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Embryotoxicity of Five Cytostatics in Fathead Minnow (*Pimephales promelas*) Larvae

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Abstract

Cytostatics are compounds used in chemotherapy, known to be genotoxic, mutagenic, and teratogenic at low concentrations. The amount of cytostatic drugs prescribed increases every year as does their release into the aquatic ecosystems, which possibly is a major concern for the health of aquatic organisms. This study aimed to evaluate the putative toxicity of five cytostatics to fathead minnow (*Pimephales promelas*) larvae: tamoxifen, capecitabine, methotrexate, cyclophosphamide, and ifosfamide. Eggs collected post-fertilization were exposed for 6 days to a range of concentrations, including one above environmental level. At all environmental concentrations, no significant difference in mortality, hatching time, length, heart rate, and presence of malformations were found. Altogether, these cytostatics do not seem embryotoxic to fish. Although, an increased proportion of complete swim bladder were found after ifosfamide's exposure, suggesting an interaction with the thyroid axis, involved in swim bladder development. Complementary work should address other endpoints, such as behavioral changes, reproductive success, and transgenerational effects.

Keywords Cytostatics · Anticancer drugs · Fish · Embryotoxicity

Cancer is one of the leading causes of death throughout the world (Nussbaumer et al. 2011), and the number one in Canada (Statistics Canada 2020). For decades, the incidence of cancerous diseases in the human population has been increasing. Cytostatics, also called anti-neoplastic drugs or anticancer drugs, are compounds commonly used in chemotherapy. Since the number of cancer incidences is increasing, this suggests that the administration of cytostatics is also in constant augmentation (Hoppe-Tichy 2010; Ferrando-Climent et al. 2014). From less than 5% a decade ago, the number of oral cancer agents in use has increased to approximately 17% by 2007, and it is now estimated that at least 25% of the existing antineoplastic agents are planned to be used as oral agents (Tadic et al. 2015). Most of these compounds prevent uncontrolled proliferation of cancer cells via DNA interaction and cell signaling (Novak et al. 2017). Due to their mode of action, they are classified as cytotoxic, genotoxic, mutagenic, and teratogenic agents, and potentially endocrine disruptors (Novak et al. 2017; Kosjek and Heath 2011). Moreover, cytostatics act unselectively on cancer cells and noncancer cells, which often cause undesirable side effects during treatments (Novak et al. 2017; Kosjek and Heath 2011).

Residues of these compounds are excreted after administration to patients into domestic and hospital wastewater (Negreira et al. 2014; Johnson et al. 2013; Zhang et al. 2013; Kosjek and Heath 2011). These residues are a mixture of parent compounds and their metabolites (Novak et al. 2017; Zhang et al. 2013). However, several studies have shown their poor elimination efficiency in conventional wastewater treatment plants (Franquet-Griell et al. 2017; Negreira et al. 2014; Zhang et al. 2013). Consequently, wastewater treatments plants are considered to be an important point source of drug contamination into the environment (Novak et al. 2017; Zhang et al. 2013; Brun et al. 2006). Cytostatics are usually present at low concentrations in the environment (sub ng/L to few µg/L; reviewed by CEAEQ (not published); Novak et al. 2017; Zhang et al. 2013; Kosjek and Heath

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2011; Brun et al. 2006). However, as their use is in constant augmentation, their environmental concentrations will likely increase in the future. Moreover, due to their unselectively toxic properties, these compounds could affect countless organisms' cells, which is a major preoccupation for environmental safety.

The aim of this study was to evaluate fish embryotoxicity of cytostatics found in Canadian surface waters and elsewhere. Five cytostatics were assessed in this study, according to their constant presence in the environment. Tamoxifen (TX) is used as an anti-estrogenic in breast cancer therapy (Zhang et al. 2013) as it inhibits the estrogen receptor binding. Methotrexate (MX) is used as an antifolic to treat several types of cancers, like non-Hodgkin's lymphoma (Nussbaumer et al. 2011) and inhibits folic acid synthesis, which is essential for DNA synthesis. Capecitabine (CAP) is used as an antipyrimidique in metastatic colorectal cancer therapy (Nussbaumer et al. 2011) and inhibits the thymidylate synthase, which blocks DNA replication. Finally, cyclophosphamide (CP) and its analogue, ifosfamide (IF), are two nitrogen mustards used to treat several types of cancers like solid tumours (Nussbaumer et al. 2011). These two nitrogen mustards are alkylating agents that form DNA adducts, which also blocks DNA synthesis. Fathead minnow (*Pimephales promelas*) present throughout North America's aquatic environments was chosen to conduct this work, as one of the species usually used in ecotoxicology assessment.

Materials and Methods

TX (CAS No: 10540-29-1, 99%), CAP (CAS No: 158798-73-3, ≥ 99%), MX (CAS No: 59-05-2, 99.5%), CP (CAS No: 50-18-0, ≥ 98%), IF (CAS No: 3778-73-2, ≥ 98%), dimethyl sulfoxide (DMSO), and 3-aminobenzoic acid ester methanesulfonate (MS-222) were purchased from Sigma. Reconstituted water (deionized water with 0.3 mM $CaSO_4$, 0.2 mM $MgSO_4$, 0.05 mM KCl, and 2.4 mM $NaHCO_3$) was prepared and stored in a regulated experimental room (25 °C, 70% humidity, and 16/8 h light/dark cycle) for all experiments. Temperature, pH, and conductivity were verified in daily to avoid undesired stress to the animals and readings were consistent.

The experimental design and measured endpoints as described follow OECD's guidelines for acute toxicity assessment (1998). CAP, CP, and IF were dissolved in reconstituted water, TX and MX were dissolved in DMSO due to their insolubility in water, the final solvent concentration was 0,01% v/v. Nominal concentrations of each cytostatic were 0.001 µg/L (or 1 ng/L), 0.1 µg/L (or 100 ng/L) and 10 µg/L as these concentrations are found within the environment (reviewed by CEAEQ). In addition, one treatment of 1000 µg/L (or 1 mg/L) was added to the experimental design to test for a high-end concentration.

Fathead minnow eggs were obtained from a colony established at the Institut National de la Recherche Scientifique (INRS; Quebec City, QC, Canada). Breeding substrate made of cut sections of polyvinyl chloride tubing (4 po diameter) were placed in culture tanks containing reproductively mature males and females in the evening prior to test initiation. The following morning, the breeding substrates were removed from the culture tanks and placed in clean reconstituted water with aeration. The eggs were removed from the tiles 4 h later. Prior to exposure, eggs were observed under a microscope to select stage 13 (or one-quarter epiboly), which appears around 10 h post-fertilization according to Delvin et al. (1996). Then, three replicates of 30 eggs each were placed in 500-mL glass-beakers with 200 mL of exposure mixture, reconstituted water or solvent controls water (0.01% DMSO). All exposure and control solutions were changed daily by transferring eggs/hatched larvae into freshly prepared solutions. All beakers were covered with Petri dishes to minimize evaporation. All treatments were analyzed by the Centre d'Expertise en Analyse Environnementale du Québec (CEAEQ; Quebec City, QC, Canada), except the lowest concentration of each treatment as the nominal concentration values were below the detection limit (15 to 28 ng/L) of available analytical equipment. All treatments were analyzed in duplicate, from stock solution, at time 0 (T0) and time 24 h (T24), by liquid chromatography coupled with mass spectrometry (LCMS, Xevo TQ-S, Waters®—LOD 2 to 3.2 ng/l, LOQ 7 to 16 ng/L) (Borgatta et al. 2016). Water sampled at T0 was stored at 4 °C for 24 h prior to being transported for chemical analyses at CEAEQ following the collection of the T24 samples. Mortality and hatching data were daily recorded and all dead egg/larva were removed daily. Then, only final mortality (over 6 days) and average hatching time were analyzed. The number of solutions that could be analyzed was limited due to budget restriction and limit of detection. For example, the 1 ng/L solution was considerably below the detection limit, so it was decided to not analyzed it. We prioritized treatment solutions rather than controls, since we considered it was more important to measure the degradation of compounds over 24 h, which is the maximum amount of time we used the solution.

After 6 days of exposure, all larvae were transferred individually to a 96–well plate. Heart rate was measured with an inverted microscope (Labomed® TCM 400). Temperature could not have affected fish heart rate over different exposure, while it remains constant for all experiments. The sampling was made over a day (always between 8 am and 6 pm) and replicates were taken for analysis randomly, so time of day seems an unlikely bias. These five experiments were conducted over three weeks by the same staff so seasonality

and staff change should also not be the issue. Larvae were then anaesthetized with 100 µL of MS-222, freshly prepared at 100 mg/L buffered with $NaHCO_3$ at 200 mg/L after removing the remaining exposure solution. Then, they were observed with a compound microscope (Nikon® SMZ18) and photographed (Nikon® digital sight DS-L3).The presence of malformations (e.g., edema, hemorrhage, tube heart, scoliosis, craniofacial deformation, and cardiac deformation), the development of swim bladder (i.e., complete, incomplete/absence), and the number of malformed larvae in each replicate were recorded (Madison et al. 2020). Pictures were analyzed with ImageJ software to measure the length.

Data analysis was performed with GraphPad Prism 8. Mortality, hatching time, heart rate, length, malformation rate, presence and number of malformations, and presence/partial development/absence of swim bladder were assessed for each treatment using analysis of variance (one-way ANOVA) after reviewing conclusive normality (Shapiro–Wilk test) and homoscedasticity (Brown-Forsythe test). Dunnett's post-hoc tests were performed to identify significant differences between treatment groups.

Results and Discussion

For all cytostatics, measured concentrations remained consistent before water change and in agreement with the nominal concentrations, except for TX and CAP at 0.1 µg/L (Table 1). Measured concentrations for TX were twice lower than nominal concentration at T0 and T24, as describe by Borgatta et al. (2016), these differences were probably due to adsorption of the molecule on glass surface. All of the following results were reported with the nominal concentration.

Table 1 Measured endpoints after exposure to five cytostatics

Treatments	Nominal levels	Measured levels T0	Measured levels T24	Mortality (%)	Hatching (day)	Heart rate (/min)	Length (mm)	Malformation (%)	Uninflated swim bladder (%)
Tamoxifen	RW	ND	ND	4.4 ± 4.7	4.8 ± 0.04	184 ± 3	5.5 ± 0.2	8.4 ± 4.2	17.6 ± 13.3
	DMSO	ND	ND	1.2 ± 2.1	4.9 ± 0.09	181 ± 3	5.4 ± 0.2	17.1 ± 6.6	22.9 ± 4.5
	0.001 µg/L	ND	ND	5.6 ± 2	5.0 ± 0.2	184 ± 2	5.4 ± 0.2	17.7 ± 9.2	29.4 ± 11.4
	0.1 µg/L	0.041 ± 0.014[a]	0.049 ± 0.0056[a]	3.2 ± 5.6	5.0 ± 0.09	180 ± 5	5.4 ± 0.2	12.8 ± 3.9	28.0 ± 15.3
	10 µg/L	3.5 ± 0.7[a]	2.8 ± 0.1[a]	3.2 ± 3.2	4.9 ± 0.1	181 ± 3	5.4 ± 0.2	18.4 ± 12.1	28.7 ± 14.4
	1000 µg/L	600 ± 600[a]	480 ± 100[a]	100 ± 0*	1.7 ± 2.1*	–	–	–	–
Capecitabine	RW	ND	ND	2.3 ± 4	4.9 ± 0.04	185 ± 6	5.4 ± 0.3	15.1 ± 4.5	19.7 ± 6.3
	0.001 µg/L	ND	ND	8.9 ± 7	4.6 ± 0.2	191 ± 1	5.5 ± 0.2	3.4 ± 6	7.1 ± 5.8
	0.1 µg/L	0.165 ± 0.021	0.170 ± 0.014	4.5 ± 2	4.6 ± 0.3	191 ± 3	5.5 ± 0.3	17.8 ± 9.6	11.9 ± 9.0
	10 µg/L	12.5 ± 2.1	13 ± 1.4	8.7 ± 8	4.8 ± 0.2	189 ± 2	5.4 ± 0.3	9.8 ± 4.4	13.9 ± 11.4
	1000 µg/L	1300 ± 700	1500 ± 100	3.3 ± 6	4.7 ± 0.09	192 ± 3	5.4 ± 0.4	21.5 ± 7.5	16.3 ± 11.6
Methotrexate	RW	ND	ND	6.7 ± 6.7	4.8 ± 0.1	187 ± 6	5.5 ± 0.2	8.5 ± 7.8	13.5 ± 8.4
	DMSO	ND	ND	5.5 ± 6.9	4.8 ± 0.2	193 ± 5	5.4 ± 0.3	9.5 ± 2.5	14.4 ± 9.6
	0.001 µg/L	ND	ND	12 ± 7	4.7 ± 0.07	194 ± 5	5.5 ± 0.2	4.0 ± 4.2	7.8 ± 4.4
	0.1 µg/L	0.115 ± 0.007	0.102 ± 0.039	11.3 ± 7.3	4.7 ± 0.2	192 ± 5	5.4 ± 0.2	4.7 ± 1.2	7.1 ± 2.8
	10 µg/L	11.5 ± 0.7	8.4 ± 0.1	3.2 ± 0.1	4.7 ± 0.06	196 ± 2	5.4 ± 0.3	6.8 ± 9.2	15.9 ± 10.1
	1000 µg/L	1100 ± 200	1150 ± 100	7.5 ± 7.4	4.7 ± 0.3	189 ± 4	5.3 ± 0.4	12.7 ± 4.8	16.5 ± 6.2
Cyclophosphamide	RW	ND	ND	2.6 ± 2.2	4.9 ± 0.1	191 ± 5	ND	14.9 ± 10.7	12.0 ± 8.4
	0.001 µg/L	ND	ND	2.5 ± 2.2	4.9 ± 0.08	189 ± 8	ND	11.9 ± 4.4	14.7 ± 5.4
	0.1 µg/L	0.12 ± 0.001	0.125 ± 0.021	4 ± 4.1	4.9 ± 0.1	192 ± 7	ND	11.5 ± 10	15.9 ± 9.6
	10 µg/L	13.5 ± 2.1	12 ± 1.4	9.9 ± 2.1	4.8 ± 0.1	193 ± 7	ND	6.8 ± 2.3	15.2 ± 9.7
	1000 µg/L	1300 ± 100	1400 ± 100	6.2 ± 2.3	4.9 ± 0.09	196 ± 3	ND	13.1 ± 1.5	15.7 ± 3.4
Ifosfamide	RW	ND	ND	2.2 ± 1.9	4.8 ± 0.06	195 ± 4	5.6 ± 0.2	9.2 ± 10.5	27.3 ± 15.0
	0.001 µg/L	ND	ND	3.4 ± 3.5	4.7 ± 0.08	191 ± 3	5.3 ± 0.3	10.4 ± .2	8.1 ± 2.3*
	0.1 µg/L	0.1 ± 0.013	0.115 ± 0.007	2.2 ± 1.9	4.7 ± 0.2	200 ± 3	5.3 ± 0.3	6.9 ± 3.4	5.7 ± 5.3*
	10 µg/L	11 ± 0	10.3 ± 1	2.2 ± 3.9	4.9 ± 0.3	196 ± 11	5.3 ± 0.3	4.9 ± 4.0	9.1 ± 8.4*
	1000 µg/L	1200 ± 0	1140 ± 200	2.1 ± 1.8	4.9 ± 0.1	198 ± 5	5.6 ± 0.2	3.3 ± 3.3	3.3 ± 3.3*

RW reconstitute water, *ND* not determined. ANOVA *$p < 0.05$

[a]Unreliability of the measured values

There were no significant differences in survival of larvae or in hatching time (Table 1) with the exposure of CAP, MX, CP and IF. For TX, 100% mortality was measured in all replicates at 1000 µg/L ($F = 43.16$; $p < 0.0001$, resp). Significant differences were measured in hatching of eggs at 1000 µg/L ($F = 7.341$; $p = 0.0023$), but this result is not robust because only 3 eggs hatched during the first 2 days of the exposure, in all 1000 µg/L replicates, then all these larvae died before the end of the exposure.

There were no significant differences in heart rate and length between treated larvae (Table 1) in the exposure of all of the molecules. Of note, no measurements of length were analyzed for the CP exposition due to a malfunction of a data storage drive.

There were no significant differences in the number of malformed larvae (Table 1) for TX, CAP, MX, CP and IF. In addition, malformation data was also analyzed per type of malformation, and number of malformations per larvae in each treatment group. No significant differences were found for any treatments (data not showed).

Finally, there were no significant differences in the development of swim bladders for TX, CAP, MX, and CP. However, a significant decrease of the proportion of fish with not fully developed swim bladders was observed for all the concentrations of IF ($F = 3.921$; $p = 0.0412$; Table 1).

Discussion

The aim of this study was to investigate the acute toxicity of five cytostatics during fathead minnows' early development. At high concentration (1 mg/L; level not found in the environment), only the TX exposure led to complete mortality, while all the other cytostatics' treatments were not toxic for the endpoints measure on the developing fish. This result for TX is in agreement with previous studies with other species. DellaGreca et al. (2007) measured 50% lethal concentration (LC_{50}) in several organisms: *Thamnocephalus platyurus*, $LC_{50;24h} = 0.40$ mg/L; *Brachionus Calyciflorus*, $LC_{50;24h} = 0.97$ mg/L; *Daphnia magna*, $LC_{50;24h} = 1.53$ mg/L. The FASS (2011) also report $LC_{50;96h}$ for fish: *Lepomis macrochirus*, $LC_{50;96h} = 0.15$ mg/L; *Oncorhynchus mykiss*, $LC_{50;96h} = 0.27/0.21$ mg/L. In chronic study in zebrafish (*Danio rerio*), high mortality (≥ 88%) was observed for larvae exposed to 1 mg/L of TX and for juveniles exposed to 0.1 mg/L (Van der Ven et al. 2007). Although, exposure of medaka's eggs (*Oryzias latipes*) to TX showed in 100% mortality at concentrations above 3125 µg/L, while 40% mortality was observed at 625 µg/L (Sun et al. 2007).

Also, some authors showed some effects in the range of the toxicology endpoints measured in the current study (No Observed Effect Concentration (NOEC) of 10 µg/L nominal and Lowest Observed Effect Concentration (LOEC) of 1000 µg/L nominal). Hatching rate and hatching time were altered at 125 and 625 µg/L, but no morphological deformation was observed (Sun et al. 2007). Wester et al. (2003) report that spawning, fertilization, hatching, survival and growth of adult zebrafish exposed to 10 to 320 µg/L of TX-citrate were reduced. Based on growth, the LOEC and the 63-day NOEC were 10 and 3.2 µg/L, respectively. Morphological changes were also observed in both the ovaries and testes starting at 10 µg/L. However, when testing for fish embryotoxicity at environmental levels of cytotatics (i.e., from 0.001 to 1000 µg/L), no toxicity was observed, and this, for all five of the cytostatics tested. For MX, CAP, CP and IF, LC_{50}, EC_{50} and NOEC reported in the literature are generally between mg/L to g/L. Henschel et al. (1997) showed $LC_{50;48h}$ of 85 mg/L in *Danio rerio* embryo exposed to MX. Straub (2010) measured a $LC_{50;48h}$ in *Daphnia magna* of 850 mg/L and $NOEC_{96h}$ of 867 mg/L in *Oncorhynchus mykiss* for CAP. Weigt et al. (2011) measured $LC_{50;72h}$ of 2,200 mg/L in *Danio rerio* for CP and 836 mg/L for IF. In fact, embryos were mostly exposed during pre-hatch development, so the selectivity of the chorionic barrier could therefore explain the absence of observed effects.

Noteworthy, only the individuals of the IF treatment were showing a fully developed and inflated swim bladder at the end of the experiment. Swim bladder is a fish organ separated in two chambers. It begins as a posterior chamber, which inflates at 5–6 days post-fertilization (dpf) and the second chamber is formed anterior to the first one, around 14 dpf (Cavallin et al. 2017; Nelson et al. 2016). Development of swim bladder is under thyroid axis control. Inhibition of the thyroid axis could lead to a decreased inflation and/or size of swim bladder and a decline of the surfactant protein production that prevents it from collapsing (Godfrey et al. 2017; Cavallin et al. 2017; Nelson et al. 2016). The presence of all fully inflated posterior swim bladder chambers in the fish exposed to IF could be a marker of a developmental acceleration. Impairment of swim bladder inflation was observed in fish exposed to dilbit for example (Alsaadi et al. 2017; Madison et al. 2017). In addition, stress response is characterized by an increase of cortisol plasma level, and moreover, cortisol acts on target tissues by binding to glucocorticoid receptors (Alsop and Vijayan 2009) and is known to work synergistically or additively with thyroid hormones (Stephen et al. 1997). Therefore, it is possible that an exposure to IF as a stress factor, even at low concentrations, increase thyroid hormone/cortisol levels in fish larvae. Also, treatment with IF in human is known to be potentially involve in development of secondary tumors as thyroid cancer (FDA 2012).

In conclusions, data suggest that the five cytostatics do not induce embryotoxicity in developing fish at environmental levels. However, because cytostatics were engineered to kill cells using molecular mechanisms of action shared

among living organisms, one needs to investigate complementary biological endpoints (e.g., DNA damage, genotoxic effect) in other species and development stage to complete previous studies and ensure entire ecosystem safety. Moreover, most of these compounds are known to be persistent in the environment. Consequently, chronic exposure to cytostatics should also be assessed to further explore for behavior, teratogenic, and/or reprotoxic effects. Finally, cytostatics are also found in mixture within the environment and could potentially act synergically and/or additively; therefore, future studies should also address these research questions.

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