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Is the step-wise tiered approach for ERA of pharmaceuticals useful for the assessment of cancer therapeutic drugs present in marine environment?

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ABSTRACT

Methotrexate (MTX) and tamoxifen (TMX) cancer therapeutic drugs have been detected within the aquatic environment. Nevertheless, MTX and TMX research is essentially bio-medically orientated, with few studies addressing the question of its toxicity in fresh water organisms, and none to its' effect in the marine environment. To the authors' knowledge, Environmental Risk Assessments (ERA) for pharmaceuticals has mainly been designed for freshwater and terrestrial environments (European Medicines Agency-EMEA guideline, 2006). Therefore, the purpose of this research was (1) to assess effect of MTX and TMX in marine organism using the EMEA guideline, (2) to develop an ERA methodology for marine environment, and (3) to evaluate the suitability of including a biomarker approach in Phase III. To reach these aims, a risk assessment of MTX and TMX was performed following EMEA guideline, including a 2-tier approach during Phase III, applying lysosomal membrane stability (LMS) as a screening biomarker in tier-1 and a battery of biochemical biomarkers in tier-2. Results from Phase II indicated that MTX was not toxic for bacteria, microalgae and sea urchin at the concentrations tested, thus no further assessment was required, while TMX indicated a possible risk. Therefore, Phase III was performed for only TMX. *Ruditapes philippinarum* were exposed during 14 days to TMX (0.1, 1, 10, 50 $\mu g\ L^{-1}$). At the end of the experiment, clams exposed to environmental concentration indicated significant changes in LMS compared to the control ($p < 0.01$); thus a second tier was applied. A significant induction of biomarkers (activity of Ethoxyresorufin O-deethylase [EROD], glutathione S-transferase [GST], glutathione peroxidase [GPX], and lipid peroxidation [LPO] levels) was observed in digestive gland tissues of clams compared with control ($p < 0.01$). Finally, this study indicated that MTX was not toxic at an environmental concentration, whilst TMX was potentially toxic for marine biota. This study has shown the necessity to create specific guidelines in order to evaluate effects of pharmaceuticals in marine environment which includes sensitive endpoints. The inadequacy of current EMEA guideline to predict chemotherapy agents toxicity in Phase II was displayed whilst the usefulness of other tests were demonstrated. The 2-tier approach, applied in Phase III, appears to be suitable for an ERA of cancer therapeutic drugs in the marine environment.

1. Introduction

Several publications have indicated the presence of pharmaceuticals within the aquatic environment at the $ng\ L^{-1}$ to $\mu g\ L^{-1}$ range, due to either direct discharge or even post waste water treatment process (Andreozzi et al., 2002; Gros et al., 2007, 2009, 2010; Quinn et al., 2008a; Zuccato et al., 2004, 2005). In addition, it has been demonstrated that at these concentrations, some pharmaceuticals produce acute and chronic effects on aquatic organisms (Fent et al., 2006; Fent, 2008; Ferrari, 2003; Quinn et al., 2009; Hernando et al., 2006; Martín-Díaz el al., 2009; Aguirre-Martínez et al., 2013a, 2013b among others). Nevertheless, for most pharmaceuticals the effect which they have on aquatic biota

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is largely unknown.

During 2006, the European Medicines Agency (EMEA) released a guideline describing how to evaluate the potential risks of pharmaceuticals products entering the environment. However, it is only focused on the environmental risks associated with the use of pharmaceuticals and not from storage, disposal, synthesis or the manufacture of these substances. The guidelines describes a step-wise tiered procedure for Environmental Risk Assessment (ERA) of pharmaceuticals with two phases. Briefly, the Phase I is a pre-screening assessment, which aims at a first estimation of exposure with an action limit of 0.01 $\mu g\ L^{-1}$. This guideline indicates that if the predicted environmental concentration (PEC) of a pharmaceutical of surface water is below this limit, it is assumed that the compound is unlikely to represent a risk for the environment. However, in some cases, the action limit may not be applicable, for example regarding endocrine disrupting compounds. If the PEC is equal to, or above 0.01 $\mu g\ L^{-1}$ then a Phase II environmental fate and effect analysis should be performed. Phase II is further divided into Tier A which gives a rapid prediction of environmental risk based on screening data. If the risk is identified at this level, then a Tier B should be performed; this tier requires extended ecotoxicity data to reduce uncertainty, this is the ultimate step in risk assessment of the EMEA guideline (Grung et al., 2008; Kampa et al., 2010).

Nevertheless, this guideline specifies that only newly authorized pharmaceuticals require an environmental assessment, and to this respect, there is little knowledge concerning the environmental risk for most chemotherapeutic agents released to the market before 2006 (Besse et al., 2012; Johnson et al., 2008). This is the case of two frequently used cancer therapeutic drugs methotrexate (MTX) tamoxifen (TMX). Methotrexate (4-amino-10-methyl-folic acid) is a commonly used anti-metabolite (folic acid antagonist) in cancer treatment and is also applied as an anti-rheumatic drug. It is not normally sold in pharmacies; but its use in medicine is widespread. This substance interacts with cell proliferation, blocking the folate dehydroreductase enzyme disrupting the synthesis of nucleic acid, which is responsible for the purine and pyrimidine synthesis (Trigg and Flanigan-Minnick, 2011). It is eliminated virtually unchanged by the kidneys (Fent et al., 2006). MTX has been found in effluents from hospital and waste water treatment plants at a concentration from 0.0021 to 0.25 $\mu g\ L^{-1}$ (Table 1). Tamoxifen is an anti-estrogen, a non-steroidal triphenylethylene derivative, which is widely and successfully used in the chemotherapy and chemoprevention of primary and recurrent breast cancer (Bergh, 2003; Custodio et al., 1993; Jordan et al., 1977; Nayfield et al., 1991; Osborne, 1998; Powles et al., 1994). More recently, this drug has been used as a prophylactic agent in women who are considered to be at a high risk of developing the disease (DellaGreca et al., 2007; Fisher et al., 2005). Like many other pharmaceuticals, it can enter the aquatic environment through municipal sewage effluents and cause adverse effects (Ashton et al., 2004; Hilton and Thomas, 2003; Mater et al., 2014; Sun et al., 2007). This is of importance since TMX has been proposed for use as a growth-promoting agent in aquaculture (Park et al., 2003) and in this context would pose an additional risk to aquatic organisms (Sun et al., 2007, 2009; Mater et al., 2014). TMX has been included on the prioritization list of bioaccumulable potential in the human body and probably in aquatic organisms (Jean et al., 2012). Assessment for this drug has been suggested by the Oslo and Paris Commission (OSPAR, 2003), Environment Agency from the U.K. (Hilton et al., 2003), and Environment Canada (2014). Moreover, as potential endocrine disruptor in European water sources, the Institute of Environment and Health (U.K.) have suggested an ERA for this drug (IEH, 2012). TMX has been found in aquatic environment at concentrations ranging from 0.004 to 0.21 $\mu g\ L^{-1}$, and in effluents from waste water treatment plants and hospital at 0.0002 and 0.037 $\mu g\ L^{-1}$ (Table 1). In addition, MTX and TMX are both included in the list of drugs that should be handled as hazardous (NIOSH, 2012, 2014).

Despite detected concentrations in the environment, most MTX and TMX research is essentially bio-medically orientated with few papers addressing the question of toxicity in aquatic organisms (Besse et al., 2012; Mater et al., 2014; Orias and Perrodin, 2013; Sun et al., 2007, 2009). Knowing that these pharmaceuticals are widely used, have been found in the environment, and are of special interest, there is a need to analyze the type of effect that they might produce in aquatic biota, taking into account their distinctive mode of action. Besse et al. (2012) suggest that these drugs should be screened and assessed for environmental risk according to the EMEA guideline released in 2006, since there is a lack of information of their ecotoxicity and more specific knowledge is required regarding the marine environment.

As previously mentioned, ERA for human pharmaceuticals should be performed according to EMEA Guideline (2006) proposed for freshwater and terrestrial environments (McVey, 2012). Nevertheless, authors believe that research should be focused on developing a risk assessment methodology in which marine environment components are included. In contrast to other pollutants, pharmaceuticals are specifically designed to have pharmacological and physiological effects on their target (i.e. humans or animals under veterinary treatment) species. However, their effects on non-target (environmentally exposed) species are difficult to predict and may often be detrimental (Hampel et al., 2014). The aims of this study were the following: (1) to assess the effects of MTX and TMX in marine organisms using the EMEA guideline, (2) to develop an ERA methodology in which marine organisms are included, (3) to evaluate the suitability of including a biomarker approach for the last phase (Phase III).

2. Materials and methods

2.1. Environmental Risk Assessment (ERA)

The ERA of the EMEA guideline use a step-wise structure (Fig. 1), including a **Phase I.** This first step is the estimation of the exposure by calculation of a predicted environmental concentration (PEC). Nevertheless, in the present study, the measured environmental concentration (MEC) was applied, which was obtained from reported data of MTX and TMX found in municipal effluents, sewage treatment plants, surface water, etc. described in Table 1. The use of MEC allows establishing more realistic ERA than PEC (Blasco and DelValls, 2008). If MEC exceeds the action limit, then further testing is required. Leading onto **Phase II,** this second step corresponds to an initial prediction of the risk applying a set of acute toxicity tests towards three species from different phyla: bioluminescence on *Aliivibrio fischeri* (Proteobacteria), growth inhibition on microalgae *Isochrysis galbana* (Haptophyta), and on sea urchin *Paracentrotus lividus* (Echinodermata) during their early life stage. In this step, the predicted no effect concentration (PNEC) is extrapolated by dividing the EC_{50} by an assessment factor of 1000. If the ratio MEC/PNEC is < 1, then no further assessment is necessary. If MEC/PNEC is > 1, an ecological hazard may occur, and so further assessment should be performed (Quinn et al., 2008a). **Phase III** includes long term exposure; in this step sensitive endpoints are included in order to evaluate the chronic effects of drugs. A 2-tier approach is applied following the methodology proposed by Viarengo et al. (2007), which was then applied to marine crabs by Aguirre-Martínez et al. (2013a, 2013b). In **Tier 1,** a sensitive, low-cost biomarker is used as "early warning" to indicate the level of stress of the organisms exposed to the contaminant. A lysosomal membrane stability test (LMS) is