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Teratogenic effects of five anticancer drugs on *Xenopus laevis* embryos

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ABSTRACT

In recent years, the environmental presence of pharmaceuticals - including anticancer drugs - is an emerging issue. Because of the lack of appropriate critical studies about anticancer drug effects in frogs, the aim of the present study was to investigate lethal and teratogenic effects of five anticancer drugs widely used in large quantities, i.e. 5-flourouracil, capecitabine, cisplatin, etoposide, and imatinib, in the embryos of the South African clawed frog, *Xenopus laevis*, using FETAX - Frog Embryo Teratogenesis Assay in *Xenopus*. None of the studied anticancer drugs induced statistically significant mortality within the concentrations tested (0.01–50 mg/L, depending on the studied compound), and no growth inhibition of embryos after a 96-h exposure was observed. Except for cisplatin, the other pharmaceuticals induced an increase of developmental malformations such as abdominal edema, axial flexure, head, eyes, gut and heart malformations with statistically significant effects observed at the highest concentrations tested (50 mg/L for 5-flourouracil; 30 mg/L for etoposide and 20 mg/L for capecitabine and imatinib). The results indicate that anticancer drugs can affect embryogenesis mechanisms.

1. Introduction

Human pharmaceuticals from different therapeutic classes constantly enter the aquatic environment indicating poor efficiencies of wastewater treatment plants in their removal. In the surface waters, these compounds can be subjected to both biotic and abiotic transformations or can bound to particulate materials and sediments generally decreasing their bioavailable concentrations. Aquatic organisms have been shown to be adversely affected by pharmaceuticals at highly variable concentrations and, in the last years, among the several classes of pharmaceuticals, anticancer drugs have become of high concern (Küster and Adler, 2014). Anticancer drug residues occur in the aquatic environment at sub-ng-μg/L concentrations, which do not pose a direct hazard to aquatic organisms. On the other hand, they can cause long-term delayed effects since these drugs are known as carcinogenic, mutagenic and teratogenic compounds affecting not only target cells but also non-tumoral cells through direct DNA damage, inhibition of cell proliferation and DNA synthesis (Xie, 2012). To date anticancer drugs consumption is increasing constantly (by 10% per year, Mater et al., 2014) because of the progression in cancer incidence rates, the developing health care systems and the higher life expectancy. Doses administered are usually calculated considering the body surface area and expressed in mg/m^2. For example, the administration of Pt compounds varies for different kinds of cancer from 20 to 100 mg/m^2, equivalent to 0.5–2.7 mg/Kg (35–196 mg/person). This means that a high percentage of this drug reaches the wastewater treatment plant and it will increase of 10% per year threating the aquatic organisms exposed for all their life span.

Several aquatic species have been employed in laboratory studies with anticancer drugs to evaluate the toxic and genotoxic effects of such compounds. Both acute and chronic toxicity of cytostatics and their metabolites were studied in organisms from different trophic levels reporting effective concentrations ranging by several orders of magnitude (Zounková et al., 2007, 2010). For example growth of cyanobacteria and algae was inhibited at mg/L concentrations (Brezovšek et al., 2014) while rotifers and crustaceans showed long term effects at much lower concentrations (Parrella et al., 2014). Furthermore, chemotherapeutic agents can cause also genotoxicity in both aquatic and terrestrial ecosystems. Concentrations in the sub-microgram/L range induced DNA damage in crustaceans (Parrella et al., 2015) or *Danio rerio* (Kovács et al., 2015a). Freshwater mussel haemocytes were less sensitive to anticancer drugs showing DNA strand breaks at concentrations in the order of μg-mg/L (Gačić et al., 2014), and the investigation of genotoxic properties in the higher plant *Tradescantia* #4430 observed significant effects at μg/L (Mišík et al., 2014). To the best of our knowledge, only recently, embryotoxic and teratogenic effects of anticancer drugs have been studied in aquatic invertebrates and vertebrates (Giari et al., 2012; Huang et al., 2012, 2011; Kovács et al., 2015a; Trendowski et al., 2014) but there is still a major lack

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of information for critical environmental risk assessment.

The aim of the present study was to investigate lethal and teratogenic effects of five anticancer drugs in the embryos from the South African clawed frog, *Xenopus laevis*. The drugs were chosen based on their different modes of action, consumption and reported occurrence in the environment, and included antimetabolites 5-flourouracil (5-FU) and its pro-drug capecitabine (CAP) which causes irreversible inhibition of thymodilate synthase, cisplatin (CDDP), a platinum-derived drug crosslinking DNA and ultimately triggering apoptosis, etoposide (ET), a topoisomerase II inhibitor, and imatinib (IM), a selective tyrosine kinase inhibitor stopping cell growth. As shown in previous studies, FETAX is reliable, sensitive and an accurate test able to detect the developmental toxicants (Chae et al., 2014; Mouche et al., 2011). The frogs *X. laevis* are easy to maintain in culture and can be stimulated by hormones every three months to lay eggs usable for embryotoxicity and teratogenicity testing (Bosisio et al., 2009; Zhang et al., 2014). Furthermore, *Xenopus* has broadly been used frequently in many laboratories worldwide to study vertebrate embryology, neurobiology and toxicology and human diseases (Woomble et al., 2016).

2. Materials and methods

2.1. Test compounds

5-FU (CAS: 51-21-8), CDDP (CAS:15663-27-1), ET (CAS: 33,419-42-0) were supplied by Sigma-Aldrich (Milano, Italy) while CAP (CAS: 220,127-57-1) and IM (CAS: 220,127-57-1) were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Safety considerations of cytostatic drugs handling

As cytostatic drugs are highly toxic compounds, their handling requires strict safety precautions in order to guarantee the best possible protection of research workers. All stock solutions were prepared under a biological safety hood with laminar airflow and absorbent paper was used to protect the work surfaces. All disposable material that was in contact with tested compounds was treated as hazardous waste.

2.3. In vitro fertilization

Males and females of *X. laevis* were bred in the same water in which the test was conducted. Water temperature was held at 21 ± 2 °C to induce the breeding behavior. 150 and 300 UI of human chorionic gonadotropin (HCG; N. V. Organon, Oss, Holland) were injected into the dorsal lymph sac of the male and the female, respectively. The eggs deposited by females about 9–12 h after injection were immediately inspected for fertility and quality by a microscope, and only normally developing eggs were used for experiments. Eggs laid in “strings” or not perfectly round were considered abnormally developing and were not used. In order to reflect conditions in nature, the eggs were not de-jellied since it is known that egg jelly protects embryos from environmental pollutants (Edginton et al., 2007).

2.4. FETAX assay

The experiments followed standard Guideline ASTM E1439-98 (1998). A stock solution (100 mg/L) of each anticancer drug was prepared in deionized water except for ET that was first dissolved in dimethyl sulphoxide (DMSO) and then diluted in deionized water (0.1% v/v in the stock solution which is a non-effective dose as reported in FETAX guideline). Fresh test solutions were prepared by serial dilution of the stock solutions with FETAX medium immediately before experiments. FETAX medium was prepared as follows: distilled water 1 L; NaCl: 625 mg; $NaHCO_3$: 96 mg; KCl: 30 mg; $CaCl_2$: 15 mg; $CaSO_4$ $2h_2O$: 60 mg; $MgSO_4$: 75 mg; pH 7.6–7.9.

Four different concentrations (dilution factor 10) for each compound were used, and the concentrations were selected by Parrella et al. (2014) according to the results from acute toxicity tests performed on aquatic organisms. For each concentration, three glass Petri dishes (45 mm diameter) each containing 10 mL of test solution and 25 embryos, selected between mid blastula (Stage 8) and early gastrula (Stage 11) to exclude the abnormal effects of spontaneous embryonic development, were used, ASTM E1439-98 (1998). A test medium (negative control) was used in addition to the test series. The embryos were incubated at 22 ± 2 °C with a 12:12 h light-dark cycle (Doumont et al., 1983; Mouche et al., 2011).

Renewal of the test solutions was done every 24 h along with the assessment of embryonic survival and removal of eventual dead embryos. The experiments were conducted for 96 h. All surviving embryos were euthanized by Tricain MS 222,100 mg/L, fixed in 3% v/v formaldehyde and examined for malformations and growth-inhibition under a light microscope. Images were analyzed with QuickPHOTO MICRO 2.3 camera.

2.5. Data analysis

FETAX assays were performed at least in three independent experiments. The results were pooled and the effective percentages were analyzed using Prism5 (Graphpad Inc., CA, USA) in order to determine the concentrations giving 50% effect by non-linear regression (log agonist vs. normalized response-variable slope). One-way ANOVA and Dunnett's multiple comparison test were used to calculate the significant differences in survival, malformation (expressed in percentage / frequency) and length (expressed in μm) from control in order to obtain the Lowest Observed Effective Concentration (LOEC). Nevertheless, this method has severe shortcomings under the conditions in which several groups are tested with only one that deviates (as it is the case for survival and growth inhibition) but it remains among the most recommended methods for the analysis of this kind of tests. Negative controls met the criteria of the FETAX acceptance (i.e. mortality and malformation rates lower than 10%).

3. Results and discussion

Despite of recent research efforts on emerging contaminants including pharmaceuticals (Loos et al., 2013), their environmental teratogenic impacts in vertebrates remain largely unknown. In the present study, we examined the in vivo lethal and sub-lethal (growth inhibitions and malformations) effects of the exposure of *Xenopus laevis* embryos to five antineoplastic drugs, 5-FU, CAP, CDDP, ET and IM. As shown by Parrella et al. (2014) by HPLC chemical analysis, anticancer drugs tested were stable in aqueous stock solutions stored at 4 °C in the dark. Continuous exposure conditions in FETAX were assured by daily exchanges of media.

As shown in Fig. 1, compounds tested at four different concentrations (mg/L) did not affect survival of the *X. laevis* embryos after 96 h exposure (ANOVA and Dunnett's test $p > 0.05$). Similar negative results were also observed for the embryo growth inhibition (Fig. 2). These weak acute toxicities are in agreement with a previous study (Kovács et al., 2015a) showing that the continuous exposure to different concentrations of 5-FU in zebrafish through two subsequent generations did not affect survival (2-week exposure) and growth (7-month exposure).

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