their toxicity appeared relevant (Fent et al., 2006).

In the European Union (EU), the environmental risk assessment on pharmaceuticals is based on a stepwise procedure. The first phase aims to estimate the predicted environmental concentration (PEC) in surface water. If the $PEC_{surface\ water}$ is below 10 ng L^{-1} and no other environmental concerns are apparent, it is assumed that the compound is unlikely to represent a risk for the environment. If the $PEC_{surface\ water}$ is equal or above 10 ng L^{-1}, then a second phase should be performed on environmental fate and effect

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http://dx.doi.org/10.1016/j.chemosphere.2014.01.057
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analysis (Directive 2001/83/EC amended by Directive 2004/27/EC). However, highly potent pharmaceuticals which can have adverse effects at trace concentrations, like hormones, directly enter Phase II irrespective of their PEC values (Christen et al., 2010). Even if most of the pharmaceutical compounds would have PEC values above the action limit, their ecotoxicity and behavior in aquatic ecosystems is still poorly understood.

France is one of the most drug-consuming countries in the world especially for psychotropic drugs (Pelissolo et al., 1996). The gap between France and other countries is especially marked for anxiolytics and hypnotics, but is lower for antidepressants (Briot, 2006). Due to the high number of pharmaceutical compounds used, it is necessary to focus on some classes. Based on the drug uptake data (2009) in Basse-Normandie (France) and on the prioritization methodology of Besse and Garric (2008), we focused on 8 antidepressants, all with PEC values higher 10 ng L^{-1} (Table 1). Antidepressants represent an important drug class associated to the treatment of clinical depression and other mental disorders in Humans. They are classified on the basis of the way they interfere with the serotonergic and norepinephrinic neurotransmitter systems. Two classes are defined: (1) Selective Serotonin Reuptake Inhibitors (SSRIs) and (2) Serotonin–Norepinephrine Reuptake Inhibitors (SNRIs). Most studies about ecotoxicological effects of antidepressants have focused on the SSRI Fluoxetine (e.g. Brooks et al., 2003; review in Nentwing, 2007; Santos et al., 2010) and few have examined the aquatic toxicity of other antidepressants (Minagh et al., 2009; Getz et al., 2011; Fong and Hoy, 2012; Fong and Molnar, 2013). Moreover, their mode of action (MOA) on non-target aquatic organisms is not well enough understood. As highlighted by Kar and Roy (2010), too little studies examine interspecies correlations of pharmaceutical toxicities, whereas these correlations can provide a tool for estimating contaminant sensitivity with known levels of uncertainty for a diversity of species. The present study aimed thus to assess the acute toxicity of 4 SSRIs (Fluoxetine, Sertraline, Paroxetine and Citalopram) and 4 SNRIs (Venlafaxine, Amitriptyline, Clomipramine, Mianserine) on the invertebrate *Daphnia magna*, and to propose a MOA using *in vitro* and *in silico* approaches. The *in vitro* approach was performed on primary cultures of abalone hemocytes (*Haliotis tuberculata*), a cell type considered as useful screening strategy for risk and impact characterization of contaminants (Parolini et al., 2011).

2. Materials and methods

2.1. Chemicals

Antidepressants used for the biotests were Venlafaxine hydrochloride, Citalopram HBr, Sertraline hydrochloride, Paroxetine hydrochloride, Amitriptyline hydrochloride, Clomipramine hydrochloride, Mianserine hydrochloride, and Fluoxetine hydrochloride; all supplied in analytical grade by Kemprotec Limited (Maltby, Middlsbrough, U.K).

Table 1
Predicted Environmental Concentrations (PEC) of 8 antidepressants estimated for the Basse Normandie Region (France) in 2009. PEC (ng L^{-1}) = (Total quantity consumed (mg)/(365 × *P* × V × D))$^{*}10^6$ with *P*: number of inhabitants in Basse Normandie (i.e. 1450 000); V: volume of waste water per capita and day (200 L); D: factor of dilution of waste water by surface water flow (i.e. 10) (from Besse and Garric, 2008). SNRI: Serotonin-Norepinephrine Reuptake Inhibitor, SSRI: Selective Serotonin Reuptake Inhibitor.

Drugs	Class	PEC values (ng L^{-1})
Venlafaxine	SNRI	187
Citalopram	SSRI	69
Sertraline	SSRI	66
Paroxetine	SSRI	62
Amitriptyline	SNRI	49
Clomipramine	SNRI	48
Mianserine	SNRI	35
Fluoxetine	SSRI	33

2.2. Daphnia acute immobilization test

Daphnia tests were conducted following the guideline NF EN ISO 6341 (1996) using the water flea *D. magna* Straus (Cladocera, Crustacea). Daphnids were bred in Elendt medium M4, pH 7.4. Experiments were run at temperatures of 20 ± 1 °C and a photoperiod of LD 16:8 in ISO medium. Twenty daphnids younger than 24 h were used for the controls and each treatment subdivided in five replicates each containing five daphnids. Culture volume was 10 mL. Immobility was observed after 24 and 48 h with the latter being the endpoint for effect calculation. A reference test with potassium dichromate ($K_2Cr_2O_7$) was performed to test the sensitivity of the daphnids. The result of this reference test was in accordance with NF EN ISO 6341 (1996) (i.e. 24 h EC_{50}-value = 1.19 ± 0.27 mg L^{-1}).

2.3. Toxicity assessment on primary cultures of Haliotis tuberculata hemocytes

Adult abalones with shell length between 9 and 11 cm were sampled by Ormasub® on the Northern Cotentin peninsula (France). Organisms were maintained in natural and continuously aerated seawater at 17 °C and fed with a mixed algal diet (*Laminaria* sp. and *Palmaria* sp.) at the Centre de Recherche en Environnement Côtier (C.R.E.C., Luc-sur-Mer, Basse-Normandie, France). Prior to their use in our study, abalones were acclimated for at least 2 weeks.

Hemocytes were cultured as previously described by Lebel et al. (1996). Briefly, after a medio–lateral incision in the abalone foot, the hemolymph was withdrawn using a 20 mL syringe with a 25G needle. Hemolymph was transferred into a sterile tube and diluted 1:4 in cooled sterile anticoagulant modified Alsever's solution (115 mM glucose; 27 mM sodium citrate; 11.5 mM EDTA; 382 mM NaCl; pH 7.5) (Bachère et al., 1988). Cell cultures were first made in artificial sterile seawater (ASSW) (pH 7.4), to allow cells to adhere onto the bottom of the culture well. Then, the ASSW was replaced by Hank's sterile 199 medium modified by the addition of 250 mM NaCl, 10 mM KCl, 25 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 10 mM Hepes (final pH 7.4), and supplemented with 2 mM L-glutamine, 100 μg mL^{-1} streptomycin, 60 μg mL^{-1} penicillin G and 2 mM concanavalin A. Cell cultures were incubated at 17 °C overnight. The culture medium was then replaced by the different antidepressant solutions. Cells were exposed during 48 h. Each concentration was tested in quadruplicate (i.e. four wells per concentration). The medium was changed every day. The cell exposure was repeated at least thrice, i.e. using at least three abalones (experiment replicates).

2.3.1. MTT assay

Cellular viability was estimated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay, which is a sensitive and quantitative colorimetric assay measuring the capacity of mitochondrial succinyl dehydrogenase in living cells to convert a yellow substrate (MTT) into a dark blue formazan product (Mosmann, 1983). This test was adapted to molluscan cell cultures by Domart-Coulon et al. (2000). Briefly, 10% (v/v) of the MTT stock solution (5 mg MTT mL^{-1} PBS 1X pH 7.4) was added to the culture dishes. After 24 h incubation at 17 °C, an equal volume of isopropanol containing 0.04 N HCl was added to each culture to dissolve the converted formazan. The absorbance was measured at a wavelength of 570 nm with a 630 nm reference.

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