

GENOTOXIC POTENCY OF CYTOSTATIC DRUG RESIDUES: THE RELEVANCE FOR AQUATIC ENVIRONMENT AND HUMANS

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Doctoral Dissertation
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GENOTOKSIČNI POTENCIAL OSTANKOV CITOSTATIKOV:
POMEN ZA VODNO OKOLJE IN LJUDI

Doktorska disertacija

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Abstract

Cytostatics, also named anticancer drugs represent a group of pharmaceuticals that is mainly used in cancer treatment. These drugs are essentially targeting processes leading to the prevention of growth and tumour cells division via interference with the genetic material of tumour cells. However, the most commonly used cytostatics are not specific enough to target only tumour cells and they affect also normal cells of exposed humans and other organisms. Residues of cytostatics are released after human consumption into the environment, where they might affect nontarget aquatic organisms and indirectly exposed humans even at the levels of sub- to few ng/L in particular during chronic exposure. Nevertheless, ecotoxicological data that would allow evaluation of the environmental and human health risks posed by cytostatic residues released into aquatic environment are scarce. In the present thesis we focused our studies on exploring (eco)toxicity and genotoxicity of several most consumed cytostatics (i.e. 5-fluorouracil; 5-FU, cisplatin; CDDP, etoposide ; ET, imatinib mesilate; IM, cyclophosphamide; CP and ifosfamide; IF), their metabolites and transformation products towards zebrafish (*Danio rerio*) liver cells (ZFL) and human derived cells (human hepatoma HepG2 cells and isolated peripheral lymphocytes) *in vitro* as single compounds and their mixtures. The studies showed that 5-FU, CDDP, ET and IM induced genotoxic effects in both *in vitro* cell models which was expected for 5-FU, CDDP and ETO and is in accordance with their mechanism of therapeutic action. Surprisingly, also IM, which was developed as specific drug that acts as selective inhibitor of aberrantly expressed tyrosine kinase in certain types of cancer cells and is not classified as genotoxic, showed genotoxic activity towards nontarget cells. The analyses of the expression of DNA damage responsive genes indicated that its genotoxic activity is probably not due to direct interaction with DNA processing. This indicates that compared to other three cytostatics chronic exposure to IM probably represent a lower hazard for aquatic organisms and humans. Our studies also showed that ZFL cells are an *in vitro* model of choice for studying environmental pollutants. The studies of the mixtures of cytostatics demonstrated that in contrast to single drugs, which exerted genotoxic effects only at high for environment irrelevant concentrations, they affected ZFL cells at low, for environmental contamination relevant concentrations at which individual compounds are inactive. In accordance, we showed that samples of hospital wastewaters that contained various cytostatics, their metabolites and transformation products induced ecotoxic and genotoxic effects in exposed crustaceans, plants and in ZFL cells. Furthermore, we demonstrated that certain metabolites and/or transformation products of CP and IF are even more toxic and hazardous than the parent compounds. To explore long-term adverse effects of 5-FU on aquatic organisms a two-generation toxicity study with zebrafish has been performed. In addition to standard toxicological endpoints, the induction of genotoxic effects and a whole genome gene expression profiling were performed. The results demonstrated that chronic exposure to environmentally relevant concentration of 5-FU (10 ng/L) induces histopathological changes in the liver and kidney, along with the genotoxic effects and deregulation of several DNA-damage-responsive genes and oncogenes, while classical ecotoxicological endpoints, such as survival, growth, hatching and reproduction, were not affected. Anyways, the observed genotoxicity and degenerative changes may lead to reduced fitness of fish populations, which may over long-term exposure over several generations lead to extinction of susceptible organisms even at very low concentrations of the drugs. Taken together the studies showed that certain highly consumed cytostatics do represent a risk for the environment and humans. Due to their genotoxic mechanism of action, environmental risk assessment for cytostatics should be mandatory irrespective of the predicted environmental concentrations. In addition to parental compounds, also their

metabolites, transformation products and complex mixtures should be included in the environmental monitoring and risk assessments.

Povzetek

Citostatiki (tudi protirakava zdravila ali antineoplastiki) predstavljajo skupino zdravil, ki se uporabljajo za zdravljenje raka. Ta zdravila v glavnem preko interakcij z genetskim materialom preprečujejo rast in razmnoževanje tumorskih celic. Genotoksični mehanizem delovanja, ki je značilen za veliko večino citostatikov, pa ni dovolj specifičen in poleg rakavih celic lahko ta zdravila prizadenejo tudi normalne celice pacientov in drugih posredno izpostavljenih ljudi ter okoljskih organizmov. Po zaužitju se ostanki citostatikov izločijo v okolje, kjer lahko predvsem zaradi kronične izpostavitve prizadenejo netarčne vodne organizme in posredno izpostavljene ljudi celo pri nizkih koncentracijah (pod ng/L). Kljub temu so ekotoksikološki podatki, ki omogočajo oceno tveganja za okolje in zdravje ljudi, pomanjkljivi. V doktorski nalogi smo se v glavnem osredotočili na (eko)toksičnost in genotoksičnost najpogostejše uporabljenih citostatikov (5-fluorouracil – 5-FU, cisplatin – CDDP, etopozid – ETO, imatinib mezilat – IM, ciklofosfamid – CP in ifosfamid – IF), njihovih metabolitov in transformacijskih produktov, kot posamezne kemikalije in v kombinaciji na izbranih *in vitro* modelih, jetrne celice rib cebric (*Danio rerio*) (ZFL) in celice humanega izvora (humane jetrne HepG2 celice in izolirani krvni periferni limfociti). Rezultati raziskav so pokazali, da 5-FU, CDDP, ETO in IM povzročajo genotoksične učinke v izbranih *in vitro* modelih, kar je bilo v skladu z našimi pričakovanji in njihovimi terapevtskimi mehanizmi, kar velja predvsem za 5-FU, CDDP in ETO. Presenetljivo je tudi, da je IM pokazal genotoksično aktivnost na netarčnih celicah, čeprav velja za bolj tarčno zdravilo, ki deluje kot selektivni inhibitor specifičnih mutiranih tirozin kinaz pri določenih vrstah raka in do sedaj skoraj ni podatkov o njegovi genotoksični aktivnosti. Vendar analiza izražanja genov odzivnih na DNA poškodbe nakazuje, da genotoksičnost IM najverjetneje ni posledica direktnih interakcij z DNA procesiranjem. Posledično predstavlja kronična izpostavitve IM manjše tveganje za posredno izpostavljene okoljske organizme in ljudi kot izpostavitve 5-FU, CDDP in ETO. Rezultati naših študij tudi kažejo, da so ZFL celice zelo občutljive na citotoksične in genotoksične učinke citostatikov in predstavljajo dober *in vitro* model za preučevanje okoljskih onesnažil. Genotoksičnost je v primeru testiranja posameznih citostatikov zaznana pri visokih okoljsko nerelevantnih koncentracijah. Nasprotno pa kombinacija več citostatikov povzroči genotoksične učinke pri nizkih, za okolje relevantnih koncentracijah, pri katerih posamezni citostatiki ne kažejo nobene aktivnosti. V nalogi smo pokazali, da bolnišnične vode, v katerih so prisotni številni citostatiki, njihovi metaboliti in transformacijski produkti, povzročijo ekotoksične in genotoksične učinke na izpostavljenih vodnih bolhah, rastlinah in ZFL celicah. Na primeru CP in IF smo pokazali, da so lahko nekateri metaboliti in transformacijski produkti bolj toksični in nevarni kot njihove izhodiščne spojine. Ti rezultati potrjujejo, da ostanki citostatikov doprinesejo k genotoksičnosti odpadnih voda. V namen preučevanja dolgodobnih škodljivih učinkov citostatikov na okoljskih organizmih smo izvedli dvogeneracijsko študijo z ribami cebricami izpostavljenimi 5-FU. Poleg standardnih ekotoksikoloških parametrov smo spremljali tudi genotoksikološke učinke in izvedli analizo izražanja celotnega genoma. Rezultati so dokazali, da kronična izpostavitve cebric okoljsko relevantnim koncentracijam 5-FU (10 ng/L) povzroči histopatološke spremembe na jetrih in ledvicah, genotoksične učinke ter deregulacijo številnih onkogenov in genov, povezanih v odgovor na DNA poškodbe. Nasprotno pa klasični ekotoksikološki parametri, kot so preživetje, rast, razmnoževanje in izleganje niso bili spremenjeni. Čeprav preživetje in razmnoževanje cebric nista bila prizadeta, pa lahko opazovane genotoksične in degenerativne poškodbe vodijo do zmanjšane fitnesa ribje populacije, ki lahko v primeru dolgodobne izpostavljenosti privede do izumrtja najobčutljivejših organizmov, tudi pri zelo nizkih okoljskih koncentracijah teh zdravil. Rezultati naših študij kažejo, da določeni pogosto uporabljeni citostatiki predstavljajo tveganje za okolje in ljudi. Glede na njihov genotoksičen mehanizem delovanja bi morala

biti ocena tveganja za citostatike obvezna ne glede na predvidene okoljske koncentracije. V okoljski monitoring in oceno tveganja pa bi poleg učinkovin (starševske spojine) morali biti vključeni tudi njihovi metaboliti in transformacijski produkti.

3.2	Assessment of the Genotoxicity of the Tyrosine Kinase Inhibitor Imatinib Mesylate in Cultured Fish and Human Cells.....	56
3.3	Influence of Selected Anti-cancer Drugs on the Induction of DNA Double-Strand Breaks and Changes in Gene Expression in Human Hepatoma HepG2 Cells	65
3.4	Ecotoxicity and Genotoxicity of Cyclophosphamide, Ifosfamide, Their Metabolites/Transformation Products and Their Mixtures	77
3.5	Cytotoxicity and Genotoxicity of Anticancer Drug Residues and Their Mixtures in Experimental Model With Zebrafish Liver Cells	88
3.6	Chemical and Toxicological Characterisation of Anticancer Drugs in Hospital and Municipal Wastewaters From Slovenia and Spain.....	97
3.7	Assessment of Toxicity and Genotoxicity of Low Doses of 5-Fluorouracil in Zebrafish (<i>Danio Rerio</i>) Two-Generation Study.....	111
4	Conclusions	125
	References	127
	Bibliography	131
	Biography	133

List of Figures

Figure 1: Mechanism of action of cisplatin.....	21
Figure 2: The metabolic pathway of activation of cyclophosphamide to DNA reactive phosphoramidate mustard.....	22
Figure 3: Mechanism of action 5-fluorouracil.....	23
Figure 4: Mechanism of action of topoisomerase inhibitors.....	25
Figure 5: Mechanism of action of imatinib mesylate.....	26
Figure 6: Algae (a), daphnia (b) zebrafish adult (c) and embryo (d).....	31
Figure 7: The image of damaged nuclei (comet assay) and binucleated cells with micronucleus, nucleoplasmic bridge and nuclear buds (micronucleus assay).....	35

Abbreviations

5-FU	... 5-fluorouracil
CBMN	... Cytokinesis block micronucleus assay
CDDP	... Cisplatin
CP	... Cyclophosphamide
EC ₅₀	... Half maximal effective concentration
EMA	... European Medicines Agency
ETO	... Etoposide
ERA	... Environmental risk assessment
FDA	... U.S. Food and Drug Administration
HepG2	... Human liver cancer cell line
HPBL	... Human peripheral blood lymphocyte
IF	... Ifosfamide
IM	... Imatinib mesylate
LC ₅₀	... Lethal concentration 50%
LOEC	... The lowest observed effect concentration
MN	... Micronuclei
MTS	... (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium)
MTT	... 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NOEC	... no observed effect concentration
NBUD	... Nuclear plasmic bud
NPB	... Nuclear plasmic bridge
PEC	... Predicted environmental concentration
STP	... Sewage treatment plant
TP	... Transformation products
ZFL	... Zebrafish liver cells

Chapter 1

Introduction

Pharmaceuticals are a broad group of synthetic chemicals with different mechanisms of action used to diagnose, treat, cure or even prevent various diseases in humans and animals. These biologically active substances have brought great benefits to humanity in terms of healthier and longer lives and improved the quality of life. Their overall consumption is substantial and it is increasing globally mainly due to population growth, aging and expiration of patents leading to less expensive and more available generics (Daughton, 2002; Kummerer, 2009).

Pharmaceuticals after their use, similar as other anthropogenic compounds, may reach wastewaters, surface waters, ground waters and even drinking waters, where they have been detected at the level of sub ng/L to few µg/L (reviewed in (Heberer, 2002; Kummerer, 2001; Kuster and Adler, 2014; Ortiz de Garcia et al., 2013)). The measured concentrations depend on consumption amounts, patterns of administration, legislation and efficiency of sewage treatment plants to remove such persistent compounds. As these features are more or less specific for each country, different concentrations between countries are expected. Nevertheless, the consumption trends of major pharmaceutical groups worldwide tend to be similar due to globalization of the pharmaceutical industry (reviewed in (Ortiz de Garcia et al., 2013)) and consequently similar pharmaceuticals are expected to occur in the environment across the globe.

Despite the low environmental concentrations, effects on non-target organisms are possible and expected especially as these compounds are designed to retain chemical stability long enough after administration to induce pharmacological response in target organs or tissues at low concentrations and are therefore resistant against degradation in the environment. However, only little is known about their effects on environmental organisms especially under conditions of chronic exposure to low, environmentally relevant concentration (Dong et al., 2013).

In addition, also humans can be unintentionally exposed to pharmaceutical residues, especially health care workers that handle these compounds and general population through contaminated drinking water. However, the data that allows human health risk assessment posed by pharmaceutical residues are scarce (Besse et al., 2012; Kosjek and Heath, 2011; Kummerer et al., 2014).

Pharmaceuticals can be classified according to the purpose of use and biological activity into different groups such as: antibiotics, analgesics, cytostatics, anti-inflammatories, estrogens and hormonal drugs, antiepileptics, cardiovascular pharmaceuticals, etc. (Kummerer, 2009). Since there are 3000 – 10000 different pharmaceuticals currently in use (Boxall et al., 2012; Dong et al., 2013), it would be impossible to experimentally assess the hazards and risks of all of these compounds in a timely manner. Therefore, different prioritization approaches have been used to identify those that are more likely to pose risk for the environment and need special attention when dealing with environmental risk (Boxall et al., 2012; Kuster and Adler, 2014). As such, several groups of pharmaceuticals have been identified. One such group are cytostatics which have been until recently constantly overlooked in risk assessments, mainly due to their low consumption amounts, low predicted environmental concentrations and scarce information regarding their occurrence in the environment (Besse et al., 2012; Kosjek and Heath, 2011; Kummerer et al., 2014). However, many of these drugs are classified as mutagenic, genotoxic, carcinogenic, embryotoxic and/or teratogenic. Furthermore it has been postulated that they can cause adverse effects in the

aquatic ecosystem and indirectly exposed humans at low concentrations (Besse et al., 2012; Kosjek and Heath, 2011; Kummerer et al., 2014; Toolaram et al., 2014).

1.1 Cytostatics and Their Pharmacodynamics and Pharmacokinetics

Cytostatics (also anticancer drugs, antineoplastic drugs, chemotherapeutics) are mainly used in chemotherapy to fight cancer, a disease involving uncontrolled multiplication of the body's own cells and spreading abnormal forms within the body. In general, these drugs prevent or disrupt tumour cell division, by interfering with DNA, promotion of cell apoptosis, or more recently by targeting the key proteins required for cell division (Besse et al., 2012; Kosjek and Heath, 2011; Kummerer et al., 2014; Xie, 2012). As most of these mechanisms of action are not very specific, cytostatics affect also patient's normal growing cells, which during the chemotherapy often cause side effects. Additionally, once present in the environment they may cause adverse effects to aquatic ecosystems and indirectly exposed humans even at environmental concentrations, especially under conditions of chronic exposure. Therefore, it was stressed out that in-depth risk assessment of these pharmaceuticals should be performed (Besse et al., 2012; Kosjek and Heath, 2011; Toolaram et al., 2014).

Anticancer drugs belong to the Class L - Antineoplastic and Immunomodulating Agents (ATC, Anatomical Therapeutic Classification) with more than 260 different drugs. According to their mechanism, these drugs can be classified into alkylating agents, antimetabolites, topoisomerase inhibitors, protein kinase inhibitors etc. Drugs that belong to the same group or even subgroup can have different chemical structures and consequently different characteristics that might be manifested in different environmental fate and effects. Therefore, risk assessment should be performed for individual drugs and not for the whole class (Kummerer, 2009).

1.1.1 Alkylating agents

Alkylating agents are used in chemotherapy for more than 70 years and still present the chemotherapy backbones for several types of cancer. Their main mechanism of action is DNA alkylation (Fleming, 1997; Kelland, 2007; Xie, 2012).

1.1.1.1 Nonclassical alkylating agents (platinum compounds)

Cisplatin (CDDP) and carboplatin are two most important drugs from this class of anticancer drugs. CDDP reached the market in 1978 and it is still among the most potent cytostatics with clinical activity against a wide variety of tumours (testicular, neck, ovarian, non small cell lung, cervical, head and esophageal cancer) (Guainazzi and Scharer, 2010; Kelland, 2007; Siddik, 2003).

After intravenous administrations, CDDP enters into the cells with passive diffusion and active transport (gated channels, transporters CTR1), but relatively slowly due to its high polarity. Once located inside the cell, the drug, which is a planar molecule of Pt(II) with two chloride ligands in cis-position, is activated by hydrolysis of one (monofunctional adduct) or two (bifunctional adduct) chloride atoms (chloride atoms are replaced with one/two molecule of water) resulting in the formation of aquated highly reactive species (Guainazzi and Scharer, 2010; Kelland, 2007). These highly reactive species mainly react with DNA molecules (covalent bound), preferentially with purine bases (guanine and to a lower extent also adenine) at N7 (nucleophilic) position, resulting in the formation of adducts and crosslinks (Figure 1) (Guainazzi and Scharer, 2010; Jamieson and Lippard, 1999; Kelland, 2007). In addition to genomic DNA, CDDP targets also mitochondrial DNA and according to some data even to a higher extent (up to 4-6 fold higher proportion of DNA adducts). This could be due to the absence of nucleotide excision repair in mitochondria, which is the main pathway for removing CDDP-induced damage in genomic DNA (Jamieson and Lippard, 1999). CDDP reacts also with several other cellular components; RNA, proteins, membrane phospholipids, and/or microfilaments but with lower affinity (Siddik, 2003).

CDDP-DNA adducts directly affect DNA integrity and block normal separation of DNA helix and thus indirectly inhibit DNA replication/synthesis and suppress transcription. Therefore, affected cells are arrested in the G2 phase of the cell cycle which at high concentrations of CDDP results in cell death. CDDP-DNA adducts also inhibit initiation and elongation of polymerase, block attaching of transcription factors and hijack proteins required for transcription. Polymerase can bypass to some extent platinum induced adducts, but during such DNA replication some additional mutations might be introduced. CDDP inhibits also the activity of telomerases and induce the shortening and degradation of telomeres (Jamieson and Lippard, 1999).

Carboplatin is a second generation analogue of CDDP and approved by FDA in 1989. It has a similar mechanism of action as CDDP. However, it showed lower anticancer activity than CDDP and more acceptable side effects (Kelland, 2007).

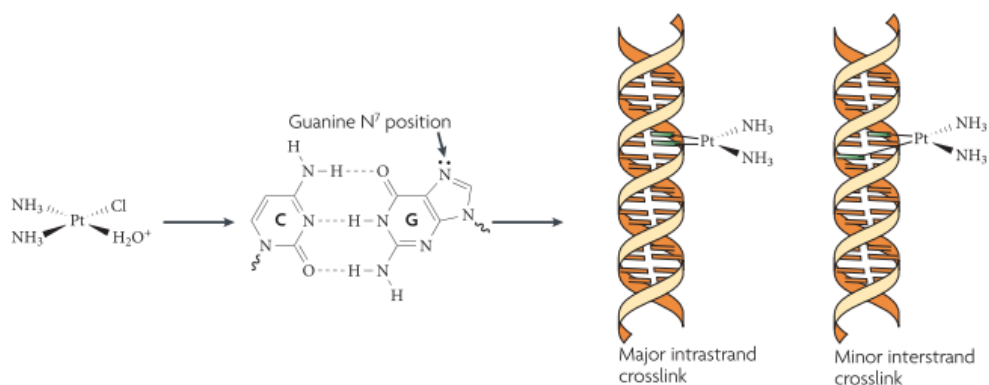


Figure 1: Mechanism of action of cisplatin (Kelland, 2007).

1.1.1.2 Classical alkylating agents – nitrogen mustards analogues

Classical alkylating agents have a similar structure as mustard gas and are mainly used for the treatment of lymphoma, leukemia, and several solid tumours. The most famous and widely used representatives are cyclophosphamide (CP) and ifosfamide (IF) (others: chlorambucil, melphalan etc.) (Besse et al., 2012; Fleming, 1997). These two drugs can be administered orally or intravenously with much better bioavailability after oral administration (85-100 %) (Fleming, 1997).

CP was approved by FDA (U.S. Food and Drug Administration) almost 60 years ago (1958) and it is still one the most effective molecules towards various cancers (Brock, 1989; Emadi et al., 2009). IF is an isomer and substitute of CP and it entered the market almost 30 years later (year 1987) in order to improve its water solubility and broaden its anticancer activity. However, it has not been proven to be more efficient than CP in any type of cancer and it has an even higher toxicity compared to CP (Brock, 1989).

CP and IF are prodrugs that need metabolic activation mainly by liver microsomal enzymes (hepatic and intracellular enzymes) such as CYP2B and CYP3A through several steps to their final active species (phosphoramidate mustard and ifosfamide mustard, respectively) (Figure 2) that are responsible for their anticancer activity (Fleming, 1997). Mechanism of action is similar as for nonclassical alkylating agents: DNA alkylation of two guanine bases at N7 within the same or complementary DNA strands forming intrastrand and interstrand crosslinks. The crosslinks are strong covalent bounds that prevent DNA separation, interfere with DNA replication, transcription and subsequently lead to cell death (Emadi et al., 2009; Fleming, 1997).

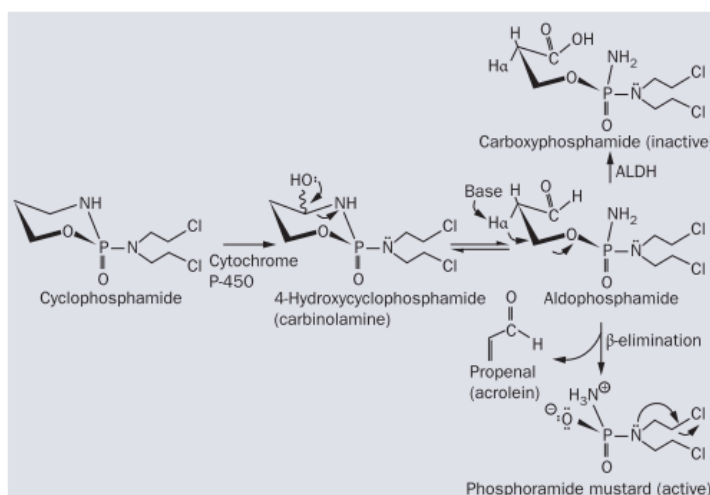


Figure 2: The metabolic pathway of activation of cyclophosphamide to DNA reactive phosphoramidate mustard (Emadi et al., 2009).

1.1.2 Antimetabolites

These drugs are widely used to treat lung, leukemia, pancreatic and colorectal cancers and are one of the oldest cytostatics (Xie, 2012). They are intentionally designed to have similar structures as some cell's own components; pyrimidine (pyrimidine analogues), purine (purine analogues) and folic acid (folic acid analogue). Nevertheless, due to their small but significant differences in the structure these drugs interfere also with normal physiological reactions in which they are involved (Kummerer et al., 2014).

1.1.2.1 Folic acid analogue/Antifolates

They are structural analogues of folates, vitamins that are essential for de novo synthesis of DNA and enter into the cell with the same transporter system as folates. Antifolates are specifically designed to target and inhibit key folate-dependent enzymes, which results in nucleotide biosynthesis inhibition and consequently inhibition of cell proliferation and further damage that in the final stage leads to cell death. In clinic around 13 different antifolates are used and among them Methotrexate is the most widely used (reviewed in (Hagner and Joerger, 2010)).

1.1.2.2 Pyrimidine analogues

The most recognizable drugs from this group are 5-fluorouracil (5-FU), capecitabine, gemcitabine, and cytarabine. They are structurally similar to pyrimidine bases, components that build DNA (Xie, 2012).

5-FU was synthesized around 60 years ago (1957) and it is still widely used as single chemotherapeutic or in combination with other cytostatics for the treatment of colorectal, breast, aerodigestive tract, head and neck cancer. It has a similar structure as uracil, but at the C-5 position hydrogen atom is replaced with fluorine atom. The drug enters into the cells with the same transporters as uracil and once inside the cell the drug is metabolized to several active metabolites which cause (Figure 3):

- inhibition of thymidylate synthase (TS), enzyme that is involved via cascade reactions into de novo source of thymidine, and thus it is necessary for DNA replication and repair. Inhibition of the enzyme leads to imbalance of other deoxynucleotides that severely disrupt DNA repair and synthesis, finally resulting in lethal DNA damage (strand breaks).

- Misincorporation of 5-FU metabolites (FdUTP and dUTP) into DNA. When cells try to repair these lesions further false nucleotide incorporations occur. Overall, futile cycles of misincorporation, excision and repair eventually lead to DNA strand breaks and cell death.
- Incorporation of 5-FU metabolites (FUTP) into RNA, disrupts protein synthesis and RNAs processing (reviewed in (Longley et al., 2003)).

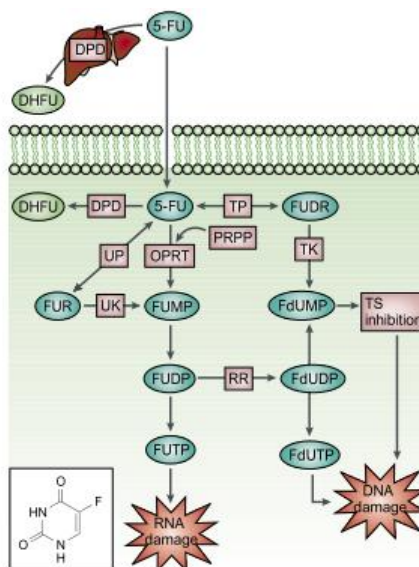


Figure 3: Mechanism of action 5-fluorouracil (Longley et al., 2003).

5-FU is rapidly degraded in the liver (up to 80 %) and thus only a small fraction of the drug is available to act antitumorigenic. In order to improve its poor bioavailability, the prodrug capecitabine has been developed. Unlike 5-FU, capecitabine is administered orally and it is absorbed unchanged through the gastrointestinal wall. It is converted to 5-FU via three-step enzymatic cascade starting in the liver. The final step occurs with enzymes (thymidine phosphorylase and uridine phosphorylase) that are more active in tumour cells than in normal, causing tumour selective activation of capecitabine to 5-FU. Additionally, capecitabine has fewer side effects than 5-FU (reviewed in (Longley et al., 2003; Walko and Lindley, 2005)).

1.1.3 Topoisomerase inhibitors

These drugs interfere with normal functions of DNA topoisomerases I and II, enzymes that are involved in controlling, modifying, and maintaining structures and topology of DNA. These processes are important during normal replication, transcription, recombination and translation of genetic material (reviewed in (Burden and Osheroff, 1998)).

Topoisomerase after the binding to DNA generates single (topoisomerase I) or double-strand breaks (topoisomerase II) in the sugar-phosphate backbone and stays attached (covalent bond) with two newly generated terminus, forming the structure known as cleavage complex. These strand breaks are in cells normally transient, at low concentrations, and short lived intermediates that are tolerated by the cells despite their potentially toxic effects. After the formation of DNA strand breaks, the DNA double helix can over- and under-wind, which results in resolving nucleic acid knots and tangles. After the DNA is relaxed, the breaks are rejoined with covalent bond and relegation favored cleavage (Burden and Osheroff, 1998; Pommier, 2013).

Topoisomerase inhibitors do not cause direct DNA damage but mainly stabilize complex DNA-enzyme (cleavage complex) that enables religation of broken DNA.

Stabilized cleavage complex is present longer than usually and when certain enzymes (helicase, polymerase) during transcription and/or replication attempt to traverse it, the cleavage complex is destroyed. This convert temporary DNA strand breaks, formed by the enzyme, into permanent DNA strand breaks. Formed brakes then become a target of different repair pathways that can further damage genetic material (reviewed in (Pommier, 2013)). When the tolerated level of strand breaks is exceeded (critical point) apoptosis occur. Overall, topoisomerase inhibitors do not directly interact with DNA but hijack cellular enzyme that itself damages DNA (reviewed in (Burden and Osheroff, 1998; Pommier, 2013)). There are two types of topoisomerase inhibitors, depending on which type of enzyme is their primary target.

1.1.3.1 Topoisomerase I inhibitors (Topotecan and irinotecan)

These inhibitors are directed toward normal function of cell cycle specific enzyme topoisomerase I. The enzyme relaxes DNA supercoiling by causing single strand break and consequently rotation of broken DNA around the cleavage complex, followed by rejoining of DNA double helix independently form ATP (Figure 4) (reviewed in (Pommier, 2013)). The first developed drug from this group is camptothecin which was removed from the market due to its severe side effects. Later its derivatives – topotecan and irinotecan were developed. These drugs mainly bind to the cleavage complex with high selectivity (do not bind to the enzyme alone and only weakly to DNA), but with low affinity. Therefore, high concentrations of the drug are required in order to observe desirable pharmacological response. Additionally, binding to the cleavage complex is reversible with short reversing time after the drug is metabolized. Therefore, prolonged infusions of relatively high concentrations of the drug must be given in order to maintain persistent cleavage complex that is then converted into DNA damage (reviewed in (Pommier, 2006)).

1.1.3.2 Topoisomerase II inhibitors

This group of cytostatics mainly targets topoisomerase II, an ATP dependent enzyme that modulates DNA topology (resolving knots and tangles) by forming transient double stranded breaks (DSBs) (Figure 4) (Burden and Osheroff, 1998; Pommier, 2013).

Among topoisomerase II inhibitors the most widely used is etoposide (ETO) which is one of the most active chemotherapeutics used as single agents in the fight against small cell lung cancer and it is also effective towards testicular cancers, lymphoma and Kaposi sarcoma (Pommier, 2013; Xie, 2012). In addition to stabilizing the cleavage complex, ETO can also bind to topoisomerase II in the absence of DNA and it enters into cleavage complex mainly through interactions with topoisomerase (reviewed in (Burden and Osheroff, 1998)).

Other important drugs from the group are doxorubicin and daunorubicin that are used in the treatment of leukemia, breast, small cell lung and ovarian cancer. In addition to stabilization of cleavage complex, these drugs also generate free radicals, intercalate between base pair of DNA, and hinder the process of strand separation, by targeting nuclear helicase (reviewed in (Pommier, 2013)).

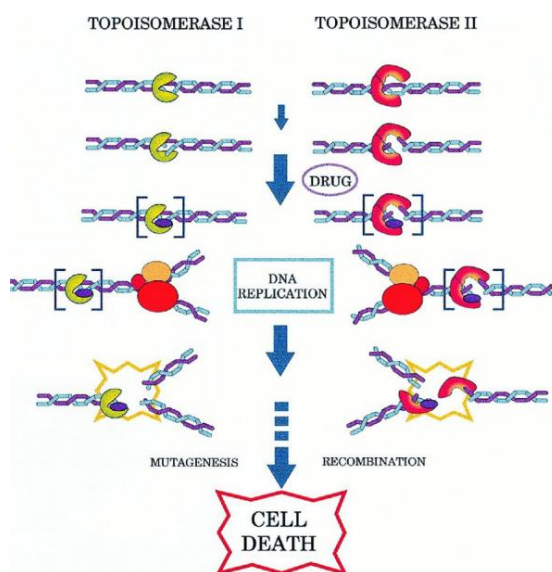


Figure 4: Mechanism of action of topoisomerase inhibitors (Pommier, 2013).

1.1.3.3 Targeted Therapies

Targeted therapy presents a new era in chemotherapy with a more personalized approach that appeared approximately 20 years ago and completely revolutionized the cancer treatment. The idea behind this revolutionary therapy is to attack cancer cells with higher selectivity on one side and to minimize damage/death of normal cells with fewer undesirable side effects on the other side. Due to their clinical success, cancer treatment moved away from the use of nonspecific conventional anticancer drugs (mainly with genotoxic activity) that do not discriminate between cancer and normal cells usually with severe side effects towards application of more targeted molecular therapies. Among all FDA approved anticancer drugs between year 2000 and 2014 79% belong to the group of targeted therapies (reviewed in (Perez-Herrero and Fernandez-Medarde, 2015)).

These drugs are developed to interact with cancer cells through different mechanisms such as stimulation of immune system, selective blocking of the signals that drive tumour cells to grow and divide uncontrollably, or targeted delivery of the drug to cancer cells only (reviewed in (Perez-Herrero and Fernandez-Medarde, 2015)) (Sawyers 2004M). According to these mechanisms of action, they can be subdivided into protein kinase inhibitors (PKIs), monoclonal antibodies, antibody drug conjugates, immune checkpoint inhibitors and hormonal agents.

1.1.3.4 Tyrosine kinase inhibitors (TKIs)

Tyrosine kinase inhibitors (TKIs) are the biggest group of drugs among targeted therapies and their number has been in the last ten years doubled, however currently they target only a small fraction of different cancer types (Levitzki, 2012). These drugs are designed to selectively target tyrosine kinases that are mutated and abnormally expressed in certain types of cancers (Arora and Scholar, 2005).

Protein tyrosine kinases (PTKs) are ATP-consuming enzymes that are activated by extracellular (transmembrane receptor; EGFR, PDGFR, VEGFR) or intracellular signals (intracellular nonreceptor ABL, SRC). After the activation, they catalyze the transfer of the phosphate group from ATP to target proteins, usually tyrosine residues. Protein phosphorylation by kinases is an important mechanism in signal transduction and regulates the majority of cellular pathways (such as cell division, cell growth, migration, differentiation, and death). Therefore, for normal cellular regulatory processes it is essential that their enzymatic activity is under tight control. In some cases PTKs can be deregulated by:

- constant autophosphorylation of PTKs in the absence of the ligand;
- specific mutations in the kinase domain (deletions, point mutations) that disrupt autoregulation of the kinase and increased sensitivity of the receptor to its ligand
- and increased or aberrant expression of receptor, ligand, or both.

This can lead to enhanced and persistent stimulation of the signal that results in cancer development, increased tumour cell growth, antiapoptotic effects, metastasis and/or promoted angiogenesis (reviewed in (Arora and Scholar, 2005)). With this, PTKs present attractive targets in chemotherapy. Most of TKIs are designed to inhibit catalytic activity of the enzyme by interfering with the ATP binding (Levitzki, 2012; Levitzki and Mishani, 2006).

First tyrosine kinase inhibitor, IM, was approved in year 2001 and was developed for treatment of chronic myeloid leukemia (CML), a disease that is a result of a reciprocal translocation of chromosome 9 and 22, cytogenetically visible as a shortened chromosome 22 (so-called Philadelphia chromosome). This translocation forms BCR-ABL fusion gene the product of which is constantly activated tyrosine kinase. This kinase results in deregulation of intracellular signaling and consequently in increased proliferation and reduced apoptosis. IM selectively and with high specificity inhibits BCR-ABL tyrosine kinase (Figure 5) and completely revolutionized outcome of this disease. As BCR-ABL kinases are absent in normal cells, IM is generally well tolerated with minimal side effects (minimally harmful to normal cells) (Druker, 2010). In addition, IM inhibits also mutated c-KIT and PDGFR kinases that play the role of gastrointestinal stromal tumor (GIST). Nevertheless, it has been shown that IM might block also PDGFR, c-KIT and c-ABL tyrosine kinase of normal cells, where these kinases play important roles, but when this happens normal cells utilize alternative pathways. Meanwhile survival of CML cells is mainly dependent on BCR-ABL, thus these cells will die when its activity is blocked (Levitzki, 2012; Levitzki and Mishani, 2006).

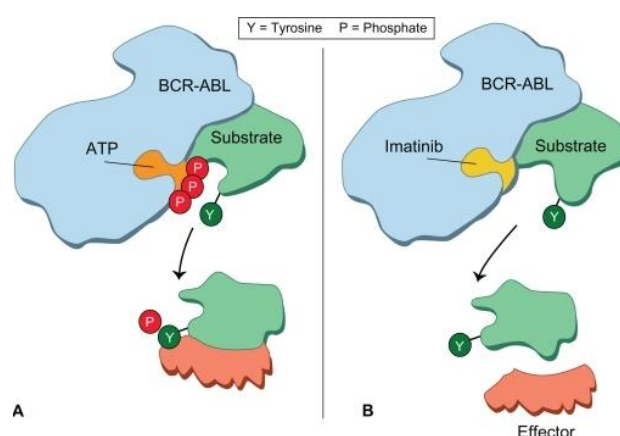


Figure 5: Mechanism of action of imatinib mesylate (Mughal and Schrieber, 2010).

1.2 Consumption Amounts and Trends

High consumption amounts of cytostatics have been reported for some countries. However, at the moment detailed data regarding the consumption amounts of all cytostatics present on the market at a national scale across a longer period that allow monitoring of trends are rare and absent for most countries with few exceptions (England, Germany, France and Spain) (Besse et al., 2012; Booker et al., 2014; Franquet-Griell et al., 2015; Kummerer et al., 2014). On the contrary, there is more available consumption data of only few cytostatics on a more local level (hospital, region) (Buerge et al., 2006; Johnson et al., 2013; Kovalova et al., 2009; Kummerer and Al-Ahmad, 2010; Martin et al., 2011; Nussbaumer et al., 2011). Surveys of cytostatics consumption are performed mainly in Europe, meanwhile there is almost no data for consumption outside of Europe (Yin et al.,

2010). Nevertheless, available consumption data are essential for identification of those drugs that are currently most likely entering into the aquatic environment (Besse et al., 2012).

The most consumed group of cytostatics by far are antimetabolites, followed by, alkylating agents, tyrosine kinase inhibitors and drugs with endocrine activity. The consumption of most classes is increasing at the local and national scale (Besse et al., 2012; Booker et al., 2014; Kummerer et al., 2014). Furthermore, it is expected that the consumption of all anticancer drugs will increase per around 10 % per year mainly due to:

- increased prevalence of new cancer cases (70 % increase of new cases over the next 2 decades, according to world health organization);
- longer chemotherapy that correlates with increased survival time in average and fewer unwanted side effects;
- improvement in cancer therapy that allows administrations of higher doses and treatment of patients that were untreated before;
- better and earlier diagnose of cancer;
- usage of cytostatics for treatment of some other diseases and
- usage of cytostatics in veterinary purposes (reviewed in (Besse et al., 2012; Booker et al., 2014; Johnson et al., 2008; Toolaram et al., 2014) (<http://www.who.int/mediacentre/factsheets/fs297/en/> 08.02.2017)).

Consumption trends for individual drugs are not so uniform and for some cytostatics also a decrease in consumption has been observed, or some even completely disappeared from the use in clinic. Such cytostatics are usually replaced with new ones, probably more efficient and/or with fewer side effects (Besse et al., 2012). However, the total number of cytostatics used in chemotherapy is increasing (Besse et al., 2012; Kummerer et al., 2014). In Germany 32 new cytostatics entered the market in period of only 6 years (from year 2006 to 2012) to altogether 102 (Kummerer et al., 2014). Interestingly, the majority of new drugs that entered the market are for target therapies (TKIs), which are already a few years after their introduction to clinical use among the most consumed cytostatics (Besse et al., 2012; Kummerer et al., 2014).

Consumption trends reveal big differences in the use of cytostatics between different countries or even more locally (regions), although rates of different cancer types are similar. For example, the grates variation was observed for 5-FU with 28-fold difference between minimum and maximum consumption. Consumption of CP is very low in Austria and Denmark and the highest in Sweden with 15-fold difference compared to European mean value. Carboplatin is almost three times more often used in the Czech Republic as is the European average (Johnson et al., 2013). Nevertheless, there are also some similarities in the use of certain drugs across Europe. For example in Germany and France the same cytostatics have been identified as top four the most consumed cytostatics in the same order; hydroxycarbamide, capecitabine, 5-FU and IM (Besse et al., 2012; Kummerer et al., 2014).

Additionally, consumption trends revealed a strong shift towards oral application carried out outside of hospitals for almost all classes of cytostatics. This shift has been observed at a national and local level and was linked with improving patients' comfort (Besse et al., 2012; Kosjek and Heath, 2011). In the period of just four years (2004 to 2008) the consumption of cytostatics in hospitals decreased from 82 % to about 35 %. The main classes used in hospitals are antimetabolites and alkylating agents, meanwhile TKIs are predominantly used outside the hospitals (Besse et al., 2012). Furthermore, approximately 75 % of oncology patients after the infusion of injection leave the hospital (Besse et al., 2012). All these trends strongly influence the possible entry routes of cytostatics into the environment.

1.3 Source of Cytostatics into the Environment

The majority of cytostatics are incompletely metabolized and thus a certain amount of unchanged parent compound is excreted together with various pharmacologically active and inactive metabolites (Besse et al., 2012; Xie, 2012). The excretion rates of unchanged parent molecules among drugs vary over the wide range from just a few % to 90 %. Additionally, differences in excretion rates are observed also for the same cytostatic and

depend on the dosage, type of administration, co-medications, and inter-patients variation (age, metabolism rate, and health) (Booker et al., 2014; Johnson et al., 2013; Kummerer et al., 2014). Therefore, it is hard to define the exact excretion value for a drug. Furthermore, often data about excretion rates and metabolites are completely missing (Kummerer et al., 2014).

The most important source of cytostatics in the environment is human consumption (Kummerer et al., 2014). After administration, cytostatic residues are largely excreted via urine and/or feces into hospital and/or municipal effluents (Figure 6). Their concentrations in effluents vary on local and national levels and strongly correlate with consumption amounts, patterns and trends, excretion rates and dilution factors (Besse et al., 2012; Kummerer et al., 2014; Zhang et al., 2013).

The first monitoring studies of cytostatic residues were focused on hospitals effluents, as it was a common impression that these compounds enter into the environment almost exclusively from hospital applications (Kovalova et al., 2012; Kummerer and Al-Ahmad, 1997; Mahnik et al., 2007; Steger-Hartmann et al., 1997; Zouunkova et al., 2007). Since hospital effluents are rarely pre-treated prior entering into the public sewer system (Verlicchi et al., 2012; Yin et al., 2010), they become an important point source of cytostatic residues into the aquatic environment (Kosjek and Heath, 2011; Zhang et al., 2013). Therefore, quantification of cytostatic residues in hospital effluents may serve as a starting point to individualize the magnitude of putative pollution problems (Mahnik et al., 2007; Yin et al., 2010).

As the consumption trends changed over time from in-patients to out-patients (the patient shortly after receiving the drug left the hospital) and oral consumption at home, household effluents became an important source as well with high environmental concern (Besse et al., 2012; Kosjek and Heath, 2011; Kummerer et al., 2014; Zhang et al., 2013). Consequently, in hospital effluents only 1.1 to 13.8 % of all consumed cytostatics are found and therefore these effluents are not considered any more as the main entry route of cytostatics into the aquatic environment (Besse et al., 2012; Ferrando-Climent et al., 2014; Kummerer and Al-Ahmad, 2010). In contrast to hospital effluents, municipal effluents have been scarcely investigated, and studies are usually limited to determinations of the most consumed cytostatics (Zhang et al., 2013). The measured concentrations are generally lower than those detected in hospital effluents (Kosjek and Heath, 2011; Martin et al., 2011). With increasing consumption at home, the introduction of cytostatics into environment became more diffuse.

Overall, the type of hospitalization strongly influences the entry routes of cytostatics into the sewage system (Besse et al., 2012). As it is expected that an increased level of cytostatics into the environment will be released in the future, it is necessary to continue surveying their presence in effluents.

1.4 Predicted Behaviour in Sewage Treatment Plants and in the Aquatic System

Usually effluents, prior entering into the environment, pass through sewage treatment plants (STPs) where the processes of removing pollutants with physical, chemical and biological processes occur. Their fate and distribution in STPs depend on the used treatment processes and physicochemical profiles of each pollutant. Furthermore, elimination processes are not related only to STPs, but to a lower extent they occur also in the environment (Kosjek and Heath, 2011).

A key factor that controls cycling of cytostatics in the aquatic media is their sorption to sewage sludge (biomass) (reviewed in (Kosjek and Heath, 2011)). However, many cytostatics are highly hydrophilic compounds with high aqueous solubility and low octanol-water partition coefficient (KOW values i.e. $< \log 2$). Due to these characteristics they will remain distributed in the water phase rather than eliminated from wastewaters by either sorption onto sewage sludge or volatilization (Booker et al., 2014; Kosjek and Heath, 2011; Zhang et al., 2013). Additionally, some cytostatics also show high mobility in sediment (Koc 8) and high potential for bioaccumulation in aquatic organisms. Nevertheless, a few cytostatics showed a potential for sorption onto sewage sludge and

sediment, but as they are poorly biodegraded they are only displaced into another compartment (Kosjek and Heath, 2011).

Another important elimination process of pollutants is their biodegradation by microorganisms (aerobic or anaerobic). Most of cytostatics have a low biodegradability or they are biodegradable only to some extent (Booker et al., 2014; Kosjek and Heath, 2011; Zhang et al., 2013). In some cases results for biodegradation are inconsistent. For example, 5-FU is classified as nonbiodegradable to almost completely biodegradable (Kosjek and Heath, 2011). Discrepancies are mainly due to different drug concentrations and the duration of exposure. At high concentrations, the drug may be cytotoxic to the microorganisms that may potentially lead to false negative results. In some studies degradation occurs only after a much longer exposure time (up to 14 days) that cannot be used in conventional STP, where the retention time (the time in which bacteria are exposed to the test compound) is between 2 and 8 h (Mahnik et al., 2007). It has been reported that after a short retention time (2h) that is typical for most STPs 11 out of 16 cytostatics remain in the effluents, meanwhile after 8 h, only 7 compounds remained in the effluent with concentrations between 10-80% of the initial concentration (Franquet-Griell et al., 2015).

The concentration of cytostatic residues can be modulated also by photo-transformation and hydrolysis, which might be at the same time also the source of new transformation products. These processes are more pronounced in surface waters than in STPs. There are only a few cytostatics that exhibit significant light absorption (direct photolysis) within the solar light spectrum or UV range (Booker et al., 2014; Kosjek and Heath, 2011). For some cytostatics indirect photolysis with photochemically derived species like the aqueous hydroxyl radical ($\text{OH}\cdot$) is even more important (Booker et al., 2014; Buerge et al., 2006). On the other hand, hydrolysis is of a minor importance for degradation of majority cytostatics. Only those drugs that contain ester and amide functional groups may be susceptible to degradation via hydrolysis (Booker et al., 2014).

1.5 Cytostatics in the Environment

Since cytostatics are not completely removed in conventional STPs, they may remain in the dissolved phase in the aqueous effluents and leave STPs only in partly reduced or even in unchanged concentrations (Kosjek and Heath, 2011; Zhang et al., 2013). Consequently STPs became an important direct source of cytostatics into the aquatic environment. However, for the majority of cytostatics the environmental burden is unknown, mainly because they have been ignored due to their low concentrations and absence of enough sensitive analytical techniques to detect such low concentrations in complex environmental matrices.

1.5.1 Measured concentrations of cytostatics in aquatic environment

Limited occurrence data show ubiquity presence of several cytostatics in the aquatic environment. The most studied drugs are those that are present on the market for a longer period and/or are consumed in higher amounts. For example, one of the most frequently detected cytostatics in European surface waters are CP and IF with range from sub-ng/L up to 20 ng/L (Buerge et al., 2006; Ferrando-Climent et al., 2014; Franquet-Griell et al., 2015; Zuccato et al., 2000). CP has been detected also in Thailand (Chao Phraja river, Bangkok) at 100-fold higher concentration, 1907 ng/L (Usawanuwat et al., 2014) which is the highest measured concentration of any cytostatics in the aquatic environment. High concentrations are also reported for hydroxycarbamide, mycophenolic acid and 5-FU; 788, 656 and 578 ng/L, respectively (Usawanuwat et al., 2014)(Franquet-Grill et al., 2016). Also tamoxifen (up to 38 ng/L), cytarabine (13 ng/L), ciprofloxacin (up to 102 ng/L), and bleomycin (17 ng/L) have been found in aquatic environment at relatively high concentrations (above 10ng/L) (Aherne et al., 1990; Ferrando-Climent et al., 2014; Martin et al., 2011). On the contrary, in some studies authors were unable to detect cytostatics in environmental samples which could be due to:

- insufficient analytical techniques;
- efficient waste water treatment procedure;

- low consumption amounts;
- inappropriate sampling;
- time of sampling; (reviewed in (Booker et al., 2014; Ferrando-Climent et al., 2014; Kosjek and Heath, 2011; Kummerer et al., 2014)).

1.5.2 Predicted environmental concentrations (PECs)

As the experimental data regarding the occurrence of cytostatics in surface waters is limited, different approaches to evaluate their approximate concentrations using the best available information have been developed. These calculated values, predicted environmental concentrations (PECs), present the expected fraction of the drug in surface waters once discharged and diluted. This approach presents an effective way to roughly identify target compounds most likely to be detected in water, before any field sampling is undertaken. However, PECs usually do not take into account the local specificities and possible hot spots and thus provide only a rough overview on a national or regional scale (Zhang et al., 2013). The accuracy of PEC values depends on the quality of data that are used in calculations, such as consumption amounts, dilution factors, excretion rates, and removal efficiency of STPs. But as each authors use slightly different values for these parameters, differences among PECs for the same cytostatic are possible (Besse et al., 2012; Franquet-Griell et al., 2015). For example, Besse and co-workers (Besse et al., 2012) calculated PECs for cytostatics used in France but they did not take into account the removal rates in STPs, as the data at the time of performing the study were scarce. Later on, research data accumulated and Franquet-Griell with co-workers (Franquet-Griell et al., 2015) recalculated PECs of relevant cytostatics considering the newest data regarding removal efficiencies of STPs and dilution factors which resulted in slightly lower PECs values.

The majority of cytostatics have PEC values much lower than 10 ng/L but for some of them also higher values have been calculated: 5-FU (30 ng/L), IM (13 ng/L), IF (12 ng/L), CP (10 ng/L), capecitabine (60 ng/L), mycophenolic acid (77.4 ng/L), bicalutamide (10.84 ng/L), gemcitabine (10 ng/L) and ciprofloxacin (51.4 ng/L) (Besse et al., 2012; Franquet-Griell et al., 2015; Gomez-Canela et al., 2014; Johnson et al., 2013; Kummerer and Al-Ahmad, 2010; Kummerer et al., 2014; Martin et al., 2014; Ortiz de Garcia et al., 2013; Rowney et al., 2009). PEC for all cytostatics, regardless of their mechanism of action can be as high as 630 ng/L (Kummerer et al., 2014). This theoretical approach has been validated by comparing the calculated values with measured environmental values and good agreement has been observed for certain cytostatics (Martin et al., 2014). However, a complete validation is at the moment difficult mainly due to limited data on the measured environmental concentrations. Nevertheless, PEC values are useful tool to prioritize cytostatics and estimate their concentration in the aquatic environment.

1.6 Effects of Cytostatic Drug Residues

The effects of cytostatics are not selective enough, and in addition to tumour cells, these drugs affect also the patient's normal growing cells and tissues, which often cause undesirable side effects, either during the therapy or even later in the lifetime, which raises concern about the health risk to occupational exposed people handling with these drugs and indirectly exposed population through drinking water (Kosjek and Heath, 2011; Kummerer et al., 2014).

Furthermore, as cytostatic residues are ubiquitously present in the environment, environmental organisms can also be affected by these compounds. Environmental organisms might exhibit the same targets as humans, causing similar pharmacodynamic effects as in humans (Bound and Voulvoulis, 2004; Kummerer et al., 2014; Toolaram et al., 2014) or some other targets that do not exist in humans causing some other secondary unexpected effects that are not pharmacologically important in the treatment of humans (Lutterbeck et al., 2015; Pichler et al., 2014; Seiler, 2002). Often specific modes of actions of cytostatics are not well known or even different modes of action occurred

simultaneously, which makes specific toxicity analysis in animals difficult to perform (Fent et al., 2006; Pichler et al., 2014).

Although the measured and predicted environmental concentrations of cytostatics are much lower (for several magnitudes of order) than therapeutically effective doses, their constant presence in the environment pose a risk to unintentionally exposed humans and environmental organisms, especially due to their highly reactive nature, low specificity and a wide-range of action (Besse et al., 2012; Kummerer et al., 2014). Many of these drugs are classified as genotoxic, mutagenic, carcinogenic, teratogenic, and/or embryotoxic (Besse et al., 2012; Kummerer et al., 2014; Parrella et al., 2015; Toolaram et al., 2014; Xie, 2012).

Overall, the presence of cytostatics residues in the environment may lead to systemic environmental effects that in the worst case could lead to the extinction of susceptible organisms, while in exposed humans increased cancer incidence and reproductive defects may occur. Thus, ecotoxicological data is needed to reliably assess the potential risks and introduce appropriate risk management.

1.6.1 Ecotoxicological potency (ecotoxicity) of cytostatics

Data on the potentially toxic effects of cytostatics towards aquatic organisms are scarce, poorly investigated and available mainly for those cytostatics that are consumed in high amounts and/or have been present on the market for many years (Booker et al., 2014; Kummerer et al., 2014).

To predict possible effects of pollutants on natural aquatic population different ecotoxicity tests are available. The principle of these tests is exposure of model aquatic organisms to increased concentrations usually of single compound for a shorter or longer period in order to evaluate the toxicity in controlled environment. The standard model organisms for aquatic effects assessment are: algae and/or cyanobacteria as primary producers, daphnids as primary consumers and fish as the vertebrate model and representative secondary consumers in the freshwater aquatic food chain (Figure 6).

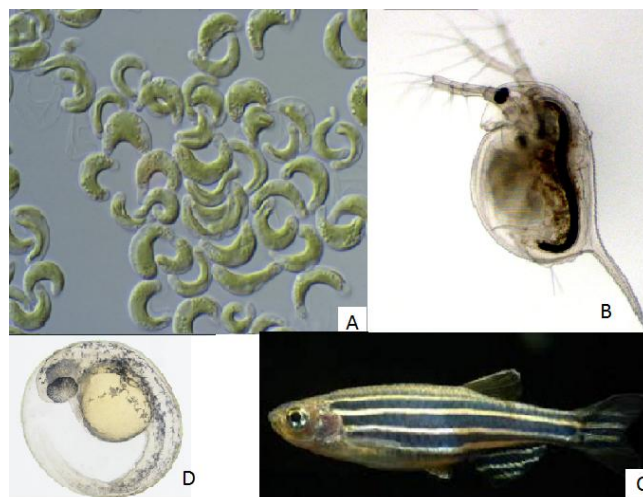


Figure 6: Algae (a, foto Dr. Tina Eleršek), daphnia (b, foto Dr. Sara Novak) zebrafish adult (c) and embryo (d, foto Matjaž Novak).

The most popular and best-described fish model species in ecotoxicology are zebrafish (*Danio rerio*). They have a number of unique features that have contributed to their popularity, such as; easy maintenance in the laboratory, small body, short generation cycle and a large number of offspring (Scholz et al., 2008). Since the exposed fish may suffer stress and pain, which is currently in conflict with animal welfare and animal right legislation, different alternatives have been developed. An example of popular alternative are experiments in which adult animals (acute fish toxicity test) are replaced with

embryos (fish embryo toxicity test). Fish embryos are not protected under various governmental definitions and it is assumed that they do not suffer such pain as adults due to less developed nervous and sensory systems. Experiments with zebrafish embryos are defined as *in vitro* test. The zebrafish embryo combines the advantages of cell cultures; easy handling and absence of animals used in the test on one side and on the other it presents a complex test system with different cell types. Zebrafish embryos develop outside the female body, they are completely transparent until 48 hours post fertilization, in five days majority organs are developed, embryonic development is very rapid and females are spawning throughout the year. Toxicity data obtained with fish embryos showed good correlation with adults and represent an attractive model for environmental risk assessment of chemicals since they offer the possibility to perform small-scale high-throughput analyses (Scholz et al., 2008).

Another popular and important alternative for the identification of potential toxicological hazards to aquatic organisms are *in vitro* experiments with fish cell lines that are even less time-consuming and costly than the embryo model and do not require animals at all. The justification of their use comes from the fact that the first interaction of a toxicant with an organism happens at the cellular level which may be then translated to higher levels of organization that can finally impact the whole organism (Fent, 2001). Depending on the nature of the study different cell lines isolated from various organs can be used. It was stressed out by several authors that fish cell lines, such as zebrafish liver (ZFL) cells, can be considered relevant for the identification of toxicological hazard to aquatic organisms (Pomati et al., 2007).

For assessing toxic properties of chemicals also different computer based techniques are available. These *in silico* toxicity predictions usually present the first step in gaining more general knowledge on toxicity of a certain chemical. Quantitative structure activity relationships (QSAR) based expert systems consist of a knowledge base comprised of structural alerts that are statistically or mechanistically related to the toxicity. The basic assumptions in QSAR modeling are that molecules with similar structure exhibit similar properties and that structural properties and/or physiochemical properties of a molecule can be used for predicting the biological activity of structurally related compounds (Kruhlak et al., 2012). QSAR modeling has nowadays been used also as a tool for prioritizing ecotoxicological impact assessments of pharmaceuticals. This approach showed good correlation with real experimentally derived results for some cytostatics (CP and IF). Nevertheless, it cannot fully replace data obtained with real experiments (reviewed in (Booker et al., 2014; Zounkova et al., 2007).

1.6.1.1 Acute toxicity

Acute toxicity describes the adverse effects of a compound towards experimental organisms that result from exposure in a short period of time (usually from 24 to 96 hours) to high for environment non-relevant concentrations of a compound. The most important endpoint evaluated with acute toxicity tests is survival rate (Brezovsek et al., 2014; Kovacs et al., 2015; Misik et al., 2014; Parrella et al., 2014; Zounkova et al., 2007), which enables evaluation of various parameters:

- lethal concentration 50 (LC50), the concentration that causes 50 % death of a test group organisms,
- the half maximal effective concentration (EC50), the concentration of a drug, which induces as an adverse response halfway between baseline and maximum,
- the no observed effect concentration (NOEC), the highest tested concentration that does not cause an effect that is statistically significantly different from control,
- the lowest observed effect concentration (LOEC), it represents the lowest concentration that provokes a statistically significant effect on the exposed population of the test organisms if compared to the untreated controls (Walker et al., 2001).

According to EC50 values, a compound can be classified as very toxic (EC50 < 1mg/L), toxic (EC50 1–10 mg/L), harmful to aquatic organisms' (EC50 10–100 mg/L) or those with EC50 above 100 mg/L as not classified (EU-Directives 93/67/ECC (Commission of the European Communities, 1996). Majority of cytostatics have low EC50 values and can be classified as toxic and/or harmful to aquatic organisms (Brezovsek et al.,

2014; Parrella et al., 2014; Zounkova et al., 2007) and only few as non harmful to aquatic organisms (EC50 values above 1000 mg/L) (Zounkova et al., 2007) (reviewed in (Ferrando-Climent et al., 2014)).

Acute toxicity values for the same drug can span over several orders of magnitude between different organisms. For example, 5-FU is very toxic to algae and cyanobacteria with low EC50 values (0.027 – 1.2 mg/L) (Brezovsek et al., 2014; Zounkova et al., 2007) and almost non toxic to daphnia and rotifers (Parrella et al., 2014). Low toxicity (EC50 2441.6 mg/L) of 5-FU was observed also in zebrafish embryos and adults (Kovacs et al., 2015). Thus, for risk assessment it is essential to study toxicity towards different organisms, since it is impossible to conclude which organisms will be the most sensitive. Additionally, the data also vary within the same species among different research groups, mainly due to different laboratory performances and the sensitivity of used clones (Brezovsek et al., 2014; Fent et al., 2006; Zounkova et al., 2007).

Acute toxicity usually occurs at much higher concentrations of cytostatics (100 – 1000 times) from those found or expected in the aquatic environment (Besse et al., 2012; Kummerer et al., 2014). However, acute toxicity experiments do not allow prediction of adverse effects that may occur during the chronic life-cycle exposure or some drug-specific side effects. Therefore, on the basis of acute toxicity data alone it could be falsely concluded that these drugs do not present risk to the environment.

1.6.1.2 Chronic toxicity

Organisms in the environment are exposed to low concentrations of pharmaceutical residues usually over a longer period of time, their entire life cycle or even through multiple generations as these drugs are released and present in the environment continuously. Long-term exposure to low environmentally relevant concentrations is unlikely to cause acute toxic effects, but it may cause subtle effects that finally impact on growth, fertility, sex ratios or reproductive behavior of exposed organisms (Crane et al., 2006).

Existing ecotoxicological data for most of cytostatics are often based merely on the evaluation of only acute toxicity, meanwhile there is virtually no data on their potential chronic effects in particular transgeneration and population studies are completely absent. Nevertheless, due to their specific mechanisms of action (interaction with DNA) it is expected that cytostatics under conditions of chronic exposure might induce a number of unexpected effects even at very low concentrations (Kosjek and Heath, 2011; Kummerer et al., 2014). Furthermore, available data show that long-term toxicity in crustaceans occurs at much lower concentration of the drug (close to those expected in the environment) than after short exposure (Parrella et al., 2014). Thus, data from chronic exposure toxicity studies are relevant for evaluating the real ecological risk of these drugs (Besse et al., 2012; Toolaram et al., 2014).

1.6.1.3 Genotoxicity of cytostatics

Genotoxic compounds have the ability to damage genetic material and in aquatic organisms that live in environment polluted with genotoxic contaminants different DNA lesions (i.e., DNA damage, micronuclei, mutations) have been observed. Furthermore, chronic exposure to genotoxicants through environment might cause in aquatic organisms increased frequency of tumours, reduction of the fertility, abnormal development, gamete loss, changes in genetic diversity and might even affect the stability of ecosystems (reviewed in Jha, 2004; Pinkney et al., 2011). Genotoxic compounds may also induce cancer and/or heritable defects in exposed humans (EMA, 2006). A general concern with genotoxic and carcinogenic compounds is that a cancer risk may exist at any level of exposure, i.e., that there is no threshold (Webb et al., 2003).

Since many cytostatics interact directly or indirectly with DNA, genotoxic effects are expected to occur also in environmentally exposed organisms (Kosjek and Heath, 2011). Therefore, in addition to standard ecotoxicological endpoints obtained with acute or chronic test (lethal and sublethal effects) hazard identification with respect to more

specific endpoints such as DNA integrity, genomic stability, and gene expression would be an important toxicological criterion given the inherent properties of cytostatics. Nevertheless, there is limited data available on using aquatic organisms for ecogenotoxicity testing and this research gap needs to be urgently filled. Only after filling these gaps, more reliable environmental risk assessments with much lower uncertainty can be performed.

1.6.1.3.1 Bacterial assays

Ecogenotoxicity of cytostatics was assessed mostly using different bacterial assays (UmuC test, SOS chromotest and Ames assay) where most of tested cytostatics showed high genotoxic potential (Toolaram et al., 2014; Xie, 2012). These *in vitro* micro-scale tests are cheap, fast and provide the possibility to perform a preliminary screening of compounds of interest. Furthermore, there is a strong correlation between observed genotoxicity in bacteria and potential genotoxicity in animals.

SOS/umuC assay has been developed and standardized to detect the genotoxicity of native, unconcentrated water samples (ISO 13829), and has been proposed as a screening test for monitoring the toxicity and genotoxicity of surface and wastewater samples in many countries (Zegura et al., 2009). The Ames test is a widely employed method that uses bacteria to test whether a given chemical can cause mutations in the DNA of the test organism by measuring reverse mutations from histidine auxotrophy to wild type (Maron and Ames, 1983). Another popular bacterial test is a SOS chromotest in which the expression of genes involved in SOS response induced by genotoxic agents in *Escherichia coli* is determined.

1.6.1.3.2 DNA strand breaks and genomic instability

DNA strand breaks and genomic instability are important genotoxic biomarkers and are evaluated by comet and micronucleus assay, respectively (Figure 7). Both assays play an important role in the detection of ecogenotoxicological effects in environmentally exposed humans and organisms. Additionally, both assays are also popular in different *in vitro* (mechanistic studies) and *in vivo* studies for the evaluation of genotoxic potential towards various model systems (Araldi et al., 2015).

Comet assay is a sensitive method for the detection of a broad spectrum of DNA damage such as single, double strand breaks, alkali labile sites, DNA-DNA and DNA-protein crosslinks on the level of single cells. Detection is based on the measurement of migration of denatured DNA during electrophoresis; damaged and fragmented DNA travels a longer distance than non-damaged DNA resulting in the comet shape of nuclei. A high level of breaks detected by the comet assay in cells exposed to a compound indicates either major DNA damage or efficient repair of DNA damage (Singh et al., 1988). In contrast to comet assay, which measures primary DNA damage, micronucleus assay reflects irreparable lesions that result from the non-repaired or inappropriately repaired primary DNA damage, which are likely to be inherited by subsequent generations of cells. With the micronucleus assay DNA damage is determined at the chromosomal level through the detection of micronuclei (MN), nuclear plasmic bridge (NPB; a biomarker of dicentric chromosomes resulting from telomere endfusions or DNA misrepair) and nuclear plasmic buds (NBUD, a biomarker to detect gene amplification and altered gene dosage events) (Fenech, 2006). Increased chromosomal damage is associated with early events in carcinogenesis (Ellinger-Ziegelbauer et al., 2009; Bonassi et al., 2011).

Furthermore, genotoxic potential of a compound can be evaluated also through detection of γ H2AX foci (phosphorylated H2AX histone), a marker of double DNA strand breaks (DSBs) which are the most detrimental DNA lesions. This method is in general more sensitive with a lower detection limit than comet and micronucleus assays. Therefore, γ -H2AX foci assay has a great potential for application in environmental toxicology (Smart et al., 2011).

Overall, for some cytostatics it has been shown that they induce genotoxic effects in different nontarget organisms and usually at lower concentrations than those causing acute effects. Cytostatics induce DNA damage in daphnids, oligochaeta and mussels,

genomic instability in higher plants and even affect the fertility of higher plants (Gacic et al., 2014; Kračun-Kolarević et al., 2015; Misik et al., 2014; Parrella et al., 2015). Surprisingly, also certain targeted cytostatics (such as TKIs) that do not express genotoxic mechanism of action showed genotoxic effects in some higher plants (Pichler et al., 2014) and daphnids (Parrella et al., 2015).

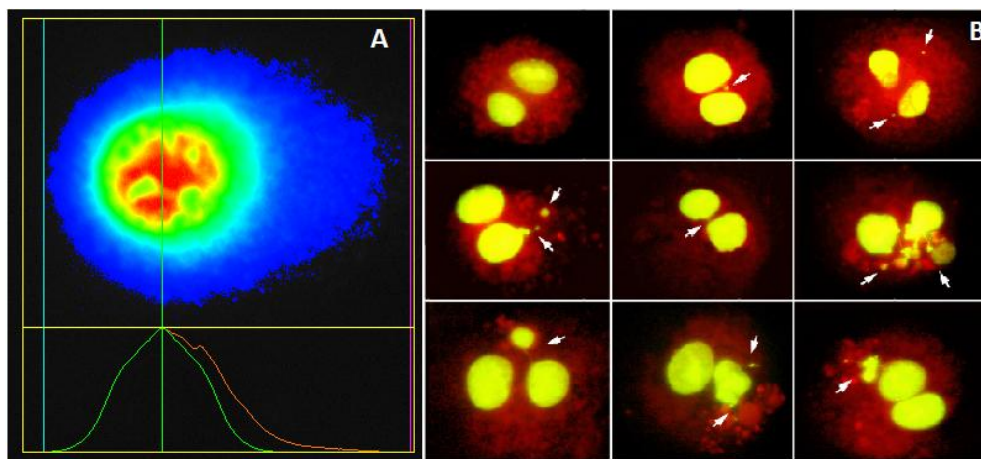


Figure 7: The image of damaged nuclei (comet assay) and binucleated cells with micronucleus, nucleoplasmic bridge and nuclear buds (micronucleus assay) (foto Assist. Prof. Bojana Žegura).

1.6.1.3.3 Toxicogenomic analysis

First interaction of a compound with a biological system occurs on a subcellular molecular level, therefore changes in gene expression may serve potentially as early warning indicators for adverse effects and as a sensitive and specific ecotoxicological endpoint where classical toxicological endpoints show no obvious adverse effects. Alteration in expression of targeted or selected series of genes can be evaluated using quantitative real-time polymerase chain reactions (qRT-PCR). Overall, ecotoxicogenomics focus on the lowest rather than the highest levels of biological organization.

The recent progress in molecular technologies and science led to the development of a more global approach, where instead of measuring just a few genes it is possible to measure changes in the expression of almost all genes in response to a toxic agent (microarrays or sequencing). This approach allows identifying and describing a pattern of expressional changes that is typical for a toxicant with a given mode of action. A comparison between the expressional profiles and the fingerprints of known model compounds can be used for identifying compounds with a similar mode of action (Fent and Sumpter, 2011). Nevertheless, toxicogenomic data with cytostatics are almost completely absent.

1.6.1.4 Toxicity of complex mixtures, metabolites and transformation products

Most research on the effects of cytostatics on environmental organisms has been conducted so far using only single drugs. However, organisms in the environment are not exposed to single cytostatics but rather continuously to complex mixtures of different cytostatics (parent compounds), their human metabolites and/or transformation products (TPs) that are formed during water treatment or under environmental conditions. In general, the knowledge about their toxicological effects is rather limited (Booker et al., 2014; Kosjek and Heath, 2011; Kummerer et al., 2014a; Xie, 2012). Nevertheless, the assessment of the toxicity of cytostatic mixtures is both a great challenge and an urgent need to achieve more progressive and proactive risk assessment.

Between compounds in mixtures different interactions are possible and they depend on the selected cytostatic, concentration of the individual drug and the applied bioassay (Brezovsek et al., 2014; Elerseck et al., 2016; Kundi et al., 2015; Misik et al., 2015; Parrella et al., 2014a):

- synergistic, the toxicity of a mixture is higher than the sum of effects of individual components;
- antagonistic, the toxicity of a mixture is lower than the sum of effects of individual components;
- additive, the toxicity of a mixture is equal to the sum of the effects of each individual component.

Existing literature data show that the mixture of cytostatics may exhibit synergistic effects. Due to these effects cytostatic residues when they occur in mixtures induce toxicity at low, for environmental contamination relevant concentrations at which individual compounds are inactive (Brezovsek et al., 2014; Elerseck et al., 2016; Kundi et al., 2015; Mater et al., 2014). These examples show that simultaneous presence of several cytostatics in the environment might result in greater toxicity to non-target organisms, than predicted based on individual active substances. Thus, environmental risk assessment that is based on ecotoxicological data for single substances can lead to underestimation of the real environmental impact of these drugs which can result in the incorrect assumption of absence of risk (Kummerer et al., 2014a).

In addition to real experiments, toxicity of cytostatic mixture can also be evaluated by using different models. For compounds with the same mode and biological site of action Concentration Addition (CA, Loewe additivity) approach can be used. On the other hand, compounds with an independent mechanism and/or different biological sites could act in the mixture according to the model of Independent Action (IA, Bliss independence) (Katsnelson et al., 2011). However, as most cytostatics have different modes of action, it is difficult to predict their interaction in mixtures.

In contrast to simple mixtures, which consist of a relatively small number of chemicals (e.g. ten or less), the complex mixtures comprise in addition to cytostatic residues also tens, hundreds or thousands other chemicals, the composition of which is qualitatively and quantitatively not fully known. Such complex mixtures are real environmental samples (effluents, surface waters), which are the most representative since they represent the media in which aquatic organisms live. Even though some cytostatics have been found in these samples, toxic response cannot be attributed specifically to these drugs (Ferk et al., 2009) as many other compounds have been identified in the samples and probably there are a lot of non identified.

On the other hand, ecotoxicity studies with metabolites and transformation products are even scarcer. Some cytostatic metabolites and TPs are less biodegradable, more polar, with a higher mobility in the aquatic cycle, more persistent and even more toxic than the parent compounds (Besse et al., 2012). Therefore, for a complete environmental risk characterization it is essential to investigate also the toxicity of the transformation products and metabolites.

1.6.1.5 Environmental risk assessment (ERA) of cytostatics

Environmental risk assessment (ERA) is a systematic procedure of predicting whether there may be a risk of adverse effects on the environment caused by a certain pollutant. In this approach predicted or measured environmental concentrations of a pollutant are compared NOECs for different environmental compartments and organisms.

For ERA of human pharmaceuticals different guidelines are available; in Europe EMA (European Medicines Agency) guideline and in the United States guideline Food and Drug Administration (FDA). Both guidelines recommend toxicity testing with aquatic species of different trophic levels, i.e. representative of algae, invertebrates and fish. The EMA guideline requires environmental assessment only for newly authorized pharmaceuticals (after year 2006), therefore most cytostatics have not been submitted to such a procedure (Besse et al., 2012). Nevertheless, the environmental impact does not provide sufficient grounds for a refusal of a drug.

ERA is a step-wise procedure consisting of two phases. In Phase I (pre-screening assessment), the environmental exposure to the drug or its metabolites is estimated based

on PEC values, irrespective of its route of administration, metabolism, pharmaceutical form and excretion rate. If the PEC is below trigger value of 0.01 µg/L no phase II in-depth risk assessment is necessary and it is assumed that the compound is unlikely to represent a risk for the environment (EMA, 2006). The FDA guideline sets a threshold of 1 µg/L. If the PEC value of a drug is equal or higher than the trigger value, then a Phase II should be performed where fate and effects are studied. Therefore Phase II is further subdivided into two parts; Tier A, in which an initial prediction of risk are performed based on relevant environmental measurements and refined PEC values (Phase II Tier A); and Tier B which focuses on the effects on fauna and flora within environmental compartments that are likely to be affected (EMA 2006). Directive 2004/27/EC also requires environmental risk assessment for human metabolites with excretion rate exceeding 10% of the total dose. Contrary, transformation products are not included in these refinements.

In some cases the action limit may not be applicable since some drugs affect the environmental organisms in concentrations lower than EMA trigger limit of 0.01 µg/L. Such drugs are those with endocrine activity and for them a tailored ERA has to be carried out due to their low effect thresholds and importance of biological functions in which they are involved (EMA, 2006). Similarly also cytostatics, especially those that interact with genetic material possess high activity, even at low environmental relevant concentrations (Kummerer et al., 2014). In addition, for genotoxic and carcinogenic compounds a safe threshold concentration with no effects cannot be given since a cancer risk may exist at any level of exposure (Webb et al., 2003). There are some proposals to exclude cytostatics from the action limit set up by EMA and FDA for the ERA (reviewed in (Kummerer et al., 2014a)). Since it is impossible to set up the safe level for genotoxic and mutagenic chemicals, it is important to get knowledge of possible overseen risk associated to their introduction into environment.

Required information for conduction of ERA is scarce and mostly available only for the most commonly-used cytostatics today. Nevertheless, available data indicate no acute adverse effects on the environment or human health because the concentrations required for observing such effects are at least three orders of magnitude higher from those measured at the moment in the environment or effluents. Therefore, their presence in the environment has been classified by several authors as low risk (Martin et al., 2014). However, the current risk assessment strategies, which are based on standard ecotoxicity tests alone, have several important limitations;

- the chronic effects (e.g. life-cycle or partial life-cycle fish tests);
- testing of sediment phase;
- drug specific side effects (genotoxicity);
- effects of metabolites and transformation products;
- effects of combined effect of complex mixtures are not taken into account (Boxall, 2004; Crane et al., 2006; Daughton and Ternes, 1999).

1.6.2 Human health issues

Cytostatic residues may present a certain risk also to indirectly exposed human population. Possible exposure routes for humans are through contaminated drinking water and occupational exposure. Possible risk to humans from exposure through consumption of contaminated drinking water containing low ng/L concentration of cytostatics as measured in surface waters is for a great majority of cytostatics completely unknown. On the other hand, more data is available regarding indirect exposure of health care workers.

1.6.2.1 Exposure from drinking water

Surface water is in some countries an important source of drinking water. In these countries drinking water is extracted from rivers or lakes, stored in reservoirs, purified, and then distributed to the community. There is a high probability that purification procedures might remove cytostatic residues that are present in surface waters, however for a great majority of cytostatics no definitive data exist yet to confirm this (Franquet-

Griell et al., 2016; Johnson et al., 2008; Rowney et al., 2009). Therefore, the possibility of cytostatics entering drinking water particularly in urbanized areas with high population density and with small rivers providing limited dilution cannot be entirely ruled out yet and drinking water becomes at risk.

One of the first attempts to assess health risk for chronic exposure to cytostatic residues present in drinking water and/or surface waters was conducted already 15 years ago (Schulman et al., 2002). The authors concluded that CP at the detected concentrations does not present appreciable risk to humans, as the measured concentrations are far below the derived safe limit (Schulman et al., 2002). Relative risk of secondary bladder cancer due to maximum possible intake by drinking water is lower than 10–3 (IF) and 10–5 (CP) compared to intake by therapy (Kummerer and Al-Ahmad, 2010). However the risk assessment based on only few published local measured concentrations of CP in water at that time and did not consider local specificities and possible hot spots. Later on more comprehensive risk assessment has been conducted with 13 different cytostatics considering concentrations under low flow conditions. Even in the case of no removal during drinking water purification it has been concluded that risk to healthy adults from this exposure is low (Rowney et al., 2009).

Although the risks may be negligible for healthy adults, more concern may be associated with some special subgroup populations, such as pregnant women, fetuses, breast-fed infants, babies, toddlers, children, and elderly people (reviewed in (Kummerer et al., 2014a; Rowney et al., 2009). Nevertheless, for compounds that directly react with the DNA, a safe threshold concentration cannot be established, thus a risk for cancer exists at any level of exposure. Additionally, consumption of cytostatics is increasing, some of them have been detected in higher concentrations than used in these risk assessments and it has not been considered that they are present in complex mixtures.

1.6.2.2 Occupational exposure

Indirect exposure to subtherapeutic concentrations of cytostatic residues can occur also in hospitals and hospital pharmacies where these drugs are prepared, administrated or treated patients are nursed. In addition to healthcare workers that handle with these drugs, also cleaners, laundry and housekeeping workers may come in contact with these drugs via the patient's excreta during their day-to-day duties. The intake mainly occurs through inhalation of aerosols, droplets and particulates and skin (dermal) absorption of spills during preparation, administration or disposal of drugs and the patient's body fluids (urine, feces and vomiting) containing these drugs (Suspiro and Prista, 2011; Villarini et al., 2016; Vyas et al., 2014). Since cytostatics are continuously administrated over a long period of time and in out-patients even at home, the risk of exposure exists also for the patient's family members that are exposed to cytostatics via the patient's excreta (Yuki et al., 2012).

Environmental monitoring studies performed in many hospitals showed widespread contamination of working environments and workers with a detectable level of cytostatics in the air, on surfaces, gloves, and on different body parts. Since dermal exposure was identified as the most important route of exposure, surface monitoring of cytostatics contamination may be used as a reliable indicator of occupational exposure to these drugs. In order to minimize the risk of occupational exposure (as low as reasonably achievable) different guidelines for handling cytostatics and protective measures, such as personal protective equipment (gloves, masks, caps, and protective eyewear) and even preparation of drugs in biological safety cabinets are required. However, this does not guarantee absolute protection of health care workers from occupational exposures and cytostatics have been detected in their urine and blood even up to concentrations of a few µg/L (up to 50 µg/L) (reviewed in (Villarini et al., 2016).

Numerous studies show direct correlations between occupational exposure to cytostatics and increased frequency of several genotoxic biomarkers of effect; sister chromatide exchange, micronucleus assay, comet assay and chromosomal aberrations (Suspiro and Prista, 2011; Vyas et al., 2014). Some of these biomarkers of effect are associated with early events in carcinogenesis and therefore justify the concern (Suspiro and Prista, 2011). Furthermore, in exposed healthcare workers several acute adverse effects, such as skin rash, light-headedness, hair loss and delayed effects on the

reproduction have been observed. Furthermore, even of greater risk are pregnant workers due to teratogenicity of several cytostatics. Overall, occupational exposure to cytostatics, despite the implication of safety measures represents a serious health risk. Therefore, further efforts to reduce indirect exposure in the working environment are recommended.

Chapter 2

Aims and Hypotheses

The cytostatic drug residues are of concern due to their potential cytotoxic, genotoxic, mutagenic, carcinogenic and teratogenic effects and might have severe consequences in non-target aquatic organisms and unintentionally exposed humans even at the level of some ng/L. Ecotoxicological data for cytostatics are limited, despite their continuous presence in the effluents and consequently in the environment. Current evidence remains inadequate to support definitive conclusions on the environmental and human risk posed by cytostatics, especially on their long-term effects in non-target organisms and humans.

The overall goal of this thesis was to provide reliable ecotoxicological data that are relevant for the environmental hazard and risk assessment of selected cytostatics with different mechanisms of action. The compounds studied were 5-fluorouracil (5-FU), cisplatin (CDDP), etoposide (ET) and imatinib mesylate (IM).

The specific goals of the thesis were:

- to determine the effects of the selected cytostatics *in vitro* in fish model (ZFL cells) and human models (HepG2 cells and HPBLs) at the level of cyto- and geno-toxic responses and at the level of gene expressions with the aim to assess the relative genotoxic potency of the tested drugs, relative susceptibility of different cell models, and to elucidate the mechanisms of their genotoxicity.
- To determine the relationship between transcript signature in liver of zebrafish chronically exposed to 5-FU and observed toxicological effects to identify molecular markers (transcript signatures) of delayed and irreversible effects.
- To assess the cyto- and geno-toxic effects of single cytostatics and their mixtures at low, for environmental contamination relevant, concentrations to identify possible combined effects (synergistic or antagonistic) in complex mixtures.
- To evaluate cytotoxic and genotoxic activity of real environmental samples on ZFL cells and correlate observed toxicity to detected cytostatics and their concentrations in the sample.

Within the present doctoral dissertation, the following hypotheses were tested in order to achieve the proposed aims:

- H1: Cytostatics with different mechanisms of chemotherapeutic action exert different cytotoxic and genotoxic potential in non-target fish and human cells.
- H2: Certain cytostatics can induce upon chronic exposure in aquatic organisms adverse effects at low environmentally relevant concentrations.
- H3: At low environmentally relevant concentrations the mixtures of cytostatics show higher toxicity and genotoxicity than single compounds at the same concentrations.

Chapter 3

Publications

3.1 Genotoxic Potential of Selected Cytostatic Drugs in Human and Zebrafish Cells

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Environmental Science and Pollution Research, 2016, 23(15): 14739-50, doi: 10.1007/s11356-015-4592-6

In this study a comparative *in vitro* toxicological characterization of three commonly used cytostatics with different mechanisms of action (5-fluorouracil [5-FU], cisplatin [CDDP] and etoposide [ET]) towards zebrafish liver (ZFL) cell line, human hepatoma (HepG2) cells and human peripheral blood lymphocytes (HPBLs). Cytotoxicity was determined with the tetrazolium-based colorimetric assay (MTT assay) and genotoxicity with the comet assay and cytokinesis block micronucleus (CBMN) assay.

The results showed different toxic and genotoxic potential of different cytostatics as well as different susceptibility of fish-derived ZFL cells compared to human-derived HepG2 cells and HPBLs to cytotoxic and genotoxic effects of selected cytostatics. ZFL cells were the most susceptible, which raises the question of a negative impact of these pharmaceuticals on fish if discharged into aquatic environments excessively. The high sensitivity of non-target ZFL cells also indicates that these cells could be a good model for testing the genotoxic potential of pharmaceuticals as well as other aquatic pollutants and could serve as an alternative to animal testing in preliminary ecotoxicological studies.

The PhD candidate performed the MTT assay, comet assay and CBMN assay on ZFL cells exposed to selected cytostatics including data analyses. He also contributed to the preparation of the manuscript.

Genotoxic potential of selected cytostatic drugs in human and zebrafish cells

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Abstract Due to their increasing use, the residues of anti-neoplastic drugs have become emerging pollutants in aquatic environments. Most of them directly or indirectly interfere with the cell's genome, which classifies them into a group of particularly dangerous compounds. The aim of the present study was to conduct a comparative in vitro toxicological characterisation of three commonly used cytostatics with different mechanisms of action (5-fluorouracil [5-FU], cisplatin [CDDP] and etoposide [ET]) towards zebrafish liver (ZFL) cell line, human hepatoma (HepG2) cells and human peripheral blood lymphocytes (HPBLs). Cytotoxicity was

determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and acridine orange/ethidium bromide staining. All three drugs induced time- and dose-dependent decreases in cell viability. The sensitivity of ZFL and HepG2 cells towards the cytotoxicity of 5-FU was comparable (half maximal inhibitory concentration (IC₅₀) 5.3 to 10.4 µg/mL). ZFL cells were more sensitive towards ET (IC₅₀ 0.4 µg/mL) and HepG2 towards CDDP (IC₅₀ 1.4 µg/mL) induced cytotoxicity. Genotoxicity was determined by comet assay and cytokinesis block micronucleus (CBMN) assay. ZFL cells were the most sensitive, and HPBLs were the least sensitive. In ZFL cells, induction of DNA strand breaks was a more sensitive genotoxicity endpoint than micronuclei (MNI) induction; the lowest effective concentration (LOEC) for DNA strand break induction was 0.001 µg/mL for ET, 0.01 µg/mL for 5-FU and 0.1 µg/mL for CDDP. In HepG2 cells, MNI induction was a more sensitive genotoxicity endpoint. The LOEC values were 0.01 µg/mL for ET, 0.1 µg/mL for 5-FU and 1 µg/mL for CDDP. The higher sensitivity of ZFL cells to cytostatic drugs raises the question of the impact of such compounds in aquatic ecosystem. Since little is known on the effect of such drugs on aquatic organisms, our results demonstrate that ZFL cells provide a relevant and sensitive tool to screen genotoxic potential of environmental pollutant in the frame of hazard assessment.

Keywords Cytostatic drugs · Environmental concentrations · Zebrafish liver cells · HepG2 cells · Human lymphocytes · Genotoxicity

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Introduction

Anti-cancer drugs are designed to kill cancer cells and fall under the group of the most dangerous compounds produced

and used. Many have been classified as carcinogenic, mutagenic and teratogenic compounds, triggering widespread concerns related to environmental exposure to the residues of these drugs (Besse et al. 2012; Negreira et al. 2014; Toolaram et al. 2014; Zhang et al. 2013). The use of these drugs in chemotherapy has increased due to increasing incidence as well as earlier diagnosis. In addition, chemotherapy is shifting from in-patient to out-patient cancer treatment leading to the presence of cytostatics not only in hospital effluents, but also in municipal wastewater treatment plant effluents (Parrella et al. 2014). With the development of sensitive analytical methods during the last years, several highly consumed cytostatic compounds have been detected in aquatic systems (Kosjek et al. 2013; Negreira et al. 2013).

The mechanisms of action (MoA) of cytostatics are mainly targeted at the prevention of growth and division of tumour cells via interference with the genetic material of the cells and cell signalling. According to their mechanism of action, they can be classified into alkylating agents, anti-metabolites, platinum complexes, intercalating agents, cytotoxic antibiotics, mitotic spindle inhibitors, topoisomerase inhibitors, protein kinase inhibitors and monoclonal antibodies (Besse et al. 2012). However, cytostatics may not be selective enough to target only tumour cells; they can also act on other types of dividing cells and tissues of exposed organisms (Besse et al. 2012; Deblonde and Hartemann 2013; Toolaram et al. 2014; Zhang et al. 2013; Zounkova et al. 2010).

The cytotoxicity and genotoxicity of cytostatics have been studied in bacteria, mammalian rodent and human cells and laboratory animals (IARC 1987; 2000; Ishidate et al. 1988; Mavournin et al. 1990). Several recent studies have shown that these compounds can induce reproductive and genotoxic effects also in non-target aquatic organisms. In crustaceans, cytostatics (5-fluorouracil (5-FU), etoposide (ET), cisplatin (CDDP), capecitabine and imatinib mesylate) inhibited reproduction at concentrations in the range of microgram per litre (Parrella et al. 2014). Inhibition of reproduction was also observed in algae, however, at higher concentrations (Brezovšek et al. 2014). In fresh water mussels *Unio pictorum* and *Unio tumidus*, 5-FU induced DNA strand breaks (Gačić et al. 2014). Induction of micronuclei (MNi) formation by 5-FU, CDDP, ET, vincristine, carboplatin and imatinib mesylate was also observed in plants (*Allium* and *Tradescantia*) (Mišić et al. 2014; Pichler et al. 2014).

The aim of the present study was to investigate the cytotoxic and genotoxic potential of the three widely used cytostatics 5-FU, ET and CDDP (Table 1) in zebrafish (*Danio rerio*) liver (ZFL) cells, human hepatoma (HepG2) cells and human peripheral blood lymphocytes (HPBLs). The ZFL cell line was selected as a model of fish-derived cells considered to be more relevant for the identification of toxicological hazard to aquatic organisms.

HepG2 cells and HPBLs were selected as cell models of human origin. The two models represent cells of cancer origin and normal primary cells, and both are commonly used in in vitro toxicity and genotoxicity studies for toxicological hazard identification.

As genotoxicity endpoints, the induction of DNA damage was determined with the comet assay (Tice et al. 2000) and the induction of genomic instability was determined with the cytokinesis block micronucleus (CBMN) assay (Fenech 2006). The two assays are considered complementary, as they measure different genotoxic endpoints. Although in vitro genotoxicity tests cannot quantitatively predict hazard to humans and wildlife, they enable the estimation of the relative genotoxic potency of the tested compounds as well as the assessment of the susceptibility of different cell models.

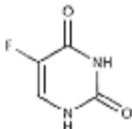
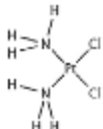
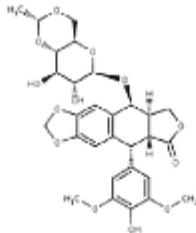
Materials and methods

Chemicals

Chromosome kit P was from Euroclone (Milano, Italy); RPMI 1640 was from Invitrogen (Carlsbad, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-fluorouracil (5-FU; MW 130.08 g/mol; CAS 51-21-8), acridine orange (AO), bleomycin, cisplatin (CDDP; MW 300.05 g/mol; CAS 15663-27-1), cytohalasin B, dimethyl sulphoxide (DMSO), ethidium bromide (EtBr), histopaque, low melting point agarose (LMP), methanol, normal melting point (NMP) agarose and William's medium E were from Sigma (St Louis, USA); etoposide (ET; MW 588.6 g/mol; CAS 100007-53-2) was from Santa Cruz Biotechnology (Dallas, USA); penicillin/streptomycin, foetal bovine serum (FBS) for HepG2 cells, L-glutamine and phosphate-buffered saline (PBS) were from PAA Laboratories (Dartmouth, USA); trypsin was from BD-Difco (Le Pont-de-Claix Cedex, France); Leibowitz L-15 and FBS for ZFL cells were obtained from ATCC, DMEM and Ham F-12 from Gibco, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and epidermal growth factor (EGF) from Invitrogen (Paisley, UK); heparinised vacutainer tubes were from Becton Dickinson (Franklin Lakes, USA); and Giemsa was from Merck (Darmstadt, Germany). All other chemical reagents were of the purest grade available, and all solutions were made using Milli-Q water.

For the selected cytostatic drugs, stock solutions were prepared in DMSO (5-FU [75 mg/mL or 576.57 mM] and ET [25 mg/mL or 42.474 mM]) and sterile Milli-Q water (CDDP [1 mg/mL or 3.33 mM]) and were aliquoted and stored at -20°C .

Table 1 The cytostatic drugs investigated in the present study

Name	5-fluorouracil	Cisplatin (cis-diammine-dichloroplatinum)	Etoposide
Abbreviation	5-FU	CDDP	ET
CAS N°	51-21-8	15663-27-1	33419-42-0
Chemical structure			
ATC code	L01BC02	L01XA01	L01CB01
MW (g/mol)	130.08	300.05	588.57

CAS N° Chemical Abstract Services number, ATC code, Anatomical Therapeutic Chemical Classification code, MW molecular weight

Toxicological characterisation

Cell models

HPBLs were obtained from two young healthy male donors. Subjects gave informed consent to participate in the study. The study was part of the project approved by the Ethics Committee and observed the ethical principles of the Declaration of Helsinki. According to the questionnaire filled out by the donors, they had not been exposed to ionizing radiation for diagnostic or therapeutic purposes or to known genotoxic chemicals that might have interfered with the results of the testing for a year before blood sampling. Blood was withdrawn by antecubital venipuncture into heparinised vacutainers containing lithium heparin as anti-coagulant under aseptic conditions. The whole blood was exposed to various concentrations of cytostatics for different periods of time. In each experiment, a non-treated solvent control and a positive control were included.

HepG2 cells were a gift from Dr. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). The cells were cultivated at 37 °C and 5 % CO₂ in William's medium E containing 15 % FBS, 2 mM L-glutamine and 100 U/mL penicillin/streptomycin.

ZFL cell line was derived from normal adult zebrafish (Ghosh et al. 1994) and was obtained from American Type Culture Collection (ATCC number: CRL-2634). Cells were cultured under humidified air atmosphere at 28 °C in a medium composed of 50 % Leibovitz L-15, 35 % DMEM and 15 % Ham F-12, supplemented with 15 mM HEPES, 0.15 g/L NaHCO₃, 0.01 mg/mL insulin, 50 ng/mL EGF, 0.1 % penicillin/streptomycin and 5 % heat inactivated FBS.

Cell viability (cytotoxicity) assays

Cytotoxicity of the tested cytostatics in HPBLs was determined by differential staining with AO and EtBr and by fluorescence microscopy (Duke and Cohen 1992). Whole blood samples were treated with 0, 0.1, 0.2, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 µg/mL concentrations of tested cytostatics and incubated for 4, 24, 48 and 72 h. After the treatment, HPBLs were isolated by histopaque density gradient centrifugation method. The slides were prepared by adding AO and EtBr (both diluted in phosphate-buffered saline, PBS) to HPBL suspension in final concentration of 100 µg/mL (1:1; v/v). A total of 100 cells per repetition were examined with an epifluorescence microscope (Olympus BX51, Tokyo, Japan). Quantitative assessments were made by determining the percentage of live and dead cells. The nuclei of live cells emitted a green fluorescence and of dead cell red fluorescence.

The viability of HepG2 and ZFL cells after exposure to cytostatics was determined with MTT assay according to Mosmann (1983), with minor modifications. This colorimetric assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. HepG2 cells were seeded onto 96-well microplates (Nunc, Naperville, USA) at a density of 4000 cells/well, while ZFL cells at a density 8000 cells/well and incubated for 24 h at 37 and 28 °C, respectively, to attach. The cell growth medium was then replaced by fresh medium containing 0, 0.29, 0.59, 1.17, 2.34, 4.69, 9.38, 18.75, 37.5, 75 and 150 µg/mL of the tested cytostatic and incubated for 4, 24, 48 and 72 h. At the end of the exposure to cytostatics, MTT was added at a final concentration of 0.5 mg/mL and the cells

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14742

Environ Sci Pollut Res (2016) 23:14739–14750

were incubated for an additional 3 h at 37 °C (HepG2 cells) and 28 °C (ZFL cells). Afterwards, the medium was removed and formazan crystals were dissolved in DMSO. The optical density (OD) was measured at 570 nm (reference filter 690 nm) with microplate spectrophotometer (Synergy Mx, BioTek, Winooski, USA). The OD difference OD570–OD690 is proportional to the concentration of formazan, which directly correlates to the number of viable cells. Viability was measured in three independent experiments each time with five replicates per treatment point. In each experiment, a solvent control (growth medium containing 0.2 v/v and 0.6 v/v % of DMSO in the case of 5-FU and ET, respectively) and growth medium control were included. There were no differences between solvent control and growth medium control observed (data not shown).

Genotoxicity

Alkaline comet assay The alkaline comet assay was carried out as described by Singh et al. (1988) with minor modifications for HPBLs (Gajski et al. 2014) and HepG2 and ZFL cells (Žegura et al. 2004). After exposure to non-cytotoxic concentrations of the selected cytostatics for 4, 24 and 48 h, the whole blood was mixed with 100 µL of 0.5 % LMP agarose and added to fully frosted slides precoated with 0.6 % NMP agarose. After solidifying, the slides were covered with 0.5 % LMP agarose, and the cells were lysed (2.5 M NaCl, 100 mM EDTA_{Na}, 10 mM Tris, 1 % sodium sarcosinate, 1 % Triton X-100, 10 % DMSO, pH 10) overnight at 4 °C. After the lysis, the slides were placed into alkaline solution (300 mM NaOH, 1 mM EDTA_{Na}, pH 13) for 20 min at 4 °C to allow DNA unwinding and electrophoresed for 20 min at 1 V/cm. Finally, the nuclei were neutralised using 0.4 M Tris buffer (pH 7.5). For the evaluation, nuclei were stained with EtBr (10 µg/mL) and analysed using an epifluorescence microscope (Zeiss, Gottingen, Germany). The percent of tail DNA was used to measure the level of DNA damage, and a total of 100 randomly captured nuclei were examined from each slide with image analysis software (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, UK).

HepG2 and ZFL cells were seeded at a density of 80,000 and 100,000 cells/well, respectively, onto 12-well tissue-culture-treated plates (Corning Costar Corporation, New York, USA) and left overnight at 37 and 28 °C, respectively, to attach. After incubation, the growth medium was replaced with fresh medium containing non-cytotoxic concentrations of the tested cytostatics. HepG2 cells were incubated for 4 and 24 h, while ZFL cells were incubated also for 72 h. The longer incubation time was chosen because ZFL cells have a longer doubling time (approximately 72 h) compared to HepG2 cells. After the incubation, HepG2 and ZFL cells were trypsinised and centrifuged at 115/259g, respectively, for

5 min. A total of 30 µL of cell suspension was mixed with 70 µL 1 % LMP agarose and added to fully frosted slides precoated with 80 µL of 1 % NMP agarose. The cells were then lysed (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % Triton X-100, pH 10) for 1 h at 4 °C, placed in electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH 13) for 20 min to allow DNA unwinding and electrophoresed for 20 min at 1 V/cm. Finally, the nuclei were neutralised using 0.4 M Tris buffer (pH 7.5). For the evaluation, nuclei were stained with EtBr (5 µg/mL) and analysed using an epifluorescence microscope (Nikon Eclipse 800, Tokyo, Japan). Three independent experiments were performed. In each experiment, images of 50 randomly selected nuclei per experimental point were analysed with image analysis software (Comet Assay IV, Perceptive Instruments Ltd.).

Benzo[a]pyrene (BaP; 50 µM) and hydrogen peroxide (H₂O₂; 1 mM) were used as positive controls in cell lines and HPBLs, respectively. In each experiment, a solvent control (growth medium containing 0.04 v/v and 0.013 v/v % of DMSO in the case of 5-FU and ET, respectively) and growth medium control were included. There were no differences between solvent control and growth medium control observed (data not shown).

CBMN assay The CBMN assay was carried out as described by Fenech and Morley (1985) with minor modifications for HPBLs (Gajski et al. 2014) and HepG2 and ZFL cells (Štraser et al. 2011). After exposure to non-cytotoxic concentrations of selected cytostatics for 24 and 48 h, whole blood (500 µL) was incubated in a Euroclone medium at 37 °C in an atmosphere of 5 % CO₂. Cytochalasin-B (6 µg/mL) was added 44 h after the culture was started. The cultures were harvested at 72 h, and the lymphocytes were fixed in methanol/acetic acid (3/1) (v/v) and air-dried. Afterwards, the slides were randomised and coded prior to analysis. The cells were stained with 5 % Giemsa solution.

In the case of HepG2 and ZFL cells, the cells were seeded on T-25 flasks (Corning Costar Corporation, Sigma) (700,000 cells/plate and 800,000 cells/plate, respectively) and incubated for 24 h under cell-line-specific conditions. The cells were then exposed to non-cytotoxic concentrations of selected cytostatics for 24 (HepG2 cells) and 72 h (ZFL cells). The different exposure times were selected because of the different doubling time of HepG2 and ZFL cells (24–28 and 72 h, respectively). After the treatment, the cells were washed twice with 1× PBS buffer; fresh medium containing cytochalasin-B (2 µg/mL) was added, and cells were incubated for additional 26 (HepG2 cells) and 48 h (ZFL cells). At the end of the exposure, floating and adherent cells were collected by trypsinisation. After centrifugation (115g), the cells were washed with 1× PBS, incubated in cold hypotonic solution (75 mM KCl) and fixed with methanol/acetic acid (3/1) (v/v) and air-dried. Afterwards, the slides were randomised and

coded prior to analysis. The cells were stained with AO (20 µg/mL).

HPBLs were analysed using a light microscope (Olympus CX41), while HepG2 and ZFL cells were analysed using an epifluorescence microscope (Nikon Eclipse 800) at $\times 400$ magnification. Micronuclei (MNi), expressed as number of micronucleated (MNed) cells, nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were counted in 1000 binucleated cells according to the HUMN project criteria published by Fenech et al. (2003). The nuclear division index (NDI) was determined by scoring 500 cells with one to four nuclei. The NDI was calculated using the formula $[M1 + 2 M2 + 3 (M3 + M4)] / 1000$, where M1–M4 represents the number of cells with one to four nuclei, respectively, and M3 and M4 are both considered to be in their third cycle (Kirsch-Volders et al. 2003).

BaP (10 µM) and bleomycin (10 µg/mL) were used as positive controls in cell lines and HPBLs, respectively. In each experiment, a solvent control (growth medium containing 0.04 v/v and 0.013 v/v % of DMSO in the case of 5-FU and ET, respectively) and growth medium control were included. There were no differences between solvent control and growth medium control observed (data not shown).

Statistical analysis

The statistical analyses were performed with Graph Pad Prism 5 (Graph Pad Software, La Jolla, USA). Non-linear regression was used to assess any dose-dependent response for 5-FU, CDDP or ET. The half maximal inhibitory concentration (IC_{50}) represents the concentration of a chemotherapeutic agent required for a 50 % inhibition of growth in vitro. For each compound, the IC_{50} values were determined using non-linear regression (log(agonist) vs. normalised response—variable slope). For the comet assay data, statistical significance between the treated groups and control was determined by one-way analysis of variance and Dunnett's multiple comparison test. The significance of CBMN assay parameters was tested using chi-square test. $P < 0.05$ was considered as statistically significant. For each endpoint effect (DNA strand breaks and MNi), the lowest-observed-effect level (LOEL) was estimated from the obtained results.

Results

Cytotoxicity

Dose- and time-dependent cytotoxic activity of 5-FU, CDDP and ET was observed in all three cell models (Fig. 1). Table 2 shows the IC_{50} values for the three cytostatics in each of the three cell models after different times of exposure. After 4 h of

exposure, none of the three drugs was cytotoxic. 5-FU was the least cytotoxic in all three cell lines, and HPBLs were the least sensitive of the three cell models. However, it should be noted that, in the HPBLs, cytotoxicity was determined with differential staining with AO and EtBr, which enables distinction between live and dead cells, while in HepG2 and ZFL cells, cytotoxicity was determined by MTT assay. The latter assay is based on measuring the activity of mitochondrial succinate dehydrogenase, which reflects the number of living cells. Therefore, only the sensitivities of ZFL and HepG2 were compared. The last column of Table 2 shows the ratio between the IC_{50} value for HepG2 cells and IC_{50} value for ZFL cells. The values higher, equal or lower than 1 represent a higher, equal or lower sensitivity of ZFL cells compared to HepG2 cells. The ratio value for 5-FU was higher than 1 after 24 h of exposure and lower than 1 after 48 h of exposure; however, considering the 95 % confidence interval for the respective IC_{50} values, this sensitivity cannot be considered as different. ZFL cells were more sensitive to exposure to ET after 48 and 72 h of exposure, while in the case of CDDP, ZFL cells were less sensitive (Table 2).

Induction of DNA strand breaks

The comet assay was performed after different periods of time of exposure. In order to enable the comparison of the responses of different cell models that exert different background levels of % tail DNA, results are shown as relative increase in % tail DNA in exposed cells over the background level in control cells (Fig. 2). As in the cytotoxicity assay, HPBLs were the least sensitive. Only ET induced a slight but significant increase in DNA strand breaks after 72 h of exposure (Fig. 2). In ZFL cells, ET and CDDP induced a significant increase in DNA strand breaks already after 4-h exposure, while after 24 and 72 h, all three cytostatics induced DNA strand breaks (Fig. 2). The most potent was ET, which, after 72 h of exposure, induced a significant increase in DNA strand breaks at concentrations ≥ 0.0001 µg/mL. Due to their faster growth and shorter doubling time, HepG2 cells were not exposed to the drugs for 72 h; their cultures become confluent and the results may not be reliable. In HepG2 cells, 5-FU did not induce DNA damage. ET induced a significant increase in DNA strand breaks after 4 and 24 h (Fig. 2). ET also induced the highest increase of DNA strand breaks over the background level of the control cells (at 10 µg/mL 4.6- and 8.3-fold, respectively) (Fig. 2). CDDP induced significant increase in DNA strand breaks after 24-h exposure at concentrations ≥ 1 µg/mL.

Induction of genomic instability

The induction of genomic instability by the selected cytostatics was evaluated by CBMN assay. In addition to MNi

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Environ Sci Pollut Res (2016) 23:14739–14750

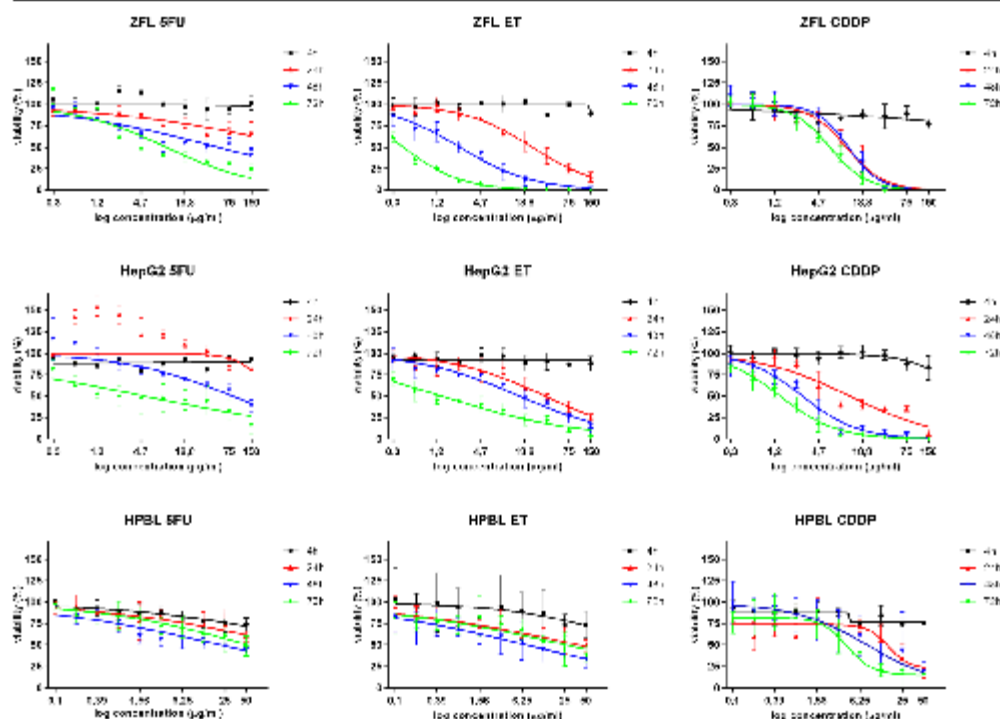


Fig. 4 The effect of the selected cytostatics on the viability of zebrafish liver (ZFL) cells, human hepatoma (HepG2) cells and human peripheral blood lymphocytes (HPBLs). The cells were exposed to graded doses of 5-fluorouracil (5-FU), etoposide (ET) and cisplatin (CDDP) over different times of exposure. The viability of HepG2 and ZFL cells was determined

by MTT assay, while the viability of HPBLs was determined by differential staining with acridine orange (AO) and ethidium bromide (EtBr). Data are mean values $\pm$ 95 % confidence intervals, presented as log dose-response curves of viability after different times of exposure (4, 24, 48 and 72 h)

induction, the frequency of NPB and NBUDs was also evaluated. Like in the comet assay, the results in Fig. 3 are presented as relative increase in MNi frequency over the background of the control cells. In HepG2 cells, all three drugs induced an increase in MNi frequency (Fig. 3)—5-FU and ET at concentrations >0.1 $\mu\text{g}/\text{mL}$ and CDDP at 1 $\mu\text{g}/\text{mL}$. It can be seen that the increase in MNi induction by 5-FU and ET was lower at the higher tested concentrations, which can be explained by cytostasis reflected by approximately a 30–40 % suppression of NDI (Fig. 3). In ZFL and HPBLs, ET and CDDP induced a significant increase in MNi frequency, while 5-FU was inactive. At the applied concentration range, the reduction of NDI, which reflects cytostatic effect of the drugs, was the lowest in HPBLs and the highest in ZFL cells (Fig. 3). In ZFL cells, 5-FU and CDDP at 10 $\mu\text{g}/\text{mL}$ and ET at 1 $\mu\text{g}/\text{mL}$ blocked cell division, which meant that there was insufficient number of binucleated cells to determine MNi induction. Of the three tested drugs, only ET induced NPBs and NBUDs in HepG2 cells and HPBLs (Fig. 4).

The positive control, bleomycin (10 $\mu\text{g}/\text{mL}$), induced 11.0 ± 0.29 - and 14.83 ± 0.08 -fold increase in the formation of MNi compared to control in HPBLs after 24 and 48 h of exposure, respectively, while BaP (10 μM) increased the formation of MNi in HepG2 and ZFL cells for 2.13 ± 0.34 - and 1.93 ± 0.24 -fold compared to control, respectively.

Discussion

In this study, we compared the susceptibility of ZFL cells and commonly used human-derived cells: HepG2 cells and HPBLs towards the cytotoxicity and genotoxicity of three cytostatics (5-FU, CDDP and ET). The results showed significant differences in the responses of the three cell lines to exposure to the selected cytostatics, which depended on the type of cytostatic (Table 2).

ZFL and HepG2 cells showed a similar sensitivity towards 5-FU-induced cytotoxicity. ZFL cells were more sensitive

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Environ Sci Pollut Res (2016) 23:14739–14750

14745

Table 2 IC₅₀ values of 5-fluorouracil (5-FU), etoposide (ET) and cisplatin (CDDP) in zebrafish (ZFL) and human hepatoma (HepG2) cells and in human peripheral blood lymphocytes (HPBLs) after 4, 24, 48 and 72 h of exposure

Exposure duration (h)	IC ₅₀ (95 % confidence interval) (µg/mL)			Sensitivity IC ₅₀ HepG2/ZFL
	ZFL	HepG2	HPBL	
5-FU				
4	>150	>150	>50	
24	>150	>150	>50	
48	53.11 (31.16–90.54)	84.72 (60.68–118.30)	21.81 (13.18–36.09)	1.60
72	10.40 (6.73–16.06)	5.27 (2.32–11.94)	>50	0.51
ET				
4	>150	>150	>50	
24	26.76 (19.94–28.31)	35.59 (25.88–48.93)	38.93 (19.79–79.69)	1.50
48	2.42 (1.97–2.97)	15.91 (12.13–20.86)	7.23 (4.43–11.80)	6.57
72	0.41 (0.37–0.44)	1.37 (0.99–1.88)	26.47 (14.33–48.91)	3.36
CDDP				
4	>150	>150	>50	
24	11.25 (9.45–13.39)	15.54 (8.91–17.65)	15.81 (10.26–24.37)	1.11
48	12.04 (10.30–14.07)	2.83 (2.37–3.39)	8.11 (1.85–35.41)	0.24
72	6.69 (5.80–7.71)	1.40 (1.19–1.65)	4.16 (3.04–5.69)	0.21

towards exposure to ET (by factors 3.3–6.6), and CDDP was more cytotoxic to HepG2 cells (by factor of 4.5). Similar IC₅₀ values for 5-FU (IC₅₀ 9.48±0.112), ET (IC₅₀ 1.31±0.013) and CDDP (IC₅₀ 0.87±0.023) for HepG2 cells have been reported by Shao et al. (2008). HPBLs seemed to be less sensitive towards the cytotoxicity of 5-FU and ET, whereas sensitivity to the cytotoxicity of CDDP was comparable to that observed in ZFL and lower from that observed in HepG2 cells. Previous studies comparing sensitivity for basal cytotoxicity endpoints such as mitochondrial dehydrogenase activity (MTT assay) of mammalian and fish cells generally suggested a similar sensitivity when exposed to a range of different chemicals (Castaño and Gómez-Lechón 2005; Papis et al. 2011). Our study indicates that this general hypothesis may not be valid for cytostatics.

All three of the tested cytostatics were genotoxic compounds; however, the mechanisms of their genotoxicity are different. 5-FU is a pyrimidine analogue. The mechanisms of 5-FU genotoxicity are the misincorporation of fluoronucleotides and inhibition of thymidylate synthase (TS), which leads to the incorporation of uracil (dUTP) and misincorporation of deoxyribonucleotides (dNTP) (Meyers et al. 2003). In ZFL cells, 5-FU induced time- and dose-dependent DNA damage, but not MNi formation, while in HepG2 cells, it induced only MNi formation. In HPBLs, 5-FU was inactive. In vitro genotoxicity of 5-FU has been shown to be difficult to detect. In HPBLs, induction of MNi was detected only at concentrations that inhibited NDI by more than 50 % (Clare et al. 2006). The difference between the responses to the clastogenic activity

of 5-FU between HPBLs and HepG2 cells may be due to, on one side, their different origin and, on the other, the fact that HPBLs are normal cells and HepG2 cells are cancer cells with an unstable genome (Dehn et al. 2004; Mohrenweiser and Jones 1998), which may contribute to their higher susceptibility. The latter may also be the reason for the lack of MNi induction in ZFL cells, which are normal immortalised cells.

ET is classified as a topoisomerase II poison. It increases the levels of topoisomerase II-induced DNA strand breaks by inhibiting the ability of topoisomerase II to ligate cleaved DNA molecules (Bromberg et al. 2003). The resulting DNA breaks initiate DNA and recombination repair pathways and generate DNA and chromosomal aberrations (McClendon and Osheroff 2007). In vitro and in vivo studies have shown that ET is a potent clastogen (Ashby et al. 1994). ET induced MNi and DNA strand breaks in all three cell lines. In addition, only ET induced an increase in NPBs and NBUDs. NPBs originate from dicentric chromosomes, which may occur due to a mis-repair of DNA breaks, telomere end fusions and could also be observed when a defective separation of sister chromatids at anaphase occurs due to decatenation failure. NBUDs represent the elimination of amplified DNA, DNA repair complexes and, possibly, excess chromosomes from aneuploid cells (Fenech 2006). The most sensitive to ET were ZFL cells. The LOEC for DNA strand break induction was 0.001 and 0.01 μg/mL for MNi induction (Table 3). Similarly, as in ZFL cells, exposure to ET in mouse lymphoma cells L5178Y induced a significant increase of MNi at concentrations ≥0.01 μg/mL (Lynch et al. 2003).



Fig. 2 Induction of DNA strand breaks in zebrafish liver (ZFL) cells, human hepatoma (HepG2) cells and human peripheral blood lymphocytes (HPBLs), after exposure to 5-fluorouracil (5-FU), etoposide (ET) and cisplatin (CDDP) at different concentrations and exposure times evaluated by alkaline comet assay. % tail DNA was determined in 50 nuclei (HepG2 and ZFL cells) per experimental point and 200 nuclei (HPBLs) per experimental point. The background level of % DNA in comet tail in ZFL cells was 4.4 ± 1.5 %, in HepG2 7.5 ± 2.5 % and in HPBLs 2.3 ± 1.1 %. Benzo[a]pyrene (BaP; 50 μ M) and hydrogen peroxide (H_2O_2 ; 1 mM) were used as positive controls (PC) in cell lines and HPBLs, respectively. Significant difference from solvent control was determined with Kruskal-Wallis with Dunn's posttest. * $P < 0.01$. Data on the figure are presented as mean values of increase of % DNA in comet tail in treated cells over the background level in control cell $\pm$ SD

CDDP interacts directly with DNA causing the alkylation of DNA and DNA-DNA and DNA-protein cross-links. It induces DNA damage and MNi formation in human and rodent in vitro and in vivo experimental systems (Blasiak et al. 2000; Duffaud et al. 1998; Filkova et al. 2014; Kliesch and Adler 1987; Kosmidar et al. 2005; Nersisyan et al. 2006; Padjas et al. 2005; Pfuhler and Wolf 1996; Wozniak et al. 2004). CDDP induced MNi formation in all of the three cell lines; however, the induction of MNi formation was, at equal concentrations, lower from that induced by ET. The increase over

the background was also lower. CDDP-induced DNA damage was observed in ZFL and HepG2 cells, and like in the case of ET, ZFL cells were the most sensitive. However, CDDP causes DNA-DNA cross-links that are not revealed in the standard comet assay. Thus, with comet assay that detects migration of broken ends of DNA molecule, the DNA damaging potential of CDDP is underestimated.

The results showed that, at the applied exposure conditions, ZFL cells were more susceptible to the genotoxic effect of the three drugs than human-derived cells. The difference in the responses may be ascribed to the differences in cell growth and kinetics and capacity of DNA repair between fish and mammals. Fish cell lines grow at lower temperatures than mammalian cells and have longer doubling time, and the studies indicated that fish cells have a lower rate and capacity of DNA repair than mammalian cells (Kienzler et al. 2013). Thus, the higher induction of DNA damage in ZFL cells compared to human-derived cells can be attributed to the differences in DNA repair systems. In addition, fish cells have a smaller genome compared to mammalian and human cells (Howe et al. 2013). Thus, it is expected that over prolonged exposure (72 vs. 24 h for ZFL and HepG2 cells, respectively), cytostatics will induce more strand breaks in fish cells than in

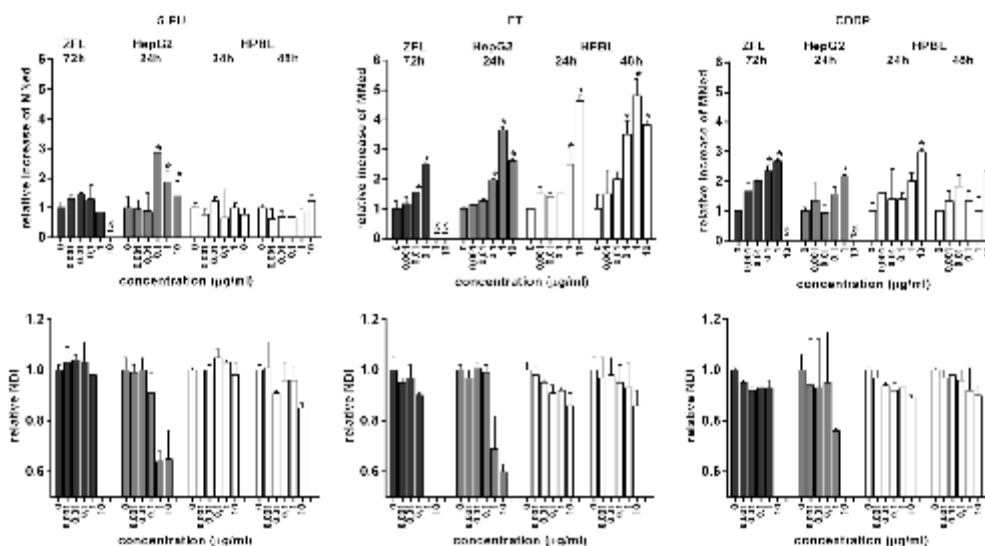


Fig. 3 Induction of micronuclei in zebrafish liver (ZFL) cells, human hepatoma (HepG2) cells and human peripheral blood lymphocytes (HPBLs), after exposure to 5-fluorouracil (5-FU), etoposide (ET) and cisplatin (CDDP) at different concentrations and exposure times evaluated by cytokinesis-block micronucleus (CBMN) assay. The number of micronucleated (MNed) cells was determined in 1000 binucleated ZFL, HepG2 or HPBL cells per experimental point. The

background level of MNed cells in ZFL was 18.0 ± 5.6 , in HepG2 cells 30.0 ± 10.1 and in HPBLs 3.7 ± 1.0 . The significant difference in the number of MNed cells between treated and control cells was determined with χ^2 test (* $P < 0.05$). The nuclear division index (NDI) was determined by scoring 500 cells with one to four nuclei. Data on the figure are presented as mean values of the relative increase of MNed cells in treated cells over the background level in control cell $\pm$ SD

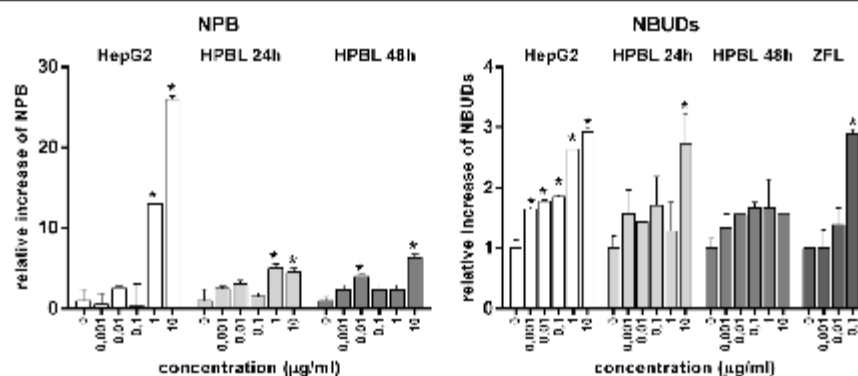


Fig. 4 Induction of nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) in zebrafish liver (ZFL) cells, human hepatoma (HepG2) cells and human peripheral blood lymphocytes (HPBLs), after exposure to etoposide (ET). The number of nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) was determined in 1000 binucleated ZFL, HepG2 or HPBL cells per experimental point. The background level of NPB cells in HepG2 cells 1.3 ± 0.6 and in HPBLs 1.3 ± 0.3 . The

background level of NBUD cells in ZFL was 26.3 ± 9.7 , in HepG2 cells 70.0 ± 31.7 and in HPBLs 4.0 ± 1.9 . The significant difference in the number of NPBs and NBUDs in 1000 binucleated cells between treated and control cells was determined with χ^2 test ($*P < 0.05$). Data on the figure are presented as mean values of the relative increase of NPBs and NBUDs in treated cells over the background level in control cell $\pm$ SD

mammalian cells. Previous studies with mammalian cells of different origin showed that their different susceptibilities to genotoxic insults can be correlated to differences in DNA damage repair capacity (Duthie and Collins 1997).

Our study showed that ZFL cells were generally more sensitive than the human-derived cells. Although the concentrations that induced genotoxic effects were much higher than the concentrations expected in the environment, the result implicates that, in an aquatic environment upon chronic exposure to

the residues of cytostatic drugs, genotoxic effects and their consequences in fish cannot be excluded. This assumption can be supported by observations from human epidemiological studies of cancer-treated patients and occupational exposure. These studies have shown chromosomal damage in lymphocytes (Burgaz et al. 2002; Chrysostomou et al. 1984; Kopjar et al. 2009) as well as in exfoliated buccal epithelial cells (Cavallo et al. 2005).

Table 3 The LOEC values for genotoxicity endpoints (DNA strand breaks and micronuclei formation, respectively) for 5-fluorouracil (5-FU), etoposide (ET) and cisplatin (CDDP) in zebrafish (ZFL) and human hepatoma (HepG2) cells and in human peripheral blood lymphocytes (HPBLs) after various times of exposure

Cells	Endpoint	Exposure (h)	LOEC ($\mu\text{g/mL}$)		
			5-FU	ET	CDDP
ZFL	Comet	4	–	10	1
		24	0.1	0.1	1
		72	0.01	0.001	0.01
HepG2	MN	72	–	0.01	0.1
	Comet	4	–	1	–
		24	–	0.1	0.1
HPBL	MN	24	0.1	0.1	1
	Comet	4	–	–	–
		24	–	10	–
		72	–	10	–
	MN	24	–	1	10
		48	–	0.1	10

– Negative

Conclusions

Our results indicated different susceptibility of fish-derived ZFL cells compared to human-derived HepG2 cells and HPBLs to cytotoxic and genotoxic effects of selected cytostatics. ZFL cells were the most susceptible, which raises the question of a negative impact of these pharmaceuticals on fish if discharged into aquatic environments excessively. The high sensitivity of non-target ZFL cells also indicates that these cells could be a good model for testing the genotoxic potential of pharmaceuticals as well as other aquatic pollutants and could serve as an alternative to animal testing in preliminary ecotoxicological studies. Since relatively little is known on the impact of cytostatics to aquatic organisms at environmentally relevant concentrations, future research that will also include *in vivo* studies on fish is warranted. Research including different cell types, especially non-target cells, and finding suitable sensitive biomarkers for the evaluation of genotoxicity of this subgroup of pharmaceuticals should be done in order to assess the risk for aquatic organisms and introduce measures to minimise the possibility of adverse effects.

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Conflict of interests None declared.

References

- Ashby J, Tinwell H, Glover P, Poorman-Allen P, Krehl R, Callander RD, Clive D (1994) Potent clastogenicity of the human carcinogen etoposide to the mouse bone marrow and mouse lymphoma L5178Y cells: comparison to Salmonella responses. *Environ Mol Mutagen* 24:51–60
- Besse JP, Latour JP, Garic J (2012) Anticancer drugs in surface waters: what can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? *Environ Int* 39:73–86
- Blaškić J, Kowalik J, Małacka-Panas E, Dżewoński J, Wojewódzka M (2000) DNA damage and repair in human lymphocytes exposed to three anticancer platinum drugs. *Teratog Carcinog Mutagen* 20: 119–131
- Brezovšek P, Elertek T, Filipič M (2014) Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Synechococcus leopoliensis*. *Water Res* 52:168–177
- Bromberg KD, Burgin AB, Osheroff N (2003) A two-drug model for etoposide action against human topoisomerase II α . *J Biol Chem* 278:7406–7412
- Burgaz S, Karahalil B, Camhi Z, Tenziglu F, Angel G, Anzoin RB, Bos RP, Hüttner E (2002) Assessment of genotoxic damage in nurses occupationally exposed to antineoplastics by the analysis of chromosomal aberrations. *Hum Exp Toxicol* 21:129–135
- Castaño A, Gómez-Lechón MJ (2005) Comparison of basal cytotoxicity data between mammalian and fish cell lines: a literature survey. *Toxicol In Vitro* 19:695–705
- Cavallo D, Ursini CL, Pemiconi B, Francesco AD, Giglio M, Rubino FM, Marinaccio A, Iavicoli S (2005) Evaluation of genotoxic effects induced by exposure to antineoplastic drugs in lymphocytes and exfoliated buccal cells of oncology nurses and pharmacy employees. *Mutat Res* 587:45–51
- Chrysostomou A, Seshadri R, Morley AA (1984) Mutation frequency in nurses and pharmacists working with cytotoxic drugs. *Aust N Z J Med* 14:831–834
- Clare MG, Lorenzon G, Akhurst LC, Marzin D, van Delft J, Montero R, Botta A, Bertens A, Cinelli S, Thybaud V, Longe E (2006) SFTG international collaborative study on in vitro micronucleus test II. Using human lymphocytes. *Mutat Res* 607:37–60
- Deblonde T, Hartemann P (2013) Environmental impact of medical prescriptions: assessing the risks and hazards of persistence, bioaccumulation and toxicity of pharmaceuticals. *Public Health* 127: 312–317
- Dehn PF, White CM, Connors DE, Shipkey G, Cumbo TA (2004) Characterization of the human hepatocellular carcinoma (hepg2) cell line as an in vitro model for cadmium toxicity studies. *In Vitro Cell Dev Biol Anim* 40:172–182
- Duffaud F, Orsière T, Baciachka-Palmaro M, Digue L, Favre R, Botta A (1998) Doxorubicin and cisplatin genotoxicity: search for a real indication using the micronucleus test. *Ann Biol Clin (Paris)* 56: 183–187
- Duke RC, Cohen JJ (1992) Morphological and biochemical assays of apoptosis. In: Coligan JE, Kruisbeal AM (eds) *Current protocols in immunology*. Wiley, New York, pp 1–3
- Duthie SJ, Collins AR (1997) The influence of cell growth, detoxifying enzymes and DNA repair on hydrogen peroxide-mediated DNA damage (measured using the comet assay) in human cells. *Free Radic Biol Med* 22:717–724
- Fenech M (2006) Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. *Mutat Res* 600:58–66
- Fenech M, Morley AA (1985) Measurement of micronuclei in lymphocytes. *Mutat Res* 147:29–36
- Fenech M, Chang WP, Kimch-Volders M, Holland N, Bonassi S, Zeiger E (2003) HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat Res* 534:65–75
- Fikrova P, Stetina R, Hrnčianik M, Hrnčianikova D, Hronek M, Zadák Z (2014) DNA crosslinks, DNA damage and repair in peripheral blood lymphocytes of non-small cell lung cancer patients treated with platinum derivatives. *Oncol Rep* 31:391–396
- Gašić Z, Kolarević S, Sunjog K, Knežević-Kolarević M, Pajunović M, Knežević-Vukčević J, Vuković-Gašić B (2014) The impact of in vivo and in vitro exposure to base analogue 5-FU on the level of DNA damage in haemocytes of freshwater mussels *Unio pictorum* and *Unio tumidus*. *Environ Pollut* 191:145–150
- Gajski G, Jelčić Z, Orešković V, Gerić M, Kollar R, Garaj-Vrhovac V (2014) Physico-chemical characterization and the in vitro genotoxicity of medical implants metal alloy (TiAlV and CoCrMo) and polyethylene particles in human lymphocytes. *Biochim Biophys Acta* 1840:565–576
- Ghosh C, Zhou YL, Colodil P (1994) Derivation and characterization of a zebrafish liver cell line. *Cell Biol Toxicol* 10:167–176
- Howe K, Clark MD, Torroja CF, Torrance J, Bertelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R, Rauch GJ, White S, Chow W, Kilián B, Quintais LT, Guernat-Assunção JA, Zhou Y, Gu Y, Yen J, Vogel JH, Eyre T, Redmond S, Banerjee R, Chi J, Fu B, Langley E, Maguire SF, Laird GK, Lloyd D, Kenyon E, Donaldson S, Selma H, Almeida-King J, Loveland J, Trevanion S, Jones M, Quail M, Wille D, Hunt A, Burton J, Sims S, McLay K, Plumb B, Davis J, Clee C, Oliver K, Clark R, Riddle C, Elliott D, Thredgold G, Haselden G, Waine D, Begum S, Mortimore B, Kerry G, Heath P, Phillimore B, Tracey A, Corby N, Dunn M, Johnson C, Wood J, Clark S, Pelan S, Griffiths G, Smith M, Glithero R, Howden P, Barker N, Lloyd C, Stevens C, Hatley J, Holt K, Panagiotidis G, Lovell J, Beasley H, Henderson C, Gordon D, Auger K, Wright D, Collins J, Raisen C, Dyer L, Leung K, Robertson L, Ambridge K, Leongamornlert D, McGuire S, Gildershorpe R, Griffiths C, Manthavadi D, Nichol S, Barker G, Whitehead S, Kay M, Brown J, Murnane C, Gay E, Humphries M, Sycamore N, Barker D, Saunders D, Wallis J, Babbage A, Hammond S, Mashreghi-Mohammadi M, Barr L, Martin S, Wray P, Ellington A, Matthews N, Ellwood M, Woodmansey R, Clark G, Cooper J, Tromans A, Giffam D, Skuce C, Pandian R, Andrews R, Harrison E, Kimberley A, Gamett J, Fosker N, Hall R, Garner P, Kelly D, Bird C, Palmer S, Gehring I, Berger A, Dooley CM, Essan-Otin Z, Eser C, Geiger H, Geisler M, Karotki L, Kim A, Konantz J, Konantz M, Oberlander M, Rudolph-Geiger S, Teucke M, Lanz C, Raddatz G, Osogawa K, Zhu B, Rapp A, Widan S, Langford C, Yang F, Schuster SC, Carter NP, Harrow J, Ning Z, Herrero J, Searle SM, Enright A, Geisler R, Plasterik RH, Lee C, Westerfield M, de Jong PJ, Zou LI, Postlethwait JH, Nüsslein-Volhard C, Hubbard TJ, Roest Croliis H, Rogers J, Stemple DL (2013) The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496:498–503

- International Agency for Research on Cancer (1987) Genetic and related effects: an updating of selected IARC monographs from volume 1 to 42: evaluation of carcinogenic risks to humans, IARC, Lyon, Supplement 6
- International Agency for Research on Cancer (2000) Some antiviral and antineoplastic drugs and other pharmaceutical agents. IARC monographs on the evaluation of carcinogenic risks to humans, vol 76. IARC, Lyon
- Ishidate M Jr, Hamois MC, Sofuni T (1988) A comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell cultures. *Mutat Res* 195:151–213
- Kienzler A, Bony S, Devaux A (2013) DNA repair activity in fish and interest in ecotoxicology: a review. *Aquat Toxicol* 134–135:47–56
- Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M, Ishidate M Jr, Kirschner S, Lorge E, Morita T, Norppa H, Surmalle J, Vanhaerwaert A, Wakata A (2003) Report from the in vitro micronucleus assay working group. *Mutat Res* 540:153–163
- Kliesch U, Adler ID (1987) Micronucleus test in bone marrow of mice treated with 1-nitropropane, 2-nitropropane and cisplatin. *Mutat Res* 192:181–184
- Kopjar N, Gamj-Vrhovac V, Kasaba V, Rozgaj R, Ramić S, Pavlica V, Zeljezić D (2009) Assessment of genotoxic risks in Croatian health care workers occupationally exposed to cytotoxic drugs: a multi-biomarker approach. *Int J Hyg Environ Health* 212:414–431
- Kojsjek T, Perko S, Žigon D, Heath E (2013) Fluorouracil in the environment: analysis, occurrence, degradation and transformation. *J Chromatogr A* 1290:62–72
- Kosmidis B, Osiecka R, Zyner E, Ochocki J (2005) Comparison between the genotoxicity of cis-Pt(II) complex of 3-amino flavone and cis-DDP in lymphocytes evaluated by the comet assay. *Drug Chem Toxicol* 28:231–244
- Lynch A, Harvey J, Aylott M, Nicholas E, Burman M, Siddiqui A, Walker S, Rees R (2003) Investigations into the concept of a threshold for topoisomerase inhibitor-induced clastogenicity. *Mutagenesis* 18:345–353
- Mavrounin KH, Blakey DH, Cimino MC, Salamone MF, Heddle JA (1990) The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res* 239:29–80
- McClendon AK, Osheroff N (2007) DNA topoisomerase II, genotoxicity, and cancer. *Mutat Res* 623:83–97
- Meyers M, Hwang A, Wagner MW, Brauning AJ, Veigl ML, Sedwick WD, Boothman DA (2003) A role for DNA mismatch repair in sensing and responding to fluoropyrimidine damage. *Oncogene* 22:7376–7388
- Milić M, Pichler C, Rainer B, Filipić M, Nemesyan A, Knasmueller S (2014) Acute toxic and genotoxic activities of widely used cytostatic drugs in higher plants: possible impact on the environment. *Environ Res* 135C:196–203
- Mohrenweiser HW, Jones IM (1998) Variation in DNA repair is a factor in cancer susceptibility: a paradigm for the promises and perils of individual and population risk estimation? *Mutat Res* 400:15–24
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63
- Negreira N, Mastroianni N, López de Alda M, Barceló D (2013) Multianalyte determination of 24 cytostatics and metabolites by liquid chromatography-electrospray-tandem mass spectrometry and study of their stability and optimum storage conditions in aqueous solution. *Talanta* 116:290–299
- Negreira N, López de Alda M, Barceló D (2014) Study of the stability of 26 cytostatic drugs and metabolites in wastewater under different conditions. *Sci Tot Environ* 482–483:389–398
- Nemesyan A, Perrone E, Roggeri P, Bolognesi C (2006) Cytogenetic toxicity of cycloplatin in human lymphocytes: detection by the micronucleus test and fluorescence in situ hybridization. *Anticancer Drugs* 17:289–295
- Padjas A, Lesisz D, Lankoff A, Banasik A, Lisowska H, Bakalarz R, Góddz S, Wojcik A (2005) Cytogenetic damage in lymphocytes of patients undergoing therapy for small cell lung cancer and ovarian carcinoma. *Toxicol Appl Pharmacol* 209:183–191
- Papis E, Davies SJ, Jha AN (2011) Relative sensitivity of fish and mammalian cells to the antibiotic, trimethoprim: cytotoxic and genotoxic responses as determined by neutral red retention, Comet and micronucleus assays. *Ecotoxicology* 20:208–217
- Parrilla A, Lavogna M, Criscuolo E, Russo C, Fiumano V, Iaidoni M (2014) Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans. *Chemosphere* 115:59–66
- Pfahler S, Wolf HU (1996) Detection of DNA-crosslinking agents with the alkaline comet assay. *Environ Mol Mutagen* 27:196–201
- Pichler C, Filipić M, Kundi M, Rainer B, Knasmueller S, Milić M (2014) Assessment of genotoxicity and acute toxic effect of the imatinib mesylate in plant bioassays. *Chemosphere* 115:54–58
- Shao ZY, Zhai BJ, Zhao CL, Hu K, Shen DM, Wu F (2008) Cytotoxic effects and in vitro reversal of multidrug resistance by therapeutic ultrasound in human hepatocarcinoma cell line (HepG2). *Ultrasonics* 48:297–302
- Singh NP, McCoy MT, Tice RR, Schneider LL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175:184–191
- Štraser A, Filipić M, Žegura B (2011) Genotoxic effects of the cyanobacterial hepatotoxin cylindrospermopsin in the HepG2 cell line. *Arch Toxicol* 85:1617–1626
- Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 35:206–221
- Toalam AP, Kümmerer K, Schneider M (2014) Environmental risk assessment of anti-cancer drugs and their transformation products: a focus on their genotoxicity characterization-state of knowledge and short comings. *Mutat Res Rev Mutat Res* 760:18–35
- Wozniak K, Czechowska A, Blasiak J (2004) Cisplatin-evoked DNA fragmentation in normal and cancer cells and its modulation by free radical scavengers and the tyrosine kinase inhibitor ST1571. *Chem Biol Interact* 147:309–318
- Žegura B, Lah TT, Filipić M (2004) The role of reactive oxygen species in microcystin-LR-induced DNA damage. *Toxicology* 200:59–68
- Zhang J, Chang VW, Giannis A, Wang JY (2013) Removal of cytostatic drugs from aquatic environment: a review. *Sci Total Environ* 445–446:281–298
- Zoukova R, Kovalova L, Blaha L, Dott W (2010) Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites. *Chemosphere* 81:253–260

3.2 Assessment of the Genotoxicity of the Tyrosine Kinase Inhibitor Imatinib Mesylate in Cultured Fish and Human Cells

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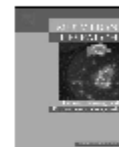
Imatinib mesylate (IM) is a widely used anticancer drug with high specificity to tumour cells and lower toxicity to normal cells causing fewer side effects. It does not target DNA, but selectively inhibits specific tyrosine kinases that are mutated in certain cancer types. Only a few studies are available that address genotoxicity of IM in nontarget environmental organisms.

In the present study cytotoxicity and genotoxicity of IM was evaluated in three different *in vitro* models that are relevant for the identification of hazard to aquatic organisms (ZFL cells) and humans (HepG2 cells and HPBLs). Additionally, the mutagenicity of IM was determined using the *Salmonella typhimurium* reverse mutation assay (Ames assay).

As expected, the results showed that IM was not mutagenic in the AMES assay. In all three cell types, dose- and time- dependent cytotoxicity of IM was observed, with ZFL cells being the most sensitive. Although ZFL cells and HPBLs are not target cells, they showed a higher sensitivity to IM cytotoxicity than cancer HepG2 cells. Surprisingly, IM induced genotoxic effects in non-target fish- and human-derived cells. DNA strand breaks were observed only in exposed ZFL and HepG2 cells. Additionally, we demonstrated for the first time that IM induces genomic instability in ZFL and HPBL cells.

High susceptibility of ZFL cells indicates that fish as non-target organisms might be affected by exposure to IM residues. Although the IM concentrations that affect ZFL cells are much higher than predicted environmental concentrations, a certain risk for environmental organisms cannot be excluded as for genotoxic compounds risk may exist at any level of exposure. In addition, considering genotoxicity of IM, an increased risk for humans at chronic exposure to low concentrations cannot be completely excluded.

The PhD candidate performed *Salmonella typhimurium* reverse mutation assay, MTT, comet and micronucleus assay on ZFL cells. He performed statistical analysis of all data and drafted the manuscript.



Assessment of the genotoxicity of the tyrosine kinase inhibitor imatinib mesylate in cultured fish and human cells



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ABSTRACT

The selective tyrosine kinase inhibitor imatinib mesylate (IM) is a widely used anticancer drug. Recent studies showing that IM can induce DNA and chromosomal damage in crustaceans and higher plants prompted us to re-examine its potential genotoxicity. IM was not mutagenic in the Ames assay (*Salmonella typhimurium*). Cytotoxicity and genotoxicity were evaluated *in vitro* in zebrafish (*Danio rerio*) liver (ZFL), human hepatoma (HepG2), and human peripheral blood lymphocytes (HPBL) cells. Genotoxicity was determined with the comet assay and with the cytokinesis-block micronucleus cytome assay. ZFL and HPBL cells showed comparable sensitivity to IM cytotoxicity, while HepG2 cells were less sensitive. At non-cytotoxic concentrations, IM induced DNA strand breaks in ZFL and HepG2 cells. An increase in the number of micronuclei was observed in ZFL and HPBL cells. In HPBL, IM also induced an increase in the number of nucleoplasmic bridges and nuclear buds. Based on the data of the concentration of IM in European countries the predicted environmental concentrations (PEC) were calculated to be in the range between 3.3 and 3.0 ng/L, which are several orders of magnitude lower than those that caused adverse effects in fish and human derived cells. However, based on the *in vitro* studies it is not possible to quantitatively predict the hazard for wildlife and humans, therefore further studies are warranted to explore the underlying molecular mechanisms of induced IM genotoxic effects as well as the studies of the occurrence of IM in the aquatic and occupational environment to establish the relevance of these observations for aquatic organisms and occupationally exposed personnel.

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1. Introduction

Imatinib mesylate (IM, Fig. 1) was the first protein kinase inhibitor used for targeted cancer chemotherapy. In contrast to cytotoxic chemotherapy drugs, protein kinase inhibitors do not target DNA. As such, they constitute a new and promising approach to cancer therapy, with high specificity to tumour cells and less toxicity to normal cells [1]. IM was developed in the mid-1990s for the treatment of BCR-ABL-associated chronic myelogenous leukaemia; it entered the market in 2001 [2] and led to a major improvement in therapeutic outcome. The mechanism of action of IM is selec-

tive inhibition of specific tyrosine kinases, including the chimeric BCR-ABL fusion oncoprotein and the transmembrane receptors KIT and PDGFR [3,4]. The Food and Drug Administration (FDA), USA, has approved IM for therapy of multiple haematological malignancies including Philadelphia chromosome-positive acute lymphocytic leukaemia and chronic myeloid leukaemia, gastrointestinal stromal tumours (GIST), and dermatofibrosarcoma protuberans.

IM is a widely used anticancer drug [5,6]. Some of the preclinical toxicological investigations required for its approval were reported by Cohen et al. [2] and in the assessment reports available from the European Medicines Agency [7,8] and FDA [9]. Genotoxicity studies demonstrated that IM is not mutagenic in the Ames test or mouse lymphoma assays. IM was clastogenic in the Chinese hamster ovary cell assay, but only at the highest concentration tested (125 µg/ml) in the presence of metabolic activation [2,7]. In the rat bone marrow micronucleus (MN) assay *in vivo*, IM was negative [7,8]. Two manufacturing intermediates, which may be present as trace impu-

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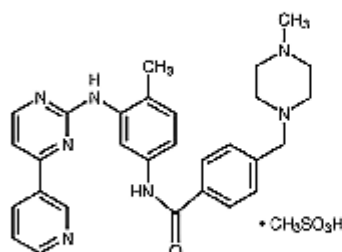


Fig. 1. Structure of Isosafrole mesylate.

rites in the final product, are mutagenic in the Ames test; one of these is also positive in the mouse lymphoma assay, but negative in the rat bone marrow MN assay [8,9]. Taking into account the negative results of the *in vivo* studies, and in the context of its therapeutic indication and effectiveness, concern with regard to IM genotoxicity has not been high [7].

Recent studies showed that IM induce DNA damage in crustaceans [10], associated with inhibition of reproduction [11]. IM also inhibited reproduction of algae and cyanobacteria [12]. In higher plants, IM induced MN formation in *Tradescantia* and *Allium* spp. and affected the fertility of plant species such as *Chenopodium* sp., *Tradescantia* sp., and *Arcobolus* sp. [13–15].

The aim of this study was to assess the *in vitro* cytotoxicity and genotoxicity of IM in cells that do not express the target aberrant tyrosine kinases: zebrafish (*Danio rerio*) liver (ZFL) cells; human hepatoma (HepG2) cells; and primary human peripheral blood lymphocytes (HPBL). ZFL cells are considered to be relevant for identification of toxicological hazards to aquatic organisms. HepG2 and HPBL cells are non-target cells of human origin that are commonly used in cytotoxicity and genotoxicity studies *in vitro* for toxicological hazard identification [16]. Genotoxicity was determined using the comet assay, which measures the induction of DNA strand breaks [17], and the cytokinesis-block micronucleus cytome (CBMN) assay, which measures the induction of genomic instability [18]. Additionally, the mutagenicity of IM was determined using the *Salmonella typhimurium* reverse mutation assay (Ames assay).

2. Materials and methods

2.1. Chemicals

IM (Lot Number A1712; purity > 98%) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A stock solution of IM (30 mg/mL; 85 mM) was prepared in sterile double-distilled water purified with the Milli-Q system (Millipore, Bedford, USA), aliquoted, and stored at -20°C .

Supplies were obtained as follows. Chromosome kit 2, Eurochem (Milan, Italy); RPMI 1640, HEPES and epidermal growth factor, hirogen (Caledon, CA, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange (AO), bleomycin, cytosine- β -D, dimethyl sulfoxide (DMSO), ethidium bromide (EBR), histopaque, low-melting-point (LMP) agarose, normal-melting-point (NMP) agarose, methanol, and Williams' medium E, Sigma Chemicals (St. Louis, MO, USA); penicillin/streptomycin, fetal bovine serum (FBS) for HepG2 cells, L-glutamine, and phosphate-buffered saline (PBS), PAA Laboratories (Darmstadt, MA, USA); trypan, BD-Difco (La Jolla, CA, USA); Leibovitz 1-15 medium and fetal bovine serum for ZFL cells, American Type Culture Collection (Manassas, VA, USA); Dulbecco's modified Eagle's medium and Ham's F-12 medium,

Gibco (Waltham, MA, USA); heparinized vacutainer tubes, Becton Dickinson (Franklin Lakes, NJ, USA); Giemsa stain, Merck (Darmstadt, Germany); lyophilized Ancestr-1284-induced male rat-thy post-mitochondrial fraction (39), Moltox (Boone, NC, USA). All other reagents were the purest grades available, and solutions were made using Milli-Q water.

2.2. *Salmonella typhimurium/microsome* (Ames) assay

IM mutagenicity was tested in the plate incorporation assay with *S. typhimurium* strains TA97a, TA98, TA100, TA102, and TA1535, with or without metabolic activation (S9) [19]. The bacterial strains were obtained from Trinova Biochem GmbH (Gießen, Germany). Briefly, IM (100 μL H 0.3125, 0.625, 1.25, 2.5, and 5 mg/mL, corresponding to doses of 31.25, 62.5, 125, 250, and 500 $\mu\text{g}/\text{plate}$, respectively), overnight culture of *S. typhimurium*, 100 μL , and phosphate buffer, 500 μL (for assays without metabolic activation) or 400 μL (for assays with metabolic activation) were added to 2 mL molten top agar containing a limited amount of histidine/biotin; these were mixed gently and poured into minimal agar plates. The plates were incubated for 48 h (TA97a, TA100, TA102, TA1535) and 72 h (TA98) at 37°C . Subsequently, the numbers of spontaneous and IM-induced His^r revertants were counted. The plates were checked for possible toxic effects of IM. Three plates were used per experimental point.

Positive controls (direct mutagenicity) were 4-nitroquinoline-N-oxide (NQO), 0.25 μg per plate, for TA97a and TA98; mitomycin C, 0.05 $\mu\text{g}/\text{plate}$, for TA102; and sodium azide (NaN₃), 0.25 and 0.125 $\mu\text{g}/\text{plate}$, respectively, for TA100 and TA1535. The positive control (S9-dependent mutagenicity) was benzo(a)pyrene (BaP), 5 and 2.5 $\mu\text{g}/\text{plate}$ for TA97a and for TA98, TA100, TA102 and TA1535, respectively.

2.3. Cell cultures

The ZFL cell line, derived from normal adult zebrafish [20], was obtained from American Type Culture Collection (ATCC, CRL-2634). The cells were cultured under a humidified air atmosphere at 28°C in a medium composed of 50% Leibovitz 1-15 medium, 50% Dulbecco's modified Eagle's medium, and 15% Ham's F-12 medium, supplemented with HEPES, 15 mM; NaHCO₃, 0.15 g/L; insulin, 0.01 mg/mL; epidermal growth factor, 50 ng/mL; penicillin/streptomycin, 100 U/mL; and 5% heat-inactivated FBS.

HepG2 cells were a gift from Dr. Frans van der Meer (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, Netherlands). The cells were cultured under a humidified air/5% CO₂ atmosphere at 37°C (Heracus HeraCell 240 incubator, Langensfeld, Germany) in Williams' medium E containing 15% FBS, L-glutamine, 3 mM, and penicillin/streptomycin, 100 U/mL.

HPBL were obtained from three young healthy male donors. The donors gave informed consent to participate in this study, which was part of a project approved by the Ethics Committee and following the ethical principles of the Declaration of Helsinki. According to the questionnaire filled out by the donors, they had not been exposed to diagnostic radiation or to known genotoxic chemicals that might have interfered with the results of the tests, for at least a year before the blood sampling. Blood was drawn by antecubital venipuncture into heparinized vacutainers containing lithium heparin as anticoagulant and under aseptic conditions. The experiments were conducted on whole blood, cultivated under a humidified air/5% CO₂ atmosphere at 37°C .

2.4. Cytotoxicity assays

Cytotoxicity of IM to ZFL and HepG2 cells was determined by measuring their viability after exposure to IM, using the MTT assay

according to Mosmann [21], with minor modifications [16]. ZFL and HepG2 cells were seeded onto 96-well microplates (Nunc, Naperville, IL, USA) at densities of 8000 and 4000 cells/well, respectively, and incubated for 24 h at 28 °C and 37 °C, respectively, to allow them to attach. The growth medium was then replaced by fresh medium containing IM (0, 0.25, 0.50, 1.17, 2.34, 4.68, 9.38, 18.75, 37.5, 75 or 150 µg/ml), and incubated for 4, 24, 48 and 72 h. At the end of the exposure, MTT (0.5 mg/ml) was added to each well and the cells were incubated for an additional 3 h at 28 °C (ZFL) or 37 °C (HepG2). The optical density was then measured at 570 nm (reference filter, 690 nm) using a microplate spectrophotometer (Synergy MX; BioTek, Winooski, VT, USA). Viability was determined in three independent experiments, each carried out with five replicates per treatment point.

Cytotoxicity of IM to HPFL was determined by differential staining with AO/EthBr using fluorescence microscopy [22]. Whole blood samples were treated with IM (0, 0.10, 0.20, 0.39, 0.78, 1.58, 3.13, 6.25, 12.5, 25 or 50 µg/ml), and incubated for 4, 24, 48 and 72 h. After treatment, HPFL were isolated by histopaque density gradient centrifugation [16]. The slides were prepared by adding AO/EthBr (both diluted in PBS) to the HPFL suspensions in final concentration of 100 µg/ml (1:1; v/v). A total of 100 cells per replication was analysed using an epifluorescence microscope (Olympus IX51, Tokyo, Japan). Quantitative assessments were made by determining the percentage of viable and dead cells, whereby the cells were classified as follows: viable cells showed a functional membrane with uniform green staining of the nucleus, and dead cells showed uniform red staining of the nucleus.

2.5. Genotoxicity

2.5.1. Alkaline comet assay

ZFL and HepG2 cells were seeded at densities of 100,000 and 50,000 cells/well, respectively, into 12-well tissue-culture-treated plates (Corning Costar Corporation, New York, NY, USA) and incubated overnight at 28 °C and 37 °C, respectively, to attach. After the incubation, the cells were treated with IM (0, 0.001, 0.01, 0.1 or 1 µg/ml), for 4 and 24 h, and in the case of the ZFL cells, also for 72 h. The longer incubation time was chosen due to the longer doubling time of the ZFL cells (approximately 72 h, according to the American Type Culture Collection) compared to the HepG2 cells (24–28 h) [20] and because we observed that, in ZFL cells, DNA damage induced by B[a]P was detected only after 72 h exposure (unpublished data). After treatment, ZFL and HepG2 cells were harvested and the formation of DNA strand breaks was determined with the alkaline comet assay as described by Singh et al. [23]. Three independent experiments were performed for each of the treatment conditions. In each experiment, images of 50 randomly selected nuclei per experimental point were analysed with the image analysis software (Comet Assay IV; Perceptiva Instruments Ltd., Boreham, Suffolk, UK).

The comet assay in HPFL was performed by exposure of whole blood samples to IM (0, 0.001, 0.01, 0.1 or 1 µg/ml) for 4, 24 and 48 h. After exposure, the alkaline comet assay was performed according to the guidelines developed by Singh et al. [23], with minor modifications, as described previously [16]. In each experiment, images of 100 randomly selected nuclei were analysed from each slide with the image analysis software (Comet Assay II; Perceptiva Instruments Ltd.).

2.5.2. Cytosine-block micronucleus cytome (CBMN) assay

The CBMN assay with ZFL and HepG2 cells was performed according to Fenech [24]. The ZFL and HepG2 cells were seeded into T-25 flasks (Corning Costar Corporation, Corning, NY, USA) at 800,000 and 700,000 cells/flask, respectively, and incubated for 24 h under the cell-line specific conditions. The cells were

then exposed to IM (0, 0.001, 0.01, 0.1, 1 or 10 µg/ml) for 24 and 48 h (HepG2 cells) and 72 h (ZFL cells). After exposure, the cells were washed twice with PBS, then fresh medium containing cytochalasin-B, 2 µg/ml, was added and the cells were incubated for additional 26 h (HepG2 cells) and 48 h (ZFL cells). The cells were then harvested, fixed and air dried. The slides were stained with AO, 20 µg/ml, and analysed under an epifluorescence microscope (Eclipse 800; Nikon, Japan) at 400× magnification.

The CBMN assay with HPFLs was performed according to Paronchi and Morley [25], with minor modifications [16]. Whole blood (500 µl) was exposed to IM (0, 0.001, 0.01, 0.1, 1 or 10 µg/ml) for 24, 48 and 72 h, added to Bursaclean medium and incubated under a humidified air/5% CO₂ atmosphere at 37 °C. Cytochalasin-B (6 µg/ml) was added 44 h after the culture was started, and after 72 h the cultures were harvested, the cells were fixed and air dried. The slides were stained with Giemsa solution and analysed under a light microscope (Olympus CK41; Tokyo, Japan) at 400× magnification.

Experiments with ZFL and HepG2 cells were performed in two independent experiments and a total of 2000 binucleated (BNC) cells with preserved cytoplasm were manually scored per experimental point (1000 BNC per each of the two experiments). With HPFLs three independent experiments were performed using the HPFLs from three donors. In total 3000 BNC were scored per experimental point (1000 BNC per donor). The numbers of micronuclei (MN); expressed as number of micronucleated (MNed) cells), nuclear buds (NBUDs), and nucleoplasmic bridges (NPBs) were determined according to standard criteria [24]. The nuclear division index (NDI) was estimated by scoring 500 cells with one to four nuclei. The NDI was calculated using the formula $[M1 + 2M2 + 3(M3 + M4)]/500$, where M1, M2, M3, and M4 represent the numbers of cells with one to four nuclei, respectively [24].

2.6. Statistical analysis

The statistical analyses were performed using Graph Pad Prism 5 (Graph Pad Software, La Jolla, CA, USA). The half-maximal inhibitory concentrations (IC₅₀) that represent the IM concentrations required for 50% inhibition of cell viability *in vitro* were determined using non-linear regression analysis (log(agonist) versus normalized response –variable slope). For the comet assay data, statistical significance between the control and the treated groups was determined by one-way analysis of variance (ANOVA; Kruskal-Wallis), and Dunnett's multiple comparison test was used for multiple comparisons versus the control. DNA damage was expressed as percentages of tail DNA. The significance of CBMN assay parameters was tested using Pearson's χ^2 test. *P*-values < 0.01 were considered statistically significant.

3. Results and discussion

We confirm that IM is not a bacterial mutagen; however, IM induced DNA strand breaks in ZFL and HepG2 cells and genomic instability in ZFL and HPFL cells. Our study shows substantial differences in the responses of the various cell types to IM exposure.

3.1. Bacterial mutagenicity

Recently, Parrella et al. [10] reported that IM induced mutations in *S. typhimurium* strains TA98 and TA100 in the absence of metabolic activation at very low doses, ranging from 0.074–2 µg/plate. We could not confirm that report. We tested the bacterial mutagenicity of IM in five different strains of *S. typhimurium* with and without metabolic activation, at doses from 0.05–0.5 mg/plate; in all strains, the responses were negative, with

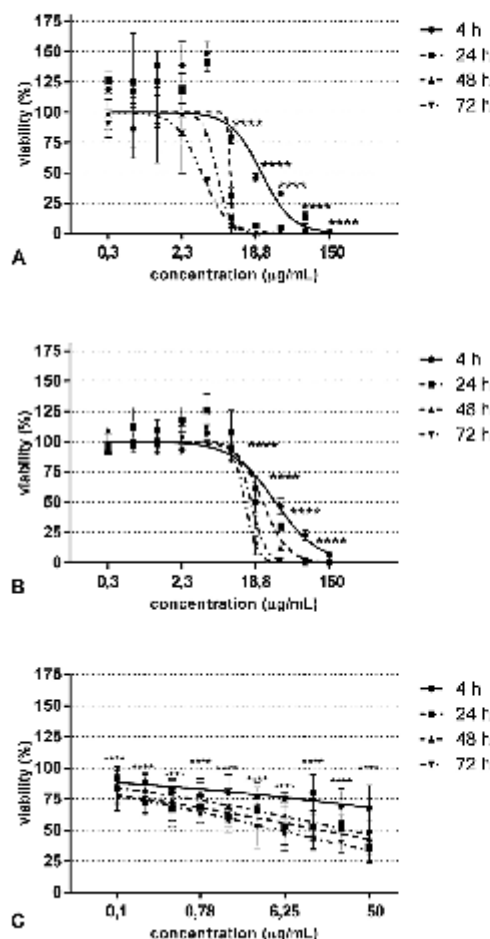


Fig. 2. Effect of IM on the viability of (a) zebrafish liver cells (ZFL), (b) human hepatoma cells (HepG2) and (c) human peripheral blood lymphocyte cells (HPBL). The viability of ZFL and HepG2 cells was determined with the MTT assay, and the viability of HPBL by ACridine differential staining. Three independent experiments, each with five replicates, were performed. The data are mean values $\pm$ SEM standard deviation intervals, presented as log dose-response curves of viability after 4, 24, 48 and 72 h exposure. * $P < 0.05$, significant difference between non-treated control and IM-treated cells (Student's t -test).

no evidence of toxicity (Supplementary Material; Table S1). Our data confirm earlier reports that IM is not a bacterial mutagen [3].

3.2. Cytotoxicity

In all three cell types, dose- and time-dependent cytotoxicity of IM was observed (Fig. 2). IM, at concentrations up to 5.38 and 18.75 μg/mL did not significantly affect the viability of ZFL and HepG2 cells, respectively. At higher concentrations, viability rapidly decreased. Viability of HPBL was significantly affected at all concentrations of IM tested (0.1–50 μg/mL).

Table 1

IC_{50} values of bacterial mutagen in zebrafish liver cells (ZFL), human hepatoma cells (HepG2) and human peripheral blood lymphocytes (HPBLs) after 4, 24, 48 and 72 h of exposure.

Exposure interval (h)	IC_{50} for bacterial cytotoxicity (SEM confidence interval) (μg/mL)	ZFL cells	HepG2 cells	HPBLs
4		37.83 (8.37–80.86)	33.53 (28.87–40.81)	ND
24		~0.1	22.85 (14.83–35.06)	15.80 (8.36–34.27)
48		8.23 (3.34–7.24)	18.43 (11.12–32.23)	29.43 (18.89–46.32)
72		4.04 (1.29–4.94)	13.64 (11.14–35.79)	3.48 (4.13–7.21)

ND, not determined.

Table 1 summarizes IM cytotoxicity IC_{50} values for each of the three cell types. After 24 and 48 h exposure, ZFL cells were the most sensitive. After 72 h exposure, ZFL and HPBL cells showed comparable sensitivities (IC_{50} values 4.0 and 5.5 μg/mL, respectively), while HepG2 cells were less sensitive (IC_{50} , 13.6 μg/mL). In contrast to these data, IM, at concentrations up to 6 μg/mL, did not affect the viability of HPBL and a human leukemia cell line that does not express BCR/ABL (CCRF-CEM cells), while human leukemia cell lines expressing BCR/ABL (K562, BV173 cells) were highly sensitive [27–29]. The IC_{50} values obtained in our study with the ZFL and HPBL cells are comparable to those obtained in several studies with cells that express specific IM targets, including small-cell lung cancer cells with aberrant expression of the KIT receptor [30] and prostate cancer cell lines and non-small cell lung cancer with aberrant expression of PDGFRs [31,32]. Interestingly, the least sensitive of the cell types tested here were HepG2 cells, which are of a cancer origin, but do not express specific IM targets. HepG2 cells have elevated endogenous expression of multidrug resistance proteins [33,34]. Also, it has been reported that in IMI cells, overexpression of multidrug resistance gene product P-glycoprotein (also known as ABCB1, MDR-1) is implicated in resistance to IM and consequently reduced efficacy of IM chemotherapy [35]. Thus, this lower sensitivity of HepG2 cells might be explained by elevated expression of multidrug resistance proteins.

3.3. Genotoxicity in fish- and human-derived cells

Genotoxicity of IM was determined with the comet and CEMM assays in ZFL, HepG2, and HPBL cells.

3.3.1. Induction of DNA strand breaks

To test IM-induced DNA damage, the comet assay was performed after short-term (4 h) and prolonged (24 h and in ZFL cells also 72 h) exposures, in order to examine both short-lived DNA damage that may be rapidly repaired as well as delayed or accumulating DNA damage. In ZFL and HepG2 cells, IM induced DNA strand breaks in dose- and time-dependent fashion (Fig. 3). In both cell lines, statistically significant increases in the percentage of tail DNA were observed after 4 h IM treatment. After 24 h exposure, the sensitivities of the ZFL and HepG2 cells were comparable, with the lowest observed effective concentration of IM (LOEC) = 1 μg/mL. In ZFL cells, a statistically significant increase in DNA strand breaks after 72 h exposure was detected at 0.01 μg/mL, indicating accumulation of IM-induced DNA strand breaks during prolonged exposure. In HPBL cells, exposure to IM did not induce DNA strand breaks (data not shown).

Information on IM induction of DNA strand breaks in non-target cells is relatively limited. In human lymphocytes and in CCRF-CEM acute lymphoblastic leukemia cells, which do not express

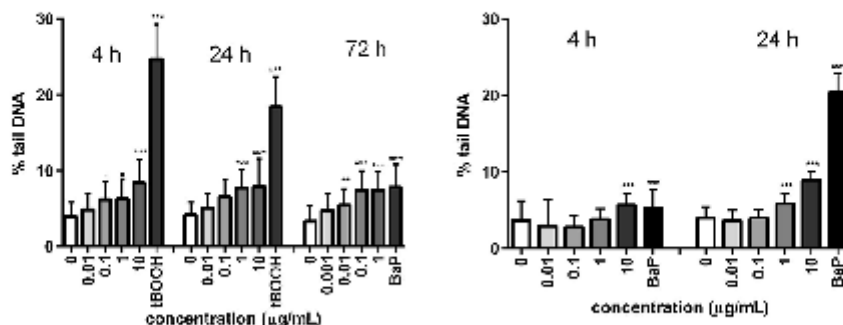


Fig. 3. IM-induced DNA damage in (a) zebrafish liver (ZFL) and (b) human hepatoma cells (HepG2). The cells were exposed to IM for 4, 24, and 72 h. DNA damage was assessed using the comet assay, and is expressed as percentage of DNA in the comet tails. Fifty cells were analyzed per experimental point in each of three independent experimental cultures. Positive controls were 1 mM hydrogen peroxide (H_2O_2) and 10 μ M benzo(a)pyrene (BaP) (as indicated). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, significant difference between control and treated cells (Mann-Whitney U-test, Dunnett's post-test).

the BCR/ABL fusion protein the target of IM, no DNA strand breaks were induced by IM [27]. In contrast, in K562 and BV173 human leukaemia cells, which express BCR/ABL, DNA strand breaks were induced by IM, 0.12 μ M, after 15 h exposure [27]. Cieschowska et al. [27] also demonstrated that the DNA damage was not due to direct interaction of IM with DNA, but rather from generation of free radicals that induce oxidation and alkylation of nucleic bases. This hypothesis that IM does not induce direct damage to DNA is further supported by the results of a recent study that showed that in HepG2 cells, IM induced formation of DNA double-strand breaks, but did not up-regulate the expression of marker genes for DNA damage responses (*CDKN1A*, *GADD45A*, *MDM2*) [38].

3.3.2. Induction of genomic instability

With the COMET assay, we demonstrated for the first time that IM induces genomic instability in ZFL and HPFL cells. In ZFL cells, exposure to IM, 1 μ M, for 72 h resulted in a significant increase in the formation of MN (Fig. 4, Supplementary material Table S2). At concentrations up to 1 μ M, the MN were not affected, while at 10 μ M, no MN were detected, which indicates complete block of cell division. This result is in line with the results of viability testing with the MTT assay (see Section 3.2). In HepG2 cells exposed to non-cytotoxic concentrations of IM for 24 and 48 h, neither induction of genomic instability nor reduction of MN was observed (data not shown).

In HPFL cells, IM induced genomic instability after 24, 48, and 72 h exposure (Fig. 5, Supplementary material Table S3–S5). In addition to MN formation, originating from broken or lagging chromosomes, formation of NBUDs and NPBs was also observed. NBUDs represent the elimination of amplified DNA, DNA repair complexes, and, possibly, nuclear chromosomes from aneuploid cells, while NPBs are considered to originate from dicentric chromosomes, which can occur due to misrepair of DNA breaks and telomere end fusion, and might also be observed during defective separation of sister chromatids at anaphase [39]. Significant increase in the frequency of genomic instability parameters was observed at concentrations ≥ 0.01 μ M, however, a clear dose dependence was not observed. At the highest concentration tested, a trend of increased MNed, NBUD, and NPB frequencies with time of exposure has also been observed. The result is in line with a previous study that reported IM-mediated induction of centrosome and chromosome aberrations and mitotic spindle defects in normal human and rodent fibroblasts propagated for 3 weeks in medium containing 3–12 μ M IM [37,38]. Clastogenic activity of IM has also been detected in Chinese hamster ovary cells [8] and higher plants

[14,15]. On the other hand, IM was negative in the rat bone marrow MN assay *in vivo* [8]. Such a discrepancy between the *in vitro* and *in vivo* clastogenicity results is not uncommon and might be attributed to differences between cultured cells and animals, e.g. *in vitro* vs. *in vivo* metabolism, metabolic inactivation, and detoxification in the intact animal, or failure of the parent compound or metabolite to reach target cells.

The primary chemotherapeutic mechanism of IM is inhibition of target tyrosine kinases (ABL, KIT, PDGFR) by blocking the ATP binding site. Fabian et al. [37,38] proposed that genotoxic activity of IM in non-target cells might be due to inhibition of the action of one or more of these targets in normal cells. ABL is involved in numerous stress signalling pathways and has a key role in cellular growth arrest, DNA repair, and apoptosis [39,40]. IM-mediated inhibition of ABL might predispose cells to replication of damaged DNA, which, by interference with DNA repair and inhibition of cell-cycle arrest and apoptosis, can lead to genomic instability. ATP-binding domains are stereochemically conserved among different kinases; thus, inhibitors directed at the ATP binding site are not completely specific for the targeted kinases. IM also inhibits the serine-threonine kinase Raf-1 [41], some other kinases [42], and even non-kinase targets (oxidoreductase 2) [43]. Conservation of the domain organization of tyrosine kinase proteins is relatively high even between distantly related organisms [44]. Therefore, IM may inhibit kinases (as yet unidentified) involved in induction of genotoxicity.

3.4. IM genotoxicity in the context of environmental and human health risks

The high susceptibility of the fish-derived ZFL cells is of relevance for the aquatic environment, as it indicates that fish, non-target organisms, might be affected by exposure to IM residues. IM is excreted through urine or faeces as a mixture of metabolites and unchanged parent compound (up to 81%), and these residues might harm non-target aquatic organisms [45,46]. The reported predicted environmental concentrations of IM, calculated from consumption data, are 0.5–5.0 ng/L [5,47,48]. In standard ecotoxicity tests with aquatic organisms, IM has been reported to inhibit growth of green algae and cyanobacteria at concentrations ranging from 0.4 [12] to 0.98 mg/L [49]. The reproduction of *Daphnia magna* after chronic exposure was inhibited at 5.6 mg/L [48], while Pavella et al. [11] reported that the reproduction of *Ceriodaphnia dubia* was inhibited at 0.27 μ M, and reproduction of *Daphnia magna* at 2.98 μ M. In fish, the LC_{50} after acute 96 h exposure was 71 mg/L for zebrafish

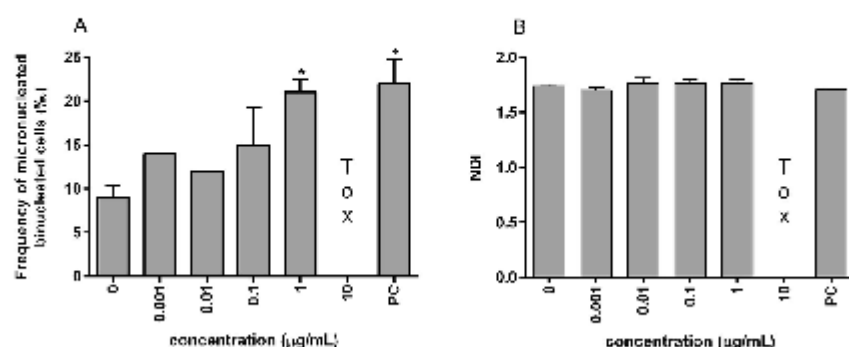


Fig. 4. NF-induced genomic instability in zebrafish liver (ZFL) cells determined by cytokinesis-block micronucleus (CBMN) assay after 72 h exposure. The frequencies of micronucleated (MNed) cells were determined in 1000 ZFL cells per replicate per experimental point. Dimethyl sulfoxide (DMSO) was used as positive control (PC). The significance of differences in the frequencies of MNed cells between treated and control cells was determined with Pearson's χ^2 test ($P < 0.01$). The nuclear division index (NDI) was determined by scoring 500 cells with one to four nuclei. Data on the figure are presented as mean values of two independent experiments $\pm$ SD.

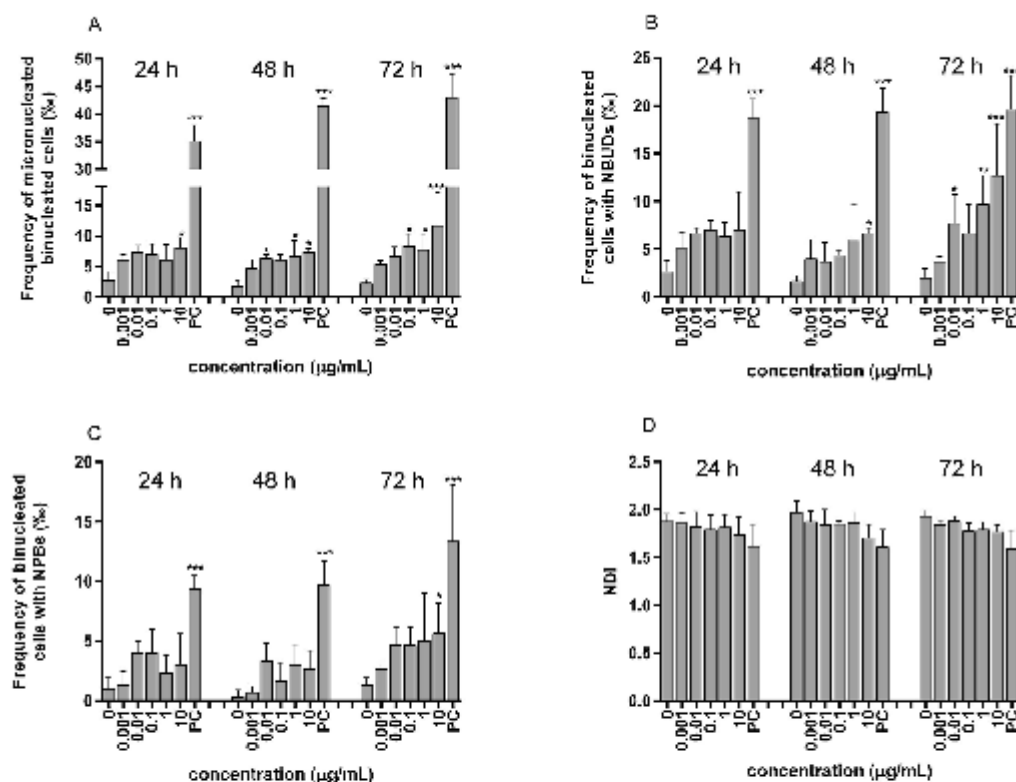


Fig. 5. NF-induced genomic instability in human peripheral blood lymphocyte cells (HPBL) determined by cytokinesis-block micronucleus (CBMN) assay after 24, 48 and 72 h exposure. The frequencies of micronucleated (MNed) cells, nucleoplasmic bodies (NPBs) and nuclear buds (NBDs) were determined in 1000 HPBL cells per donor per experimental point. Dimethyl sulfoxide (DMSO) was used as positive control (PC). The significance of differences in the frequencies of MNed cells, NPBs, and NBDs between treated and control cells was determined with Pearson's χ^2 test ($P < 0.01$, $**P < 0.001$, $***P < 0.0001$). The nuclear division index (NDI) was determined by scoring 500 cells with one to four nuclei. Data on the figure are presented as mean values of three independent experiments $\pm$ SD.

Healthy individuals may be unintentionally exposed to DM, predominantly through occupational exposure. Environmental monitoring studies performed in hospitals and pharmacies demonstrated potential exposure of healthcare workers via contamination of work surfaces, equipment, and urine of patients treated with anticancer drugs [53,54]. Numerous studies have reported trace amounts of some anticancer drugs in the urine of healthcare workers and an increased frequency of genotoxicity indicators in exposed workers in comparison to non-exposed population [55]. Recent meta-analysis of the results of published studies reporting the use of the COMET assay in HPSL cells as a biomarker of genotoxic risk in health care workers exposed to anticancer drugs confirmed an association between exposure and increased frequency of MN [56]. Here, we have demonstrated that DM induces MN formation in HPSL cells *in vitro*. It cannot be excluded that DM may present a health risk to unintentionally exposed healthcare workers. However, information regarding work-surface contamination and possible exposure of healthcare workers to DM is lacking, which prevents risk estimation.

The results of the present study demonstrate BM-induced genotoxic effects in non-target fish- and human-derived cells. The concentrations that caused adverse effects in these cells are higher than those that can at present be expected in the environment. However, considering that BM exerts genotoxic activity, an increased risk for wildlife and human health at chronic exposure to low concentrations cannot be completely excluded. This warrants further investigation to clarify the molecular mechanisms involved in BM genotoxicity. Additionally, data on the occurrence and levels of BM in aquatic and occupational environment are needed to characterize the risk for wildlife and humans upon potential continuous exposure to low doses of BM.

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- [1] A. Rogers, R.M. Schepker, Role of targeting ligands inhibitors in cancer therapy, *J. Pharmacokinet. Exp. Ther.* 315 (2005) 971–978.
- [2] M.J. Collins, C. Williams, J.R. Johnson, J. Ross, J. Calabrese, A. Schenck, K. Swann, J. Leighton, S.S. Chen, K. Wood, M. Kaufman, E. Chen, T. Kim, A.M. Wilson, W. Frazier, Apparent accuracy for intracellular molecular analysis in the treatment of chronic myelogenous leukaemia, *Clin. Cancer Res.* 10 (2002) 965–969.
- [3] E. Kaufman, T. O'Reilly, J. Wood, Pharmacology of imatinib (STI-571), *Ber. J. Cancer* 38 (Suppl. 5) (2002) 558–559.
- [4] Z. Polster, E. Maier, B. Buchberger, W. Gross-Gessl, G. Penedich, J. Ueffelsch, J. Zanker, T. O'Reilly, P. Knauer, B. Löffler, H. Peter, J. Zimmermann, T. Meyer, G. Caswell, P. Finet, F.W.G. Heitkamp, Protein kinases as targets for anticancer agents: New inhibitors in clinical drugs, *Pharmacol. Ther.* 83 (2004) 79–96.
- [5] J.T. Moore, J.F. Lawrence, J. Gerke, Anticancer drugs in surface waters: What can we say about the occurrence and environmental significance of cytotoxic, cytostatic and anti-metabolic therapy drugs? *Environ. Int.* 35 (2011) 73–86.
- [6] K. Knechtner, A. Nies, A. Schuster, A. Stein, I. Blank, Anticancerous compounds in the aquatic environment: Assessment of apical toxicity, *Environ. Sci. Pollut. Res.* 23 (2016) 14591–14604.
- [7] European Medical Agency (EMA). EMA: EMA scientific discussion (2005). http://www.ema.europa.eu/docs/en_GB/document_library/EU_Scientific_Discussion/Human/D00406/WP/C000222.pdf [Accessed on 5 November 2016].
- [8] The Center for drug evaluation and research, Application number: D04 31-22, Pharmacology/toxic review(s), (2001) http://www.accessdata.fda.gov/drugsatfda_docs/newdrugs/2001/21-222_Civarc.pharm.PZ.pdf [Accessed on 5 November 2016].
- [9] European Medical Agency (EMA). EMA: EMA summary of product characteristics (2016) http://www.ema.europa.eu/docs/en_GB/document_library/Human/D00406/WP/C000222.pdf [Accessed on 5 November 2016].
- [10] A. Furlani, M. Lavergne, E. Colonna, C. Mizog, M. Ishidori, Bio-geochemistry of the water cycle using cement away in epinephrine, *J. Hazard. Mater.* 390 (2015) 372–380.
- [11] A. Furlani, M. Lavergne, E. Colonna, C. Mizog, V. Picot, M. Ishidori, Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans, *Chemosphere* 115 (2014) 58–66.
- [12] P. Sawrowski, T. Kowalski, M. Witek, Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchneriella subcapitata* in the context of the genotoxigenicity *Spermatocystis vaginalis*, *Water Res.* 53 (2011) 168–177.
- [13] M. Mohle, M. Witek, A. Sawrowski, K. Milojkovic, K. Knapczyk, M. Knapczyk, Analysis of combined effect of cytostatic drugs on microorganisms detection by the *Vibrifluorescence*, *Environ. Sci. Pollut. Res.* 23 (2016) 14592–14599.
- [14] C. Fischer, M. Witek, M. Knapczyk, K. Knapczyk, A. Knapczyk, Assessment of genotoxicity and acute toxic effect of the breast cancer in plant biotransformation, *Chemosphere* 315 (2014) 54–60.
- [15] C.A. Isidoroff, R.L. Nye, E.L. Stachurski, K. Knapczyk, Evaluation of the toxic effects of four anti-cancer drugs in plant biotransformation and its pathway for screening in the context of waste water reuse for irrigation, *Chemosphere* 135 (2015) 402–410.
- [16] G. Gold, M. Gerke, B. Ziegler, M. Pankaj, J. Meule, D. Holzer, M. Witek, V. Gross-Wilde, M. Witek, Genotoxic potential of selected cytostatic drugs in human and zebrafish cells, *Toxicol. Sci. Pollut. Res.* 7 (2016) 14796–14806.
- [17] A. Asquero, A.R. Collins, The essential cancer assay: a comprehensive guide to assessing DNA damage and repair, *Arch. Toxicol.* 87 (2014) 948–968.
- [18] M. Penach, M. Hirsch-Vadinou, A.Z. Tsampanis, J. Bourdieu, J.M. Chast, J. Ferry, M. Moraga, Th. Bascand, J.D. Tardieu, P. Thérèse, Molecular mechanisms of micronucleus, micronucleus bridge and nuclear body formation in mammalian and human cells, *Mutagenesis* 36 (2011) 121–132.
- [19] R.H. Morris, R.J. Ames, Revised methods for the *Salmonella mutagenicity test*, *Mutat. Res.* 113 (1983) 173–215.
- [20] C. Ghosh, Y.L. Zhou, P. Collins, Derivation and characterization of a metabolite liver cell line, *Cell Biol. Toxicol.* 10 (1994) 147–176.
- [21] T. Macmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and sprouting assays, *J. Immunol. Methods* 65 (1983) 55–63.
- [22] E.C. Deitz, J.L. Cohen, Morphological and biochemical assays of apoptosis, In: J.L. Collins, A.M. Brubaker (Eds.), Current Protocols in Immunology, Wiley, New York 1998, pp. 3–3.
- [23] K.P. Singh, M.P. McCoy, R.E. Tins, L.I. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, *Exp. Cell Res.* 779 (1984) 144–151.
- [24] M. Penach, The in vitro micronucleus technique, *Mutat. Res.* 455 (2000) 81–95.
- [25] M. Penach, A.M. Maraz, Measurement of micronuclei in lymphocytes, *Mutat. Res.* 147 (1985) 29–38.
- [26] M. Knapczyk, V. Gross-Wilde, T. Polak, M. Asquero, S. Albertini, D. Knapczyk, M. Knapczyk, M. Witek, P. Ziegler, K. Knapczyk, K. Knapczyk, J. Knapczyk, A. Knapczyk, A. Witek, Report from the in vitro micronucleus assay working group, *Mutat. Res.* 546 (2003) 153–163.

3.3 Influence of Selected Anti-cancer Drugs on the Induction of DNA Double-Strand Breaks and Changes in Gene Expression in Human Hepatoma HepG2 Cells

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In the present study the genotoxic potential of four widely used cytostatics (5-fluorouracil (5-FU), etoposide (ETO), cisplatin (CDDP) and imatinib mesylate (IM)) has been evaluated in terms of induction of double strand breaks (DSBs) and analysis of the changes in the expression of genes involved in the response to DNA damage (CDKN1A, GADD45A, MDM2), apoptosis (BAX, BCL2) and oncogenesis (MYC, JUN) in an experimental model with human hepatoma HepG2 cells.

After 24 h of exposure all four cytostatics at non-cytotoxic concentrations induced a significant increase in the formation of DNA double strand breaks (DSBs), with IM being the least effective. The analysis of the changes in the gene expressions showed that 5-FU, CDDP and ET upregulated the genes involved in DNA damage response, which is in line with their mechanism of action. On the contrary, IM did not change the mRNA level of the studied genes, showing a different mechanism of action that probably does not involve direct interaction with DNA processing.

Concentrations that caused genotoxic effects in nontarget HepG2 cells are close to those measured in treated patients during the therapy, therefore increased risk for development of delayed adverse effects in patients cannot be completely excluded. Because IM is not likely to directly interact with genetic material, exposure to IM during chemotherapy represents lower risk for delayed effects such as secondary cancer, reproductive effects and heritable diseases than chemotherapy with 5-FU, ET and CDDP.

The PhD candidate performed MTT assay and DSB detection with the gamma-H2AX assay. He also performed all cell treatments and mRNA expression analyses. He drafted the manuscript.

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DOI 10.1007/s11356-015-5420-8



FATE AND EFFECTS OF THE RESIDUES OF ANTICANCER DRUGS IN THE ENVIRONMENT

Influence of selected anti-cancer drugs on the induction of DNA double-strand breaks and changes in gene expression in human hepatoma HepG2 cells

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Abstract In chemotherapy, various anti-cancer drugs with different mechanisms of action are used and may represent different risk of undesirable delayed side effects in treated patients as well as in occupationally exposed populations. The aim of the present study was to evaluate genotoxic potential of four widely used anti-cancer drugs with different mechanisms of action: 5-fluorouracil (5-FU), cisplatin (CDDP) and etoposide (ET) that cause cell death by targeting DNA function and imatinib mesylate (IM) that inhibits targeted protein kinases in cancer cells in an experimental model with human hepatoma HepG2 cells. After 24 h of exposure all four anti-cancer drugs at non-cytotoxic concentrations induced significant increase in formation of DNA double strand breaks (DSBs), with IM being the least effective. The analysis of the changes in the expression of genes involved in the response to DNA damage (*CDKN1A*, *GADD45A*, *MDM2*), apoptosis (*BAX*, *BCL2*) and oncogenesis (*MYC*, *JUN*) showed that 5-FU, CDDP and ET upregulated the genes involved in

DNA damage response, while the anti-apoptotic gene *BCL2* and oncogene *MYC* were downregulated. On the contrary, IM did not change the mRNA level of the studied genes, showing different mechanism of action that probably does not involve direct interaction with DNA processing. Genotoxic effects of the tested anti-cancer drugs were observed at their therapeutic concentrations that may consequently lead to increased risk for development of delayed adverse effects in patients. In addition, considering the genotoxic mechanism of action of 5-FU, CDDP and ET an increased risk can also not be excluded in occupationally exposed populations. The results also indicate that exposure to 5-FU, CDDP and ET represent a higher risk for delayed effects such as cancer, reproductive effects and heritable disease than exposure to IM.

Keywords Anti-cancer drugs · Gene expression · Genotoxicity · H2AX · HepG2 cells · Therapeutic concentrations

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Introduction

Anti-cancer drugs that are used to treat cancer significantly contributed to the increased survival and better life quality of cancer patients and their use is rapidly growing, due to earlier diagnosis of the disease and due to increasing incidence of cancer (US National Institute of Health, www.cancer.gov). Many of these drugs are designed to act via interactions with DNA function and cell signalling and thus may not affect only cancer cells but also normal dividing cells. Consequently, many of these drugs are genotoxic, mutagenic, carcinogenic, teratogenic, and/or embryotoxic (Kummerer et al. 2000; Christensen 1998; Besse et al. 2012; Zoukova et al. 2010; Brezovsek et al. 2014; Parrella et al. 2014, 2015) and upon unintentional exposure may be dangerous for humans as well as other organisms.

The aim of the present study was to compare genotoxic potential of four widely used cytostatics at therapeutic doses, namely fluoropyrimidine 5-fluorouracil (5-FU), cisplatin (or cis-diamminedichloroplatinum; CDDP), etoposide (ET) and imatinib mesylate (IM) in the experimental model with human hepatoma HepG2 cells, which is extensively used in the *in vitro* cytotoxicity and genotoxicity studies (Viegas et al. 2012; Pezdirc et al. 2013; Straser et al. 2013; Zhao et al. 2013; Fang et al. 2012). The main advantage of the experimental model with HepG2 cells are retained inducibility and activities of several phase I and phase II xenobiotic metabolizing enzymes presented in human hepatocytes (Knausmuller et al. 2004). Another important advantage that makes these cells particularly suitable for genotoxicity studies is the expression of wild-type tumour suppressor TP53 (Bressac et al. 1990) which makes them an appropriate model also for the studies of P53-regulated response to DNA damage at the level of gene transcription and translation (van Delft et al. 2004).

According to their mechanisms of action, the selected compounds represent different classes of anti-cancer drugs. The pyrimidin analogue 5-FU belongs to the group of anti-metabolites. It blocks DNA synthesis and replication, by inhibition of thymidylate synthase and incorporation of its metabolites into DNA and RNA (Longley et al. 2003). CDDP belongs to the group of platinum-derived drugs. It causes the alkylation of DNA and DNA-DNA and DNA-protein cross-links that block DNA transcription and replication, which leads to cell death (Gonzalez et al. 2001). A topoisomerase inhibitor ET inhibits enzyme topoisomerase II and causes an increase in DNA and chromosomal breakage leading towards cell death (Pommier 2013). IM was the first of the protein kinase inhibitors, which were developed for targeted chemotherapy. IM does not target DNA but selectively inhibits certain oncogenic mutant protein tyrosine kinases, including the chimeric BCR-ABL fusion oncoprotein of chronic myeloid leukaemia, the transmembrane receptor KIT and platelet-derived growth factor

(PDGF) receptors (Buchdunger et al. 2000; Fabbro et al. 2002).

The cytotoxicity of the four drugs has been evaluated by the MTT assay whereas the genotoxic potential has been evaluated by analysing formation of phosphorylated H2AX histones (γ H2AX) in the exposed HepG2 cells. Formation of the γ H2AX is increasingly used in genotoxicity studies as a marker of double-stranded breaks (DSBs) induced by DNA-damaging compounds (Rogakou et al. 1999; Celeste et al. 2003) that also enables reliable discrimination of chemicals with genotoxic potential from non-genotoxins (Smart et al. 2011). To enable an insight into the understanding of possible mechanism of genotoxicity of the four tested drugs, we also studied alterations in the expression of selected genes involved in DNA damage response, apoptosis and oncogenesis that can provide an early and global answer to toxic events (Brown and Botstein 1999; Ellinger-Ziegelbauer et al. 2005; Ellinger-Ziegelbauer et al. 2009).

Materials and methods

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluoropyrimidine 5-fluorouracil (5-FU), cis-diamminedichloroplatinum (CDDP), dimethylsulphoxide (DMSO), methanol, glycogen and William's medium E were from Sigma (St. Louis, USA); etoposide (ET) and imatinib mesylate (IM) were from Santa Cruz Biotechnology (Dallas, USA); penicillin/streptomycin, foetal bovine serum (FBS), L-glutamine and phosphate-buffered saline (PBS) were from PAA Laboratories (Dartmouth, USA) and TRIzol reagent was from Invitrogen, Paisley, Scotland, UK. cDNA High Capacity Archive Kit, TaqMan Universal PCR Master Mix and the Taqman Gene Expression Assays were from Applied Biosystems, NJ, USA. Mouse monoclonal IgG1, Anti-phospho-Histone H2AX (Ser139) and FITC conjugate were from Milipore, Billerica, MA, USA. All other chemical reagents were of the purest grade available and all solutions were made using Milli-Q water.

For the selected anti-cancer drugs, stock solutions were prepared in DMSO (5-FU and ET) or sterile Milli-Q water (CDDP and IM) and were aliquoted and stored at -20°C .

Cell culture

HepG2 cells were a gift from Dr. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). The cells were grown at 37°C and 5 % CO_2 in William's medium E (Sigma, St. Louis, USA) containing 15 % foetal bovine serum, 2 mM L-glutamine and 100 U/mL penicillin/streptomycin.

Cell viability (cytotoxicity) assays

The cytotoxicity of anti-cancer drugs was determined with MTT assay, which measures the activity of mitochondrial succinate dehydrogenase, according to Mosmann (1983) with minor modifications. The HepG2 cells were seeded onto 96-well microplates (Nunc, Naperville, IL, USA) at a density of 4000 cells per well and incubated for 24 h at 37 °C and 5 % CO₂ to attach. The growth medium was replaced with fresh medium containing 0, 0.29, 0.59, 1.17, 2.34, 4.69, 9.38, 18.75, 37.5, 75 and 150 µg/mL of tested anti-cancer drugs and incubated for additional 24 h. At the end of the exposure to anti-cancer drugs, MTT was added to a final concentration of 0.5 mg/mL and the cells were incubated for additional 3 h at 37 °C. Afterwards, the medium was removed and formazan crystals were dissolved in DMSO. The optical density (OD) was measured at 570 nm (reference filter 690 nm) with microplate spectrofluorimeter (SynergyMx, BioTek, Winooski, USA). The difference OD₅₇₀–OD₆₉₀ is proportional to the concentration of formazan, which directly correlates to the number of the viable cells. The viability was measured in at least three independent experiments each time with five replicates per treatment point.

DSB detection–γH2AX foci analysis

The cells were seeded on T25 plates (Corning Costar Corporation, Corning, NY, USA) at a density of 0.8×10^6 per plate, left to attach overnight and subsequently exposed to anti-cancer drugs (0.0001, 0.001, 0.01, 0.1 and 1 µg/mL) for 24 h. In each experiment, a vehicle control (DMSO in case of 5-FU and ET or sterile Mill-Q water in case of CDDP and IM) was included. According to the manufacturer's protocol, an etoposide (1 µg/mL) as a positive control was used. At the end of the exposure, floating and adherent cells were collected by trypsinization. For the fixation, the cells were centrifuged at 115g, 4 °C for 5 min, washed twice with ice-cold PBS, resuspended in 0.5-mL cold PBS and ethanol (1.5 mL) was added drop wise into the cell pellet, while vortexing. The cells were fixed at 4 °C overnight and stored at –20 °C till analysis. Fixed cells were centrifuged at 259g for 10 min, washed twice with ice-cold 1× PBS, resuspended in 0.5-mL 1× PBS containing 2000-fold diluted anti-γH2AX antibodies, mixed and incubated at 4 °C for 30 min in the dark. Labelled cells were then washed twice and resuspended in 0.3 mL of 1× PBS. Flow cytometric analysis was carried out on a FACSCalibur flow cytometer (BD Biosciences Pharmingen™, San Diego, CA, USA) as described by Straer et al. (2013). FITC intensity, corresponding to DSBs, was detected in the FL1-H channel. In each sample 10^4 events were recorded. Independent experiments were repeated three times.

For further analysis, the raw data (FITC intensities of each cell, FL1-H intensity), obtained from the CellQuest Pro

software (BD Biosciences), was converted from the .fcs to the .csv format, using the program FCSExtract, which is available on the following website: <http://research.stowers-institute.org/efg/ScientificSoftware/Utility/FCSExtract/index.htm>.

Gene expression analysis

Cells were seeded on T-25 plates (Corning Costar Corporation, Corning, NY, USA) at a density of 10^6 cells per plate and incubated for 24 h at 37 °C and 5 % CO₂ in humidified atmosphere. The cells were washed with 1× PBS, exposed to non-cytotoxic concentrations (0.00001, 0.01 and 1 µg/mL) of anti-cancer drugs and incubated for 24 h. After the incubation, cells were washed twice with 1× PBS and total RNA was isolated using TRIzol reagent according to the manufacturer's protocol with minor modifications. Glycogen (20 µg/mL) was added to the cell lysate. The RNA was incubated with isopropyl alcohol overnight at –20 °C to precipitate. All solutions needed for RNA isolation were prepared in RNase-free water. Independent experiments were performed in duplicates and repeated three times.

The RNA was transcribed to cDNA using 1 µg of total RNA and cDNA High Capacity Archive Kit, according to the manufacturer's protocol. Gene expression was quantified by RT-qPCR using TaqMan Universal PCR Master Mix and the following Taqman Gene Expression Assays: *MDM2* (*MDM2* proto-oncogene, E3 ubiquitin protein ligase), Hs00234753_m1; *GADD45A* (growth arrest and DNA damage-inducible gene, alpha), Hs00169255_m1 and *CDKN1A* (cyclin-dependent kinase inhibitor 1A) Hs00355782_m1; *BAX* (*BCL2* associated X protein) Hs99999001_m1; *BCL2* (B-cell CLL/lymphoma 2), *JUN* (jun proto-oncogene; Hs99999141_s1) and *MYC* (v-myc avian myelocytomatosis viral oncogene homolog, Hs00153408_m1). *GAPDH* was used as a reference gene. Reactions (in triplicates) were set in Viia7 real-time PCR system (Applied Biosystems, USA) according to manufacturer's protocols. Genomic DNA contamination was controlled by no-RT controls. The standard curve method was used for relative gene expression quantification and the transcript accumulation of each gene was normalized to *GAPDH* gene expression. Target gene expression in treated cultures was divided by average expression of vehicle control samples for each treatment separately.

Statistical analysis

Logistic regression was used in to assess any dose-dependent response for 5-FU, CDDP, ET or IM and possible differences in viability between the drugs 24 h after submission. The half maximal inhibitory concentration (IC₅₀) that represents the concentration of a chemotherapeutic agent that is required

for 50 % inhibition of growth in vitro was determined for each compound using logistic regression coefficients. Besides determining the IC_{50} , logistic regression was used in order to calculate the odds, which are the measure of likelihood for the cells dying taking into consideration the increasing concentrations of different chemicals. The odds are expressed as the ratio of the probability of the event happening (i.e. cells dying) against the probability of the event not happening (i.e. cells surviving). For the γ H2AX foci analysis, the statistical significance between treated groups and the vehicle control was determined with a linear mixed-effects model. The model enabled us to take into account the individual differences between individual replicates. Calculations were done with the statistical program R (R Development Core Team) and its packages reshape (Wickham 2007), ggplot2 (Wickham 2009), MASS (Venables and Ripley 2002) and nlme (Pinheiro et al. 2012). To compare the genotoxic potential of the tested drugs, the relative increase of the γ H2AX mean fluorescence of the exposed cells over that of the negative control cells was calculated and is expressed as induction factor (IF). For the gene expression, statistical significance between treated groups and control was determined by two-tailed Student's *t* test with GraphPad Prism 5 (GraphPad software).

Results

Cell viability

The viability of HepG2 cells after the exposure to 5-FU, CDDP, ET and IM for 24 h was determined by MIT assay. At the tested concentration range, CDDP, ET and IM reduced cell viability in a dose-dependent manner (Fig. 1), while 5-FU was not cytotoxic. The IC_{50} values (Table 1) show that the most cytotoxic was CDDP followed by IM and ET. From the shapes of the dose-response curves, it can be seen that CDDP and ET reduced cell viability already at the lowest tested concentration. With increasing concentrations of the drugs, the reduction of cell viability gradually increased. This is reflected in relatively low viability odds (Table 1), which is the ratio of the probability of cells dying against the probability of the cells surviving. IM at the lowest concentrations did not significantly affect cell viability whereas at concentration $>10 \mu\text{g/L}$, the toxicity rapidly increased with increasing concentration. This is reflected in significantly higher viability odds of IM compared to ET and CDDP (Table 1).

Formation of γ H2AX

The induction of DSBs by the selected anti-cancer drug in HepG2 cells was determined within the range of non-cytotoxic concentrations (0.0001–1 $\mu\text{g/mL}$) indirectly

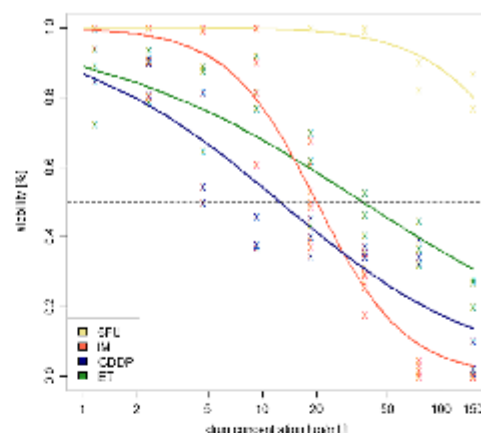


Fig. 1 The dose-response curve representing the biological response (% of viable cells) to increasing concentrations (0, 0.29, 0.59, 1.17, 2.34, 4.69, 9.38, 18.75, 37.5, 75 and 150 $\mu\text{g/mL}$) of four anti-cancer drugs—fluoropyrimidine 5-fluorouracil (5-FU), etoposide (ET), cisplatin (CDDP) and imatinib mesylate (IM). Each experiment was repeated at least three times and is represented with a symbol (x) on the plot. The dose-response relationship was fitted using logistic regression and IC_{50} values were calculated from the fitted regression line for each chemical separately (represented with colours as indicated in the figure legend). The viability ratio of 0.5 (50 % viable cells) is represented with a dashed line. Concentration of chemicals is represented on log-scale

through the detection of γ H2AX foci by measuring the fluorescent signal of individual cells using flow cytometry. 5-FU and CDDP induced significant increase in γ H2AX foci at the highest two concentrations (1 and 0.1 $\mu\text{g/L}$), while in cells exposed to ET and IM, significant increase in γ H2AX signal was detected only at the highest tested concentration (1 $\mu\text{g/mL}$) (Fig. 2). Based on the IF (induction factor) calculations, 5-FU, CDDP and ET showed comparable potency of γ H2AX induction, while IM showed only marginal induction of γ H2AX foci (Table 2).

Table 1 IC_{50} values of 5-fluorouracil (5-FU), etoposide (ET) cisplatin (CDDP) and imatinib mesylate (IM) in human hepatoma (HepG2) cells after 24 h of exposure

Anti-cancer drug	IC_{50} (95 % confidence interval) $\mu\text{g/mL}$	The viability odds ^a
5-FU	>150	n.d.
CDDP	15.54 (8.91–17.65)	1.5
ET	35.59 (25.88–48.93)	5.5
IM	23.63 (18.72–29.83)	16.3

^aInterpreted as 'A decrease of odds of cell surviving at an increase of compound concentration for 1 $\mu\text{g/mL}$ '

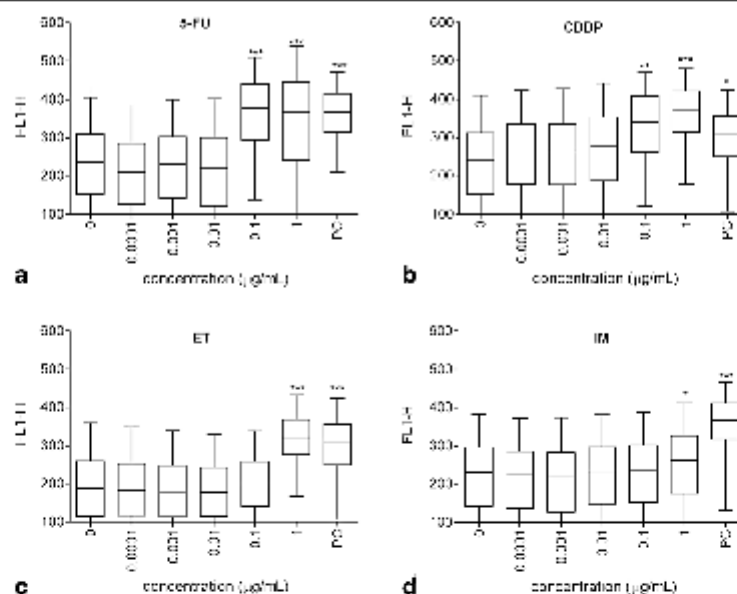


Fig. 2 Induction of double-strand breaks (DSBs) by fluoropyrimidine 5-fluorouracil (5-FU) (a), cisplatin (or cis-diamminedichloroplatinum; CDDP) (b), etoposide (ET) (c) and imatinib mesylate (IM) (d). HepG2 cells were incubated for 24 h with anti-cancer drugs (0.0001, 0.001, 0.01, 0.1 and 1 µg/mL), and the presence of DSBs was analysed by flow cytometry, indirectly through the detection of γ H2AX foci. In each experiment, a vehicle control (0) and a positive control (PC, 1 µg/mL

etoposide, 24 h) were included. Distribution of the fluorescent signals of individual cells in the samples is presented as quantile box plots. The experiments were repeated three times and 10^4 events were recorded in each sample. Significant differences between anti-cancer drug-treated cells and the vehicle control (0) is indicated by * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

Effect of anti-cancer drugs on the expression of the selected genes

The expressions of DNA damage response genes *CDKN1A*, *GADD45A* and *MDM2* were significantly upregulated in cells exposed to 1 µg/mL of 5-FU and CDDP, while ET upregulated expression of *CDKN1A* and *GADD45A*, but not *MDM2* (Fig. 3). The expression of pro-apoptotic gene (*BAX*) was not affected by exposure to the tested drugs whereas the expression of the anti-apoptotic gene *BCL2* was downregulated in cells exposed to 1 µg/mL of 5-FU, CDDP and ET (Fig. 3). The expression of the oncogene *JUN* was upregulated only in cells exposed to 5-FU, while *MYC* oncogene was downregulated by 5-FU, CDDP and ET. No changes in the expression of the selected genes were observed in cells exposed to IM (Fig. 3).

Discussion

In this study, we evaluated cytotoxic and genotoxic potential of four anti-cancer drugs (5-FU, CDDP, ET and IM) with

different mechanisms of action in HepG2 cells. The results of cytotoxicity testing showed that the most cytotoxic was CDDP followed by IM and ET, while 5-FU did not affect viability of HepG2 cells at tested concentrations. The results obtained with CDDP, ET and 5-FU are in agreement with previous studies with HepG2 cells as well as with squamous

Table 2 Induction of γ H2AX fluorescence by 5-fluorouracil (5-FU), etoposide (ET) cisplatin (CDDP) and imatinib mesylate (IM) in human hepatoma (HepG2) cells after 24 h of exposure expressed as induction factor (IF)

	5-FU		CDDP		ET		IM	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
NC	1.00	0.23	1.00	0.17	1.00	0.06	1.00	0.01
0.1	0.95	0.22	1.11	0.11	1.15	0.15	0.98	0.05
1	1.04	0.19	1.11	0.18	1.12	0.11	0.96	0.04
10	0.96	0.28	1.16	0.19	1.10	0.13	1.03	0.08
100	1.65	0.18	1.39	0.22	1.22	0.13	1.05	0.11
1000	1.56	0.12	1.53	0.13	1.90	0.15	1.15	0.12

IF = mean γ H2AX fluorescence in exposed cells/mean γ H2AX fluorescence in control cells

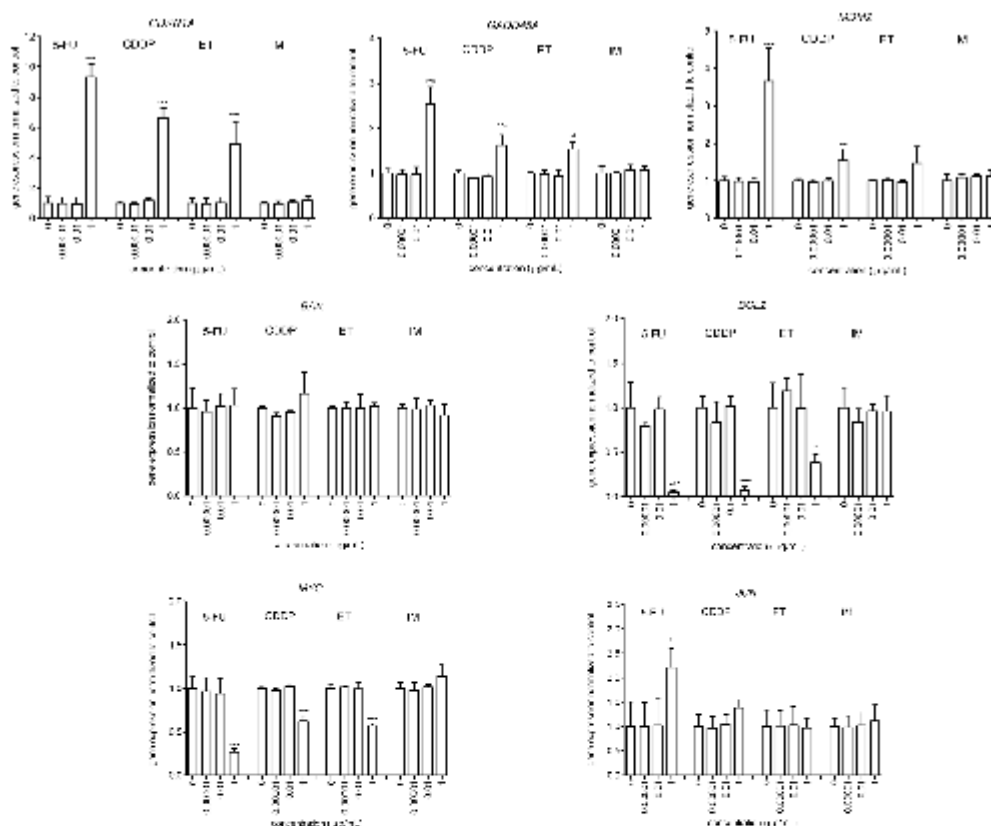


Fig. 3 Changes in the expression of the genes involved in DNA damage response (*CDKN1A*, *GADD45A* and *MDM2*), oncogenesis (*MYC* and *JUN*) and apoptosis (*BCL2* and *BAX*) after the exposure of HepG2 cells to fluoropyrimidine 5-fluorouracil (5-FU), etoposide (ET), cisplatin (or cis-diamminedichloroplatinum; CDDP) and imatinib mesylate (IM)

(0.0001, 0.01 and 1 µg/mL) for 24 h. Relative gene expression (normalized to average gene expression in vehicle controls±SD; n=3) is shown. Significant differences between anti-cancer drug-treated cells and the vehicle control (0) is indicated by **p*<0.05, ***p*<0.01 and ****p*<0.001

cell carcinoma cell line and isolated human peripheral blood lymphocytes in which CDDP had the greatest cytotoxic potential followed by ET and 5-FU (Shao et al. 2008; Okamura et al. 2004; Gajski et al. 2015). Interestingly, the cytotoxicity of IM was in our study comparable to that of ET and CDDP although HepG2 cells do not express the specific kinase targets of IM. However, IM inhibits several kinases (i.e. c-ABL, c-KIT, PDGFR) (Fabarius et al. 2005, 2007; Deininger et al. 2005; Karaman et al. 2008) as well as some non-kinase targets (i.e. oxidoreductase NQO2) (Bartscheff et al. 2007), that in normal cells function as physiological proteins. This explains the observed cytotoxicity of IM in the non-target HepG2 cells.

The genotoxic potential was evaluated in terms of induction of DSBs at low concentrations (0.0001–1 µg/mL) that are not cytotoxic to HepG2 cells. The results showed that 5-FU,

CDDP and ET, which belong to the classical anti-cancer drugs that directly interact with DNA function, induced formation of γH2AX foci. Similar observations were reported also by other researchers, who demonstrated that 5-FU (Ikeda et al. 2010; Matsuzaki et al. 2010), CDDP (Clingen et al. 2008; Huang et al. 2004; Ikeda et al. 2010; Matsuzaki et al. 2010; Olive and Banath 2009) and ET (Smart et al. 2008; Matsuzaki et al. 2010; Muslimovic et al. 2009) can induce phosphorylation of histone H2AX in different cell lines. All three drugs also induce chromosomal aberrations in vitro (Gajski et al. 2015).

Exposure of HepG2 cells to IM, a selective inhibitor of specific tyrosine kinases that are overexpressed in certain types of cancer induced at the highest tested concentration a slight although significant increase in the formation of γH2AX foci. Previously, it has been shown that IM can

induce phosphorylation of H2AX in cells expressing specific targets for IM (PDGFR, BCR-ABL, c-KIT) (Liu et al. 2007; Dong et al. 2014; Zhang et al. 2012), whereas it did not induce γ H2AX foci in human colon cancer isogenic cell lines (Ferrandiz et al. 2009). Furthermore, Czechowska et al. (2005) demonstrated that IM induced DNA damage (DNA alkali-labile sites and strand breaks) only in leukemic cells expressing specific mutated tyrosine kinase targets (BCR/ABL) but not in normal lymphocytes. On the other hand, it has been reported that IM induced chromosome and centrosome aberrations in human and mammalian normal cells (Fabarius et al. 2005, 2007), formation of micronuclei and reduced fertility in higher plants (Pichler et al. 2014). It has been hypothesized that one or more ABL-related tyrosine kinases, which are inhibited by IM play a role in DNA repair processes and in the control of cell cycle progression and may affect the ubiquitination of centrosomal components leading to extra centrosomal duplication and mitotic spindle catastrophe (Fabarius et al. 2005, 2007) indicating aneuploid potential of IM.

To further elucidate the mechanisms of genotoxic activity of the tested drugs, we have measured the changes in the expression of the genes involved in DNA damage response (*CDKN1A*, *GADD45A*, *MDM2*), apoptosis (*BAX*, *BCL2*) and oncogenesis (*MYC*, *JUN*) at the same time point as the formation of γ H2AX foci was determined. Expression of *CDKN1A*, *GADD45A* and *MDM2* have been shown to be specifically upregulated by genotoxic carcinogens (Elinger-Ziegelbauer et al. 2005) and have been successfully used as molecular markers of genotoxic activity (Zegura et al. 2008; Petkovic et al. 2011; Pezdir et al. 2013; Blagus et al. 2014). *CDKN1A* (p21) is P53 downstream regulated inhibitor of cyclin-dependent kinases (CDKs) that inhibits the cell cycle at the G1–S and G2–M transition, until DNA damage can be repaired or apoptosis is initiated (Vogelstein et al. 2000; Michael and Oren 2003). In addition to that, *CDKN1A* is directly involved in DNA repair, including NER (Cazzalini et al. 2010). The *GADD45A* gene can be induced by DNA-damaging agents and growth arrest signals through P53-dependent or P53-independent pathways (Zhan 2005). It plays a role in the control of cell cycle G2–M checkpoint, the DNA repair process and apoptosis (Zhan 2005; Jin et al. 2000; Wang et al. 1999). *MDM2* is E3 ubiquitin ligase that mediates ubiquitination of P53 and its proteasomal degradation and it is upregulated by elevated levels of p53 (Vogelstein et al. 2000; Michael and Oren 2003). *MDM2* enhances the tumorigenic potential of the cells and promotes cell survival and cell cycle progression (Deb 2003). The expression of *CDKN1A* and *GADD45A* has been upregulated by 5-FU, CDDP and ET, meanwhile the expression of *MDM2* was upregulated by 5-FU and CDDP, with 5-FU being the most potent inducer. These results corroborate with several other studies in which upregulation of these genes as well as proteins by these drugs

has been detected in different cells (Kondo et al. 1995; Hershberger et al. 2002; Delmastro et al. 1997; Smith et al. 1996; Vaskivuo et al. 2006; Sermeus et al. 2008; de Angelis et al. 2006; Malmlof et al. 2008).

In response to DNA damage, P53 regulates apoptosis by regulating the transcription of anti- and pro-apoptotic genes of BCL2 family (Adams and Cory 2007; Perfettini et al. 2002; Hanahan and Weinberg 2011). The anti-apoptotic BCL2 family members such as *BCL2* and *BCL2L1* maintain cell survival by inhibiting pro-apoptotic BCL2 family members such as *BAX* and *BAK* (Youle and Strasser 2008). In HepG2 cells, 5-FU, CDDP and ET decreased mRNA level of the anti-apoptotic gene *BCL2*, while the expression of *BAX* was not affected. Similar result was reported by Singh et al. (2006) who showed that exposure to 5-FU and carboplatin in HPV-positive carcinoma downregulated of *BCL2* protein without affecting the level of pro-apoptotic *BAX* protein. The ratio of death agonist BAX to antagonist BCL2 determines whether a cell will respond to an apoptotic signal (Laffon et al. 2001; Woo et al. 2000; Zegura et al. 2008). In our study, the ratio was increased indicating that exposure to these drugs can activate apoptosis cascade. The result is in line with several previous studies that showed that exposure of cells to 5-FU, CDDP and ET increased the ratio of *BAX/BCL2* expression (Liu et al. 2013; Sawada et al. 2000; Singh et al. 2006).

It has been shown that the cellular oncogene *MYC* could be the preferential target of some anti-cancer drugs in human tumour cell lines (reviewed in Pourquier et al. 1998). Genes of the *MYC* family genes are key regulators of cell proliferation, and their deregulation contributes to the genesis of most human tumours (Adhikary and Eilers 2005; Meyer and Penn 2008). It was shown that mouse *jun* can protect hepatocytes from apoptosis, as hepatocytes lacking *jun* showed increased sensitivity to TNF α -induced apoptosis indicating that c-jun antagonizes the pro-apoptotic activity of P53 in liver tumour (Eferl et al. 2003). The results of our study showed the upregulation of *JUN* after the treatment with 5-FU and downregulation of *MYC* after the treatment with 5-FU, CDDP and ET. Downregulation of *MYC* at transcriptional and translational level after the induction of DNA damage by 5-FU, CDDP and ET was already reported for different cell lines (Vaskivuo et al. 2006; Zhao et al. 2008; Pourquier et al. 1998) and has been suggested to generate a signal leading to programmed cell death (Pourquier et al. 1998).

Contrary to 5-FU, CDDP and ET, IM at non-cytotoxic concentrations did not change the mRNA levels of the studied P53 regulated DNA damage responsive and apoptotic genes, neither it changed the expression of proto-oncogenes in HepG2 cells. Although it induced a slight increase in the formation of γ H2AX foci, these results point out to a different molecular mechanism involved in its potential genotoxic activity. On the other hand, microarray analysis showed that IM at high concentration (6 μ g/mL) in target gastrointestinal

stromal tumour cells (GIST-T1) affected the expression of *MDM2*, *MYC* and *JUN* genes and upregulated the DNA damage response regulator gene *p53*, suggesting DNA damage response, that was confirmed with the comet assay and immunoblotting of H2AX (Ochs et al. 2009). In patients with Ph-positive chronic myeloid leukaemia, IM at a therapeutic dose of 400 mg/day had no uniform influence on gene expression patterns in their normal (non-target) Ph-negative progenitor cells (Neumann et al. 2005); therefore, the molecular mechanisms by which IM affects genomic and genetic stability in normal cells remain unknown.

The cell death is essential process for elimination of the cells that have suffered serious damage, such as DSBs, if this damage is not repaired. Thus, the cell death it is an important defence against the emergence of cancer and other diseases. Therefore, the chemicals with high genotoxicity and low cytotoxicity display a higher probability to cause certain diseases, since the damage to DNA is an initial event in the pathogenesis of disease. Such chemical is 5-FU, which showed in our study the highest genotoxic potential and the lowest cytotoxicity (hypothetically higher survival of damaged cells), among all tested anti-cancer drugs.

Our study showed that all studied anti-cancer drugs expressed genotoxic activity at concentrations, which are in the range of measured concentrations of these drugs in the plasma of treated patients. In some cases, plasma concentration of the anti-cancer drugs can reach even a higher level than those used in our study (reviewed in Schulz and Schmoldt 2003; Urien and Lokiec 2004). Therefore, patients receiving therapeutic doses of these drugs could, besides well-known short-term side effects, also suffer from long-term and delayed side effects such as secondary cancer or reproductive disorders that can be expressed later in the life of patients. The most indicative information that corroborates the hypothesis of delayed effect after exposure to anti-cancer drugs originates from surveys of survivors of childhood cancer treatment. In patients surviving 30 years after primary diagnosis, the adverse outcomes such as subsequent malignant neoplasm, cardiopulmonary dysfunction, avascular necrosis, endocrinopathies and neurocognitive disorders were associated with therapeutic exposures (Landier et al. 2015). In addition, several epidemiological studies showed a correlation between occupational exposure of health care workers to anti-cancer drugs and chromosomal damage in lymphocytes (Kopjar et al. 2009) as well as in exfoliated buccal epithelial cells (Cavallo et al. 2005). These studies also indicate a potential increased risk for genotoxicity-related disease and disorders, even at secondary exposure to much lower concentrations of these drugs. Although the information regarding the concentrations of anti-cancer drugs in body fluids in occupationally exposed personnel is limited, the maximal measured concentration of 5-FU metabolite (α -fluoro- β -alanine) in the urine of a nurse administering the drug (1140 μ g/L; 1.14 μ g/

mL) (Cavallo et al. 2005) was even higher from the highest concentration tested in our study.

The results from the present study show that 5-FU, CDDP, ET and IM exert different cytotoxic activity in HepG2 cells. The most cytotoxic was CDDP, followed by IM and ET, while 5-FU was not cytotoxic at the tested concentrations. At the non-cytotoxic concentrations, 5-FU, CDDP and ET all directly interfered with DNA processing induced DSBs and also showed a pattern of the changes in the expression of DNA damage, apoptosis and proto-oncogenes that is typical for genotoxic compounds. On the contrary, IM at non-cytotoxic concentration only slightly induced formation of DSBs and had no effect on the expression of selected genes, showing different mechanism of action that most probably does not involve direct interaction with DNA processing. These results indicate that exposure of patients to therapeutic doses or unintentionally exposed medical staff to low concentrations, however chronically, to these drugs represent an increased risk for the development of delayed adverse effects.

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Conflict of interest The authors declare that they have no competing interests.

References

- Adams JM, Cory S (2007) The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 26:1324–1337
- Adhikary S, Eilers M (2005) Transcriptional regulation and transformation by MYC proteins. *Nat Rev Mol Cell Bio* 6:635–645
- Bantscheff M, Eberhard D, Abraham Y, Bastuck S, Boesche M, Hobson S, Mathieson T, Perin J, Rada M, Rau C, Reader V, Sweetman G, Bauer A, Bourmeester T, Hopf C, Kruse U, Neubauer G, Ramsden N, Rick J, Kuster B, Drewes G (2007) Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat Biotechnol* 25:1035–1044
- Besse JP, Latour JF, Garric J (2012) Anticancer drugs in surface waters. What can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? *Environ Int* 39:73–86
- Blagus T, Zager V, Cemazar M, Sersa G, Kamernsek U, Zegum B, Nunic J, Filipic M (2014) A cell-based biosensor system HepG2CDKN1A-DsRed for rapid and simple detection of genotoxic agents. *Biosens Bioelectron* 61:102–111
- Bressan B, Galvin KM, Liang TJ, Iselbacher KJ, Wands JR, Ostroff M (1990) Abnormal structure and expression of P53 gene in human hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 87:1973–1977
- Brezovsek P, Elerssek T, Filipic M (2014) Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Synechococcus leopoliensis*. *Water Res* 52:168–177
- Brown PO, Botstein D (1999) Exploring the new world of the genome with DNA microarrays. *Nat Genet* 21:33–37

- Buchdunger E, Cioffi CL, Law N, Stover D, Ohno-Jones S, Druker BJ, Lydon NB (2000) Abl protein-tyrosine kinase inhibitor STI571 inhibits *in vitro* signal transduction mediated by c-Kit and platelet-derived growth factor receptors. *J Pharmacol Exp Ther* 295:139–145
- Cavallo D, Ursini CL, Pemiconi B, Di Francesco A, Giglio M, Rubino FM, Marinaccio A, Iavicoli S (2005) Evaluation of genotoxic effects induced by exposure to antineoplastic drugs in lymphocytes and exfoliated buccal cells of oncology nurses and pharmacy employees. *Mutat Res-Gen Tox En* 587:45–51
- Cazzalini O, Scovassi AI, Savio M, Stivala LA, Prosperi E (2010) Multiple roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response. *Mutat Res-Rev Mutat* 704:12–20
- Celeste A, Fernandez-Capetillo O, Knahlek MJ, Fitch DR, Staudt DW, Lee A, Bonner RF, Bonner WM, Nussenzweig A (2003) Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat Cell Biol* 5:675–U51
- Christensen FM (1998) Pharmaceuticals in the environment—a human risk? *Regul Toxicol Pharm* 28:212–221
- Clingen PH, Wu JYH, Miller J, Mistry N, Chin F, Wynne P, Price KM, Hartley JA (2008) Histone H2AX phosphorylation as a molecular pharmacological marker for DNA interstrand crosslink cancer chemotherapy. *Biochem Pharmacol* 76:19–27
- Czechowska A, Poplawski T, Drzewoski J, Blasink J (2005) Imatinib (STI571) induces DNA damage in BCR/ABL-expressing leukemic cells but not in normal lymphocytes. *Chem Biol Interact* 152:139–50
- De Angelis PM, Svendsrud DH, Knvik KL, Stokke T (2006) Cellular response to 5-fluorouracil (5-FU) in 5-FU-resistant colon cancer cell lines during treatment and recovery. *Mol Cancer* 5:20
- Deb SP (2003) Cell cycle regulatory functions of the human oncoprotein MDM2. *Mol Cancer Res* 1:1009–1016
- Delmastro DA, Li J, Vaisman A, Solle M, Chaney SG (1997) DNA damage-inducible gene expression following platinum treatment in human ovarian carcinoma cell lines. *Cancer Chemother Pharmacol* 39:245–53
- Deininger M, Buchdunger E, Druker BJ (2005) The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood* 105(7):2640–53
- Dong YQ, Xiong M, Duan LN, Liu Z, Niu TH, Luo Y, Wu XP, Xu CS, Lu CR (2014) H2AX phosphorylation regulated by p38 is involved in Bim expression and apoptosis in chronic myelogenous leukemia cells induced by imatinib. *Apoptosis* 19:1281–1292
- Eferl R, Ricci R, Kenner L, Zenz R, David JP, Rath M, Wagner EF (2003) Liver tumor development: c-Jun antagonizes the proapoptotic activity of p53. *Cell* 112:181–192
- Ellinger-Ziegelbauer H, Stuart B, Wahle B, Bomann W, Ahr HJ (2005) Comparison of the expression profiles induced by genotoxic and nongenotoxic carcinogens in rat liver. *Mutat Res-Fund Mol M* 575:61–84
- Ellinger-Ziegelbauer H, Aubrecht J, Kleinjans JC, Ahr HJ (2009) Application of toxicogenomics to study mechanisms of genotoxicity and carcinogenicity. *Toxicol Lett* 186:36–44
- Fabianus A, Giehl M, Frank O, Duesberg P, Hochhaus A, Hehlmann R, Seifarth W (2005) Induction of centrosome and chromosome aberrations by imatinib *in vitro*. *Leukemia* 19:1573–1578
- Fabianus A, Giehl M, Frank O, Spiess B, Zheng C, Muller MC, Weiss C, Duesberg P, Hehlmann R, Hochhaus A, Seifarth W (2007) Centrosome aberrations after nilotinib and imatinib treatment *in vitro* are associated with mitotic spindle defects and genetic instability. *Brit J Haematol* 138:369–373
- Fabbro D, Ruetz S, Buchdunger E, Cowan-Jacob SW, Fendrich G, Liebetanz J, Mestan J, O'Reilly T, Traxler P, Chaudhuri B, Fretz H, Zimmermann J, Meyer T, Caravatti G, Furet P, Manley PW (2002) Protein kinases as targets for anticancer agents: from inhibitors to useful drugs. *Pharmacol Therapeut* 93:79–98
- Fang L, Zhang HB, Li H, Fu Y, Yang GS (2012) miR-548c-3p inhibits proliferation and migration and promotes apoptosis in CD90(+) HepG2 cells. *Radiol Oncol* 46:233–241
- Fernandez N, Martin-Perez J, Blanco R, Donertas D, Weber A, Eilers M, Dotto P, Delgado MD, Leon J (2009) HCT116 cells deficient in p21(Waf1) are hypersensitive to tyrosine kinase inhibitors and adriamycin through a mechanism unrelated to p21 and dependent on p53. *DNA Repair* 8:390–399
- Gajski G, Geric M, Zegam B, Novak M, Nunic J, Bajrektarevic D, Ganj-Vrhovac V, Filipic M (2015) Genotoxic potential of selected cytostatic drugs in human and zebrafish cells. *Environ Sci Pollut Res Int*. doi:10.1007/s11356-015-4592-6
- Gonzalez VM, Fuentes MA, Alonso C, Perez JM (2001) Is cisplatin-induced cell death always produced by apoptosis? *Mol Pharmacol* 59:657–663
- Hershberger PA, McGuire TF, Yu WD, Zukowski EG, Schellens JHM, Egorin MJ, Trump DL, Johnson CS (2002) Cisplatin potentiates 1, 25-dihydroxyvitamin D-3-induced apoptosis in association with increased mitogen-activated protein kinase kinase 1 (MEKK-1) expression. *Mol Cancer Ther* 1:821–829
- Huang X, Okafuji M, Traganos F, Luther E, Holden E, Darzynkiewicz Z (2004) Assessment of histone H2AX phosphorylation induced by DNA topoisomerase I and II inhibitors to topotecan and mitoxantrone and by the DNA cross-linking agent cisplatin. *Cytom Part A* 58A:99–110
- Ikeda M, Kurose A, Takatori E, Sugiyama T, Traganos F, Darzynkiewicz Z, Sawai T (2010) DNA damage detected with gamma H2AX in endometrioid adenocarcinoma cell lines. *Int J Oncol* 36:1081–1088
- Jin SQ, Antinore MJ, Lung FT, Dong X, Zhao HC, Fan FY, Colchagie AB, Blanck P, Roller PP, Fornace AJ, Zhan QM (2000) The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. *J Biol Chem* 275:16602–16608
- Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri B, Davis ML, Edeen PT, Faraoni R, Floyd M, Hunt JP, Lockhart DJ, Milanov ZV, Morrison MJ, Pallares G, Patel HK, Pritchard S, Wodicka LM, Zarrinkar PP (2008) A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol* 26:127–132
- Knasmüller S, Mersch-Sundermann V, Kevekordes S, Darmoudi F, Huber WW, Hoelzl C, Bichler J, Majer BJ (2004) Use of human-derived liver cell lines for the detection of environmental and dietary genotoxins: current state of knowledge. *Toxicology* 198:315–328
- Kondo S, Bameet GH, Ham H, Morimura T, Takeuchi J (1995) Mdm2 protein confers the resistance of a human glioblastoma cell-line to cisplatin-induced apoptosis. *Oncogene* 10:2001–2006
- Kopjar N, Ganj-Vrhovac V, Kasuba V, Rozgaj R, Ramic S, Pavlica V, Zeljenic D (2009) Assessment of genotoxic risks in Croatian health care workers occupationally exposed to cytotoxic drugs: a multi-biomarker approach. *Int J Hyg Envir Heal* 212:414–431
- Kummerer K, Al-Ahmad A, Bertram B, Wiesler M (2000) Biodegradability of antineoplastic compounds in screening tests: influence of glucosidation and of stereochemistry. *Chemosphere* 40:767–773
- Laffon B, Pasaro E, Mendez J (2001) Effects of styrene-7,8-oxide over p53, p21, bcl-2 and bax expression in human lymphocyte cultures. *Mutagenesis* 16:127–132
- Landier W, Armenian S, Bhatia S (2015) Late effects of childhood cancer and its treatment. *Pediatr Clin N Am* 62:275–+
- Liu CL, Lin YP, Hu ML (2013) Fucosanthin enhances cisplatin-induced cytotoxicity via NF kappa B-mediated pathway and downregulates DNA repair gene expression in human hepatoma HepG2 cells. *Mar Drugs* 11:50–66
- Liu Y, Tseng M, Pestreau SA, Rossi F, Antonescu C, Besmer P, Fletcher JA, Duensing S, Duensing A (2007) Histone H2AX is a mediator of

- gastrointestinal stromal tumor cell apoptosis following treatment with imatinib mesylate. *Cancer Res* 67:2685–2692
- Longley DB, Harkin DP, Johnston PG (2003) 5-Fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 3:330–338
- Malmfö M, Pajjarvi G, Hogberg J, Stenius U (2008) Mdm2 as a sensitive and mechanistically informative marker for genotoxicity induced by benzo[a]pyrene and dibenzo[a,h]pyrene. *Toxicol Sci* 102:232–240
- Matsuzaki K, Harada A, Takeiri A, Tanaka K, Mishima M (2010) Whole cell-ELISA to measure the gamma H2AX response of six aneugens and eight DNA-damaging chemicals. *Mutat Res-Gen Tox En* 700: 71–79
- Meyer N, Penn LZ (2008) MYC—timeline reflecting on 25 years with MYC. *Nat Rev Cancer* 8:976–990
- Michael D, Oren M (2003) The p53-Mdm2 module and the ubiquitin system. *Semin Cancer Biol* 13:49–58
- Muslimovic A, Nystrom S, Gao Y, Hammarsten O (2009) Numerical analysis of etoposide induced DNA breaks. *PLoS One* 4
- Neumann F, Teutsch N, Kliszewski S, Bork S, Bross B, Schimkus N, Roes N, Gerding U, Hildebrandt B, Royer-Pokora B, Eiks R, Gattermann N, Haas R, Krennert R (2005) Gene expression profiling of Philadelphia chromosome (Ph)-negative CD34+ hematopoietic stem and progenitor cells of patients with Ph-positive CML in major molecular remission during therapy with imatinib. *Leukemia* 19:458–460
- Ochs MF, Rink L, Tam C, Mburu S, Taguchi T, Eisenberg B, Godwin AK (2009) Detection of treatment-induced changes in signaling pathways in gastrointestinal stromal tumors using transcriptomic data. *Cancer Res* 69:9125–9132
- Okamura M, Hashimoto K, Shimada J, Sakagami H (2004) Apoptosis-inducing activity of cisplatin (CDDP) against human hepatoma and oral squamous cell carcinoma cell lines. *Anticancer Res* 24:655–661
- Olive PL, Banath JP (2009) Kinetics of H2AX phosphorylation after exposure to cisplatin. *Cytom Part B-Clin Cy* 76B:79–90
- Parrella A, Lavorgna M, Crisuolo E, Russo C, Isidori M (2014) Estrogenic activity and cytotoxicity of six anticancer drugs detected in water systems. *Sci Total Environ* 485:216–222
- Parrella A, Lavorgna M, Crisuolo E, Russo C, Isidori M (2015) Ecogenotoxicity of six anticancer drugs using comet assay in daphnia. *J Hazard Mater* 286:573–580
- Perfettini JL, Reed JC, Israel N, Martinou JC, Dautry-Varsat A, Ojcius DM (2002) Role of Bcl-2 family members in caspase-independent apoptosis during chlamydia infection. *Infect Immun* 70:55–61
- Petkovic J, Zegura B, Stevanovic M, Drnovsek N, Uskokovic D, Novak S, Filipic M (2011) DNA damage and alterations in expression of DNA damage responsive genes induced by TiO2 nanoparticles in human hepatoma HepG2 cells. *Nanotoxicology* 5:341–353
- Pezdic M, Zegura B, Filipic M (2013) Genotoxicity and induction of DNA damage responsive genes by food-borne heterocyclic aromatic amines in human hepatoma HepG2 cells. *Food Chem Toxicol* 59: 386–394
- Pichler C, Filipic M, Kundi M, Rainer B, Krasmaeller S, Misik M (2014) Assessment of genotoxicity and acute toxic effect of the imatinib mesylate in plant bioassays. *Chemosphere* 115:54–8
- Pinheiro J, Bates D, DebRoy S, Sarkar DR (2012) Development Core Team nlme: linear and nonlinear mixed effects models, R Package Version 3.1-103; R Foundation for Statistical Computing, Vienna, Austria
- Pommier Y (2013) Drugging topoisomerases: lessons and challenges. *ACS Chem Biol* 8:82–95
- Pouquier P, Montaudon D, Huet S, Larue A, Clary A, Robert J (1998) Doxorubicin-induced alterations of c-myc and c-jun gene expression in rat glioblastoma cells: role of c-jun in drug resistance and cell death. *Biochem Pharmacol* 55:1963–1971
- Rogakou EP, Boon C, Redon C, Bonner WM (1999) Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J Cell Biol* 146:905–915
- Sawada N, Nakashima S, Banno Y, Yamakawa H, Hayashi K, Takemura K, Nishimura Y, Sakai N, Nozawa Y (2000) Ordering of ceramide formation, caspase activation, and Bax/Bcl-2 expression during etoposide-induced apoptosis in C6 glioma cells. *Cell Death Differ* 7:761–772
- Schulz M, Schmoldt A (2003) Therapeutic and toxic blood concentrations of more than 800 drugs and other xenobiotics. *Pharmazie* 58: 447–474
- Sermes A, Coise JP, Crespin M, Mainfroid V, de Longueville F, Ninane N, Raes M, Remacle J, Michiels C (2008) Hypoxia induces protection against etoposide-induced apoptosis: molecular profiling of changes in gene expression and transcription factor activity. *Mol Cancer* 7
- Shao ZY, Zhai BJ, Zhao CL, Hu K, Shen DM, Wu F (2008) Cytotoxic effects and in vitro reversal of multidrug resistance by therapeutic ultrasound in human hepatocarcinoma cell line (HepG2). *Ultrasonics* 48:297–302
- Singh S, Chhipa RR, Vijayakumar MV, Bhat MK (2006) DNA damaging drugs-induced down-regulation of Bcl-2 is essential for induction of apoptosis in high-risk HPV-positive HEp-2 and KB cells. *Cancer Lett* 236:213–221
- Smart DJ, Halicka HD, Schmuck G, Traganos F, Darzynkiewicz Z, Williams GM (2008) Assessment of DNA double-strand breaks and gamma H2AX induced by the topoisomerase II poisons etoposide and mitoxantrone. *Mutat Res-Fund Mol M* 641:43–47
- Smart DJ, Ahmed KP, Harvey JS, Lynch AM (2011) Genotoxicity screening via the gamma H2AX by flow assay. *Mutat Res-Fund Mol M* 715:25–31
- Smith ML, Kontny HU, Zhan QM, Sreenath A, O'Connor PM, Fornace AJ (1996) Antisense GADD45 expression results in decreased DNA repair and sensitizes cells to uv-irradiation or cisplatin. *Oncogene* 13:2255–2263
- Stasser A, Filipic M, Novak M, Zegura B (2013) Double strand breaks and cell-cycle arrest induced by the cyanobacterial toxin cylindrospermopsin in HepG2 cells. *Mar Drugs* 11:3077–3090
- Uien S, Lokiec F (2004) Population pharmacokinetics of total and unbound plasma cisplatin in adult patients. *Brit J Clin Pharmacol* 57: 756–763
- van Delft JHM, van Aken E, van Breda SGJ, Herwijnen MH, Staal YCM, Kleinjans JCS (2004) Discrimination of genotoxic from non-genotoxic carcinogens by gene expression profiling (vol 25, pg 1265, 2004). *Carcinogenesis* 25:2525–2525
- Vaskivuo L, Rysa J, Koivupera J, Myllynen P, Vaskivuo T, Chvalova K, Serpi R, Savolainen ER, Puistola U, Vahakangas K (2006) Azidothymidine and cisplatin increase p14ARF expression in OVCAR-3 ovarian cancer cell line. *Toxicol Appl Pharm* 216:89–97
- Venables WN, Ripley BD (2002) Modern applied statistics with S, 4th edn. USA, New York
- Viegas O, Zegura B, Pezdric M, Novak M, Ferreira IM, Pinho Q, Filipic M (2012) Protective effects of xanthohumol against the genotoxicity of heterocyclic aromatic amines MeIQx and PhIP in bacteria and in human hepatoma (HepG2) cells. *Food Chem Toxicol* 50(3–4):949–955
- Vogelstein B, Lane D, Levine AJ (2000) Surfing the p53 network. *Nature* 408:307–310
- Wang XW, Zhan QM, Coursen JD, Khan MA, Kontny HU, Yu LJ, Hollander MC, O'Connor PM, Fornace AJ, Harris CC (1999) GADD45 induction of a G(2)/M cell cycle checkpoint. *Proc Natl Acad Sci U S A* 96:3706–3711
- Wickham H (2007) Reshaping data with the reshape package. *J Stat Softw* 21:1–20

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14761

- Wickham H (2009) *ggplot2: elegant graphics for data analysis*. Springer New York
- Woo M, Hakem R, Mak TW (2000) Executionary pathway for apoptosis: lessons from mutant mice. *Cell Res* 10:267–278
- Youle RJ, Strasser A (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Bio* 9:47–59
- Zegum B, Zajc I, Lah TT, Filipic M (2008) Patterns of microcystin-LR induced alteration of the expression of genes involved in response to DNA damage and apoptosis. *Toxicol* 51:615–23
- Zhan QM (2005) Gadd45a, a p53-and BRCA1-regulated stress protein, in cellular response to DNA damage. *Mutat Res-Fund Mol M* 569: 133–143
- Zhang YJ, Lu CR, Cao Y, Luo Y, Bao RF, Yan S, Xue M, Zhu F, Wang Z, Duan LN (2012) Imatinib induces H2AX phosphorylation and apoptosis in chronic myelogenous leukemia cells in vitro via caspase-3/Mst1 pathway. *Acta Pharmacol Sin* 33:551–557
- Zhao HY, Ooyama A, Yamamoto M, Ikeda R, Hamaguchi M, Tabata S, Funakawa T, Che XF, Iwashita K, Oka T, Fukushima M, Nakagawa M, Ono M, Kuwano M, Akiyama S (2008) Down regulation of c-Myc and induction of an angiogenesis inhibitor, thrombospondin-1, by 5-FU in human colon cancer KM12C cells. *Cancer Lett* 270: 156–63
- Zhao J, Gao Z, Zhang H, Wang ZH, Song L, Ma JX, Pei SY, Wang CJ (2013) The potential value of the neutral comet assay and gamma H2AX foci assay in assessing the radiosensitivity of carbon beam in human tumor cell lines. *Radiol Oncol* 47:247–257
- Zoumkova R, Kovalova L, Blaha L, Dott W (2010) *Chemosphere* 81: 253–260

3.4 Ecotoxicity and Genotoxicity of Cyclophosphamide, Ifosfamide, Their Metabolites/Transformation Products and Their Mixtures

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Ecotoxicity data of human metabolites and transformation products is scarce and for most cytostatics almost completely absent. Furthermore, they occur in the environment as a complex mixture. Therefore, ecotoxicity and genotoxicity of two commonly used cytostatics, cyclophosphamide (CP) and ifosfamide (IF) and their human metabolites/transformation products (TPs), carboxy-cyclophosphamide (CPCOOH), keto-cyclophosphamide (ketoCP) and N-dechloroethyl-cyclophosphamide (NdCP) was evaluated as individual compounds and as a complex mixture. Ecotoxicity was studied in terms of growth inhibition of primary producers, alga *Pseudokirchneriella subcapitata* and cyanobacterium *Synechococcus leopoliensis*. Among the investigated compounds only CPCOOH revealed significant ecotoxicity (*S. leopoliensis* EC₅₀ = 17.1 mg/L) and according to EU Directive 93/67/EEC it can be classified as harmful to aquatic organisms (EC₅₀ 10–100 mg/L). Meanwhile all other compounds can be classified as non-toxic towards both phytoplankton species (LC₅₀ >320 mg/L). The mixture showed higher growth inhibition of *S. leopoliensis* (EC₅₀ = 11.5 mg/L) than predicted by the concentration addition model (EC₅₀ = 21.1 mg/L) which indicates the potentiating effects of the CPCOOH toxicity.

Genotoxic activity of the investigated compounds and their mixture has been evaluated with SOS/Umu assay with *Salmonella typhimurium*. The results confirmed the genotoxicity of CP in the presence of external metabolic activation, whereas CPCOOH showed genotoxic activity (320 mg/L) also without metabolic activation indicating that this compound is a direct acting genotoxin. Such genotoxicants can directly interact with DNA of environmental organisms and can also be a responsible mechanism for observed toxicity of CPCOOH to *S. leopoliensis*. Genotoxic activity was observed also for a complex mixture in the presence of metabolic activation, however at approximately ten-fold lower concentrations than after exposure to CPCOOH.

To our knowledge, this is the first study describing the ecotoxicity and genotoxicity of the commonly used cytostatics CP and IF, their known metabolites/TPs and their mixture. The results point out to the importance of toxicological evaluation and monitoring of drug metabolites as they may be for certain aquatic species more hazardous than parent compounds.

The PhD candidate collaborated in performing genotoxicity assays and data evaluation



Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites/transformation products and their mixtures

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ABSTRACT

Cyclophosphamide (CP) and ifosfamide (IF) are commonly used cytostatic drugs that repress cell division by interaction with DNA. The present study investigates the ecotoxicity and genotoxicity of CP, IF, their human metabolites/transformation products (TPs) carboxy-cyclophosphamide (CPCOOH), keto-cyclophosphamide (ketoCP) and N-dechloroethyl-cyclophosphamide (NdCP) as individual compounds and as mixture. The two parent compounds (CP and IF), at concentrations up to 320 mg L⁻¹, were non-toxic towards the alga *Pseudokirchneriella subcapitata* and cyanobacterium *Synechococcus leopoliensis*. Further ecotoxicity studies of metabolites/TPs and a mixture of parent compounds and metabolites/TPs performed in cyanobacteria *S. leopoliensis*, showed that only CPCOOH (EC₅₀ = 171 mg L⁻¹) was toxic. The measured toxicity (EC₅₀ = 11.5 mg L⁻¹) of the mixture was lower from the toxicity predicted by concentration addition model (EC₅₀ = 21.1 mg L⁻¹) indicating potentiating effects of the CPCOOH toxicity. The SOS/umuC assay with *Salmonella typhimurium* revealed genotoxic activity of CP, CPCOOH and the mixture in the presence of S9 metabolic activation. Only CPCOOH was genotoxic also in the absence of metabolic activation indicating that this compound is a direct acting genotoxin. This finding is of particular importance as in the environment such compounds can directly affect DNA of non-target organisms and also explains toxicity of CPCOOH against cyanobacteria *S. leopoliensis*. The degradation study with UV irradiation of samples containing CP and IF showed efficient degradation of both compounds and remained non-toxic towards *S. leopoliensis*, suggesting that no stable TPs with adverse effects were formed. To our knowledge, this is the first study describing the ecotoxicity and genotoxicity of the commonly used cytostatics CP and IF, their known metabolites/TPs and their mixture. The results indicate the importance of toxicological evaluation and monitoring of drug metabolites as they may be for certain aquatic species more hazardous than parent compounds.

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Abbreviations: AFLB1, aflatoxin B1; CP, cyclophosphamide; CPCOOH, carboxy-cyclophosphamide; DOC, dissolved organic carbon; EC₅₀, the concentration that gives half-maximal response (50%); HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; H₂O₂, hydrogen peroxide; IF, ifosfamide; ketoCP, keto-cyclophosphamide; NdCP, N-dechloroethyl-cyclophosphamide; NADP, nicotinamide adenine dinucleotide phosphate; NOEC, no observed effect concentration; NQNO, 4-nitroquinoline-N-oxide; ONPG, O-nitrophenyl-β-D-galactopyranoside; S9, rat liver post-mitochondrial fraction; TP, transformation product.

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1. Introduction

Cyclophosphamide (CP) and ifosfamide (IF) are two commonly prescribed chemotherapeutic drugs. They belong to a group of alkylating agents and their primary mode of action is based on their interaction with DNA resulting in repressed cell division (Besse et al., 2012). After the excretion from the human body the unaltered parent compounds and their human metabolites end up in the sewerage system where they eventually reach a municipal wastewater treatment plant, where according to several published studies they are not completely removed (Buerge et al., 2006; Delgado et al., 2011; Kiffmeyer et al., 1998; Kovalova et al., 2012; Köhler et al., 2012; Kummerer et al., 2000, 1997; Steger-

Hartmann et al., 1997). Once released into the aqueous environment, the parent compounds and their pharmacologically active metabolites and/or transformation products (TPs), formed during water treatment or under environmental conditions, might have a negative impact on aquatic biota (Kümmerer et al., 2000; Lenz et al., 2007). Cyclophosphamide and IF have been determined in ng L^{-1} to $\mu\text{g L}^{-1}$ levels in hospital wastewaters (Ferrando-Climent et al., 2013; Gómez-Canela et al., 2012; Kim et al., 2009a; Negreira et al., 2014, 2013a, 2013b; Steger-Hartmann et al., 1996; Yin et al., 2010) and in ng L^{-1} quantities in wastewater treatment plant influents and effluents (Buerge et al., 2006; Kümmerer et al., 1997; Ternes, 1998; Zuccato et al., 2005) and surface waters (Buerge et al., 2006; Moldovan, 2006; Valcárcel et al., 2011). Although several CP and IF human metabolites are known to be excreted by patients receiving chemotherapy, their presence in the environment is rarely reported. Only one CP metabolite/TP, namely carboxy-cyclophosphamide (CPCOOH), has been qualitatively determined in a Spanish hospital wastewater (Ferrando-Climent et al., 2013).

To reduce the levels of CP and IF in wastewaters, various wastewater treatment technologies can be applied including biological treatment, ozonation, UV irradiation and advanced oxidation processes such as ozonation/ H_2O_2 , UV/ H_2O_2 and UV/ TiO_2 (Chen et al., 2008; Delgado et al., 2011; Fernández et al., 2010; Kim et al., 2009b; Kovalova et al., 2012; Kümmerer et al., 1997; Lester et al., 2011; Lin et al., 2014; Steger-Hartmann et al., 1997; Wols et al., 2013), where parent compound removal was used as a parameter for evaluating treatment efficiency. However, during water treatment (biological and abiotic), compounds are exposed to several degradation factors, e.g. microbes, reactive oxygen species, sunlight, UV light, ozone and chemical oxidative agents that may generate potentially toxic TPs. Identification of these structurally diverse compounds is a challenging task, where TPs may have a different toxic potential from that of the parent compound, which must be taken into account. In addition, the toxicity studies of TPs require authentic standards that are often not commercially available and have to be custom synthesized. Therefore, a more rational approach is to evaluate the changes in the toxicity of sample before and after treatment (influent and effluent), which considers the change in the whole toxicity of the sample. Studies describing degradation of CP and IF reported either a lower or similar toxicity of treated samples in comparison to the untreated control (Delgado et al., 2011, 2010; Hansel et al., 1996; Lin et al., 2015; Lutterbeck et al., 2015) with the exception of a recent study by (Lin et al., 2015b), who reported a slight increase in the acute toxicity of ozone treated samples containing either CP or IF.

When evaluating adverse effects of compounds in the environment it is important to study their toxicity on organisms from different trophic levels: phytoplankton, invertebrate and vertebrate species (Zoukova et al., 2010). In the aquatic ecosystems phytoplankton species are the main primary producers at the bottom of the food chain and any disturbance of these phytoplankton communities can affect communities at higher trophic levels (Geis et al., 2000; Pfleeger et al., 1990). To assess the toxicity of environmental pollutants towards aquatic primary producers, tests based on the OECD Guidelines for Testing of Chemicals (i.e. Freshwater Alga and Cyanobacteria, Growth Inhibition Test) are commonly performed (OECD TG 201, 2011). A literature overview revealed that only two CP growth inhibition studies have been performed using the alga *Pseudokirchneriella subcapitata*, both showing low toxicity with no observed effect concentration (NOEC) $> 100 \text{ mg L}^{-1}$ (Grung et al., 2008; Zoukova et al., 2007). No published ecotoxicity studies, to the authors knowledge, have included IF and the human metabolites/TPs of CP and IF. On the other hand, the review paper by Toolaram et al. (2014) reveals

genotoxic activity of CP, IF and some of their human metabolites (phosphoramidate mustard, 4-OH-peroxy-cyclophosphamide, non-nitrogen mustard, CPCOOH and keto-cyclophosphamide; ketoCP), with certain metabolites being more potent than the parent compounds (Toolaram et al., 2014). In the aquatic environment, organisms are exposed to a complex mixture of numerous compounds including cytostatic drugs and their metabolites, which may exert higher adverse effects on non-target organisms than expected based on the measured effect of the exposure to a single compound. Therefore it is important to study both, the effects of pure compounds and even more the effects of their mixtures (Brain et al., 2004). Recently published studies describe toxic activities of mixtures of several cytostatic drugs (Brezovšek et al., 2013, 2014; Parrella et al., 2014), however, to the best of our knowledge, the toxic potential of mixture of CP, IF and their metabolites/TPs has not been investigated yet.

The aim of this study was to evaluate ecotoxicity and genotoxicity of the parent cytostatics CP and IF and their commercially available human metabolites/TPs: CPCOOH, ketoCP and N-dechloroethyl-cyclophosphamide and its structural analogue 3-dechloroethyl-ifosfamide (NdCP) and their mixture by determining the effect on growth of primary producers (alga *P. subcapitata* and cyanobacteria *Synechococcus leopoliensis*) and SOS/umuC response of *Salmonella typhimurium* TA1535/pSK1002, respectively. Furthermore, the measured influence of the mixture of CP, IF and the three metabolites/TPs on growth of *S. leopoliensis* was compared to the predicted effect determined with the concentration addition (CA) model (Loewe, 1927; Loewe and Muischnek, 1926). Finally, to evaluate the treatment efficiency of CP and IF by UV irradiation, the degradation along with ecotoxicity of potential stable TPs formed during this treatment was assessed on *S. leopoliensis*.

2. Materials and methods

2.1. Standards, reagents and chemicals

Cyclophosphamide, IF, CPCOOH, ketoCP and NdCP were handled in compliance with the internal safety standards for working with anti-neoplastic drugs (Eitel et al., 2000). All wastes were disposed of as hazardous waste. The operator protected themselves against UV irradiation during experiments by wearing protective clothes and UV-protective glasses.

Cyclophosphamide (99%) and IF (99%) were purchased from Sigma Aldrich (Hong Kong, China). Carboxy-cyclophosphamide, ketoCP and NdCP (a structural analogue of CP and IF metabolites/TPs) were obtained from Niomech - IT GmbH (Bielefeld, Germany). Chemical structures, CAS numbers and the molecular weights of the investigated compounds are presented in Table 1. Stock solutions for ecotoxicity bioassays were prepared in OECD medium and filtered through $0.2 \mu\text{m}$ cellulose-nitrate filters (Sartorius Stedim Biotech, Göttingen, Germany). The concentrations in the stock solutions were as follows: CP: 3.06 mg mL^{-1} ; IF: 1.01 mg mL^{-1} ; CPCOOH: 5.43 mg mL^{-1} ; ketoCP: 0.55 mg mL^{-1} and NdCP: 5.56 mg mL^{-1} . For the UV irradiation experiments, stock solutions of CP and IF were prepared in MilliQ water (Millipore Ltd, Darmstadt, Germany) at 16.05 and 16.16 mg mL^{-1} , respectively. The solutions were sterilized by filtration ($0.2 \mu\text{m}$ cellulose-nitrate filters). The stock solutions used for genotoxicity testing were prepared either in dimethylsulfoxide (DMSO; metabolites/TPs) or in sterile bidistilled water (CP and IF). The concentrations of stock solutions were 20 mg mL^{-1} and 50 mg mL^{-1} for parent compounds and metabolites/TPs, respectively. The solvent DMSO was purchased from Sigma-Aldrich (Steinheim, Germany) and was of analytical grade purity.

Table 1

Names, abbreviations, structures, CAS numbers and molecular weights (MW) of investigated compounds.

Name and abbreviation	Structure	CAS	MW
cyclophosphamide CP		50-18-0	198.59
ifosfamide IF		3778-73-2	198.59
carboxycyclophosphamide CPCOOH		22788-18-7	293.08
4-ketocyclophosphamide ketoCP		27046-19-1	275.07
N-dechloroethylcyclophosphamide or 3-dechloroethylifosfamide NdCP		36761-83-8	198.59

The chemicals used for SOS/umuC bioassay, i.e. ampicillin, glucose-6-phosphate (disodium salt), D-(+)-glucose (anhydrous), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium dodecyl sulphate, Na₂CO₃, MgCl₂·H₂O, Na₂HPO₄, Na₂HPO₄·2H₂O, NaH₂PO₄·H₂O, aflatoxin B1 (AFB1) and 4-nitroquinoline-N-oxide (NQNO) were obtained from Sigma-Aldrich (St. Louis, USA), nicotinamide adenine dinucleotide phosphate (NADP) and NaCl from Merck (Darmstadt, Germany), while O-nitrophenyl-β-D-galactopyranoside (ONPG), β-mercaptoethanol, KCl and MgSO₄·7H₂O were purchased from Fluka (Buchs, Switzerland). Lyophilized Aroclor 1254 induced male rat liver post-mitochondrial fraction (S9) was obtained from Moltox (Boone, USA) and trypton from BD Biosciences (Sparks, USA).

We assessed the stability of all investigated compounds under the experimental conditions. Detailed descriptions and results are presented in SM-1a. The stability of the parent compounds in HPLC

water (J. T. Baker, Deventer, Netherlands) at 4 °C was confirmed by [Negreira et al. \(2013b\)](#), whereas the stability of metabolites/TPs was investigated as part of this study (Supplementary Material; SM-1b).

2.2. Bioassays

2.2.1. Ecotoxicity on green alga *P. subcapitata* and the cyanobacterium *S. leopoliensis*

Green alga *P. subcapitata* SAG 61.81 and the cyanobacterium *S. leopoliensis* SAG 1402-1 were obtained from the algae collection SAG - Sammlung von Algenkulturen Universität Göttingen (SM-2). The organisms were cultivated in an OECD medium (pH = 8.1, composition of the medium is described in the Annex of [OECD TG 201 \(2011\)](#)), according to the Guidelines in [OECD TG 201 \(2011\)](#). The pH of the medium was measured at the beginning and at the end of experiments and did not change significantly during the

performance of the experiments. The bioassays were conducted in glass Erlenmeyer flasks ($V_{\text{final}} = 20$ mL) under 24 h day⁻¹ light irradiation (cooled lamps Sylvania GRO-Lux F 18W/GRO-T8; intensity: 80–120 $\mu\text{E m}^{-2} \text{s}^{-1}$), with constant shaking at 100 rpm (GFL 3017, Burgwedel, Germany) and kept at 24 ± 2 °C. The V_{final} consisted of the OECD medium, tested organism and a solution of either the five investigated compounds, a mixture of all five compounds or the UV-irradiated samples. According to OECD TG 201, the initial cell densities were $5 \times 10^3 - 5 \times 10^4$ cells mL⁻¹ and $5 \times 10^4 - 5 \times 10^5$ cells mL⁻¹ for the algae and cyanobacteria, respectively (OECD TG 201, 2011). Cell growth was determined after 24, 48 and 72 h of exposure to the parent compounds (CP and IF), their metabolites/TPs and their mixture using a flow cytometer (BD FACSCalibur, Sparks, MD, USA), whereas in the case of UV-irradiated samples it was determined by cell counting under an inverted light microscope (Nikon Edipse 80i, Nikon, Japan) as described elsewhere (Elersek, 2012). The algal/cyanobacterial growth rate, expressed as the logarithmic increase in cell number within 72 h of exposure to the test compounds, a mixture of compounds or the UV-irradiated samples was compared to the control cultures exposed under the same experimental conditions. The parent compounds' ecotoxicity was assessed for both species, whereas metabolites/TPs, mixtures and UV-irradiated samples were tested only towards *S. leopoliensis*. The experiments for determining the ecotoxicity of individual compounds and their mixture were performed three times independently, each time in triplicates, while, the preliminary experiments for determining the ecotoxicity of control and UV-irradiated samples were performed twice in duplicate. The experiment for determining the ecotoxicity of a CP and IF mixture (UV experiments) was performed once in triplicate. According to the OECD guidelines, the ecotoxicity of each compound was determined at five concentrations as geometric series with a factor 3.2 (3.05, 9.77, 31.25, 100 and 320 mg L⁻¹). Standard solutions were prepared in sterilized MilliQ water. The toxicity of the UV-irradiated samples was evaluated as follows; 10 mL of UV-irradiated sample (medium was sterilized MilliQ water) was added to 10 mL of 2 M OECD medium (concentrated by factor 2) with pre-culture of *S. leopoliensis* to give a final volume of 20 mL with 1 M OECD medium (as used within all the performed experiments). The initial amount of CP and IF during the UV experiments (10 mg L⁻¹) was diluted at 1:1 ratio for the bioassays to give a final concentration of CP and IF at 5 mg L⁻¹.

2.2.2. Genotoxicity on *S. typhimurium* TA1535/pSK1002

The evaluation of genotoxic activity of the investigated compounds and their mixture was assessed by SOS/umuC assay according to Reifferscheid et al. (1991). The genotoxicity of individual compounds and their mixture was evaluated using the same concentrations as in ecotoxicity bioassays (3.05, 9.77, 31.25, 100 and 320 mg L⁻¹ for individual compounds and 6, 7.9, 10.5, 17.1 and 37.2 mg L⁻¹ for the mixture). The genotoxicity tests were conducted with (+S9) and without (-S9) metabolic activation in a triplicate on a microtiter plates, where a genetically modified *S. typhimurium* TA1535, carrying a multicopy plasmid pSK1002 with fused umuC-lacZ genes, was used as the tested organism. Briefly, the overnight bacterial culture was diluted in a ratio 1:10 with fresh TGA medium and agitated (300 rpm) at 37 °C for 1.5 h until the bacteria reached the exponential growth phase. The bioassay was performed with the following mixture: 10 μL of solution containing compound/mixture in DMSO or bidistilled water and 90 μL of bacterial culture. In the case of metabolic activation, 10 μL of the tested compound was incubated with 90 μL of the S9 bacterial culture mixture, containing 10% of the S9 extract. The microtiter plates were further incubated at 37 °C for 2 h with shaking. Afterwards, the incubation mixture was diluted with fresh TGA medium (1:10) and incubated

at 37 °C for additional 2 h with shaking. The bacterial growth rate was determined by measuring absorbance at 600 nm. The β -galactosidase activity (stopped with Na₂CO₃ after 30 min of incubation at 25 °C and shaking) was assessed using ONPG as a substrate, where the absorbance of the o-nitrophenol was measured (at 420 nm).

4-nitroquinoline-N-oxide (0.25 mg L⁻¹) and AFB1 (5 mg L⁻¹) were used as a positive controls without and with metabolic activation, respectively. In addition, distilled water and DMSO (the same amount as in tested samples) were used as the negative and solvent control.

2.3. Experimental and predicted ecotoxicity of the mixture

The ecotoxic activity of the mixture of CP, IF, ketoCP, NdCP and CPCOOH was tested at concentration in the mass ratio 1:1:1:1:1. The concentrations were based on EC₅₀₋₉₀ values calculated for CPCOOH (see Chapter 3.1, Fig. 2, Table 2), namely 6, 7.9, 10.5, 17.1 and 37.2 mg L⁻¹ since CPCOOH was the only compound that significantly inhibited the growth of *S. leopoliensis* within the working concentration range, while other tested compounds did not have any effect at concentrations up to 320 mg L⁻¹. Moreover, by applying the same concentration of each compound in the mixture it was possible to compare the effect of individual compound (i.e. CPCOOH) to the effect of the mixture (see Chapter 3.1, Fig. 2, Table 2). The obtained results were compared to the CA model as described by Loewe (1927), originally by Loewe and Muischnek (1926). The CA model is based on the assumption that when the investigated compounds share similar sites and modes of action, the effect of a mixture remains constant if one compound is replaced by another (Faust et al., 2003). The model was used to predict ecotoxicity since the investigated parent compounds and their human metabolites/TPs share similar structures and modes of action, i.e. interaction with DNA (alkylation of DNA).

2.4. UV irradiation of samples

Experiments were performed in a cylindrical glass reactor containing 760 mL of sterilized aqueous solution (MilliQ) spiked with the mixture of CP and IF (10 mg L⁻¹ each). The solution was stirred continuously in the dark using a magnetic stirrer (200 rpm). A monochromatic low pressure (LP) mercury lamp (Photochemical reactors LTD, Berkshire, UK) was introduced into the reactor and kept separate from the test solution using a quartz cooling well. Sample temperature was maintained at 20 °C. The LP lamp (6 W) as an irradiation source emits 90% of its radiation at 254 nm. The UV intensity, measured using ferrioxalate actinometry as described in manufacturers' instructions, was 0.78 $\mu\text{E m}^{-2} \text{s}^{-1}$.

The removal of CP and IF during UV irradiation was assessed by GC/MS as described elsewhere (Česen et al., 2015). Briefly, samples (0.1 mL) were dried under nitrogen at 35 °C to dryness and further derivatised with trifluoroacetic anhydride in ethyl acetate (for 1 h at 60 °C) for further chemical analysis. The removal was calculated as the ratio between CP and IF concentrations in UV irradiated vs. non-irradiated samples at the following specified times: 6, 12, 24, 36 and 48 h. The DOC (dissolved organic carbon) values were analysed using the Hach Lange cuvette method (LCK385) in combination with a DR/2800 spectrophotometer (Hach Lange, Düsseldorf, Germany) in order to assess mineralization degree of CP and IF.

2.5. Data evaluation

Data analysis and statistical evaluation of the toxicities were performed using Microsoft Excel 2010 and Prism 5 (GraphPad Inc.,

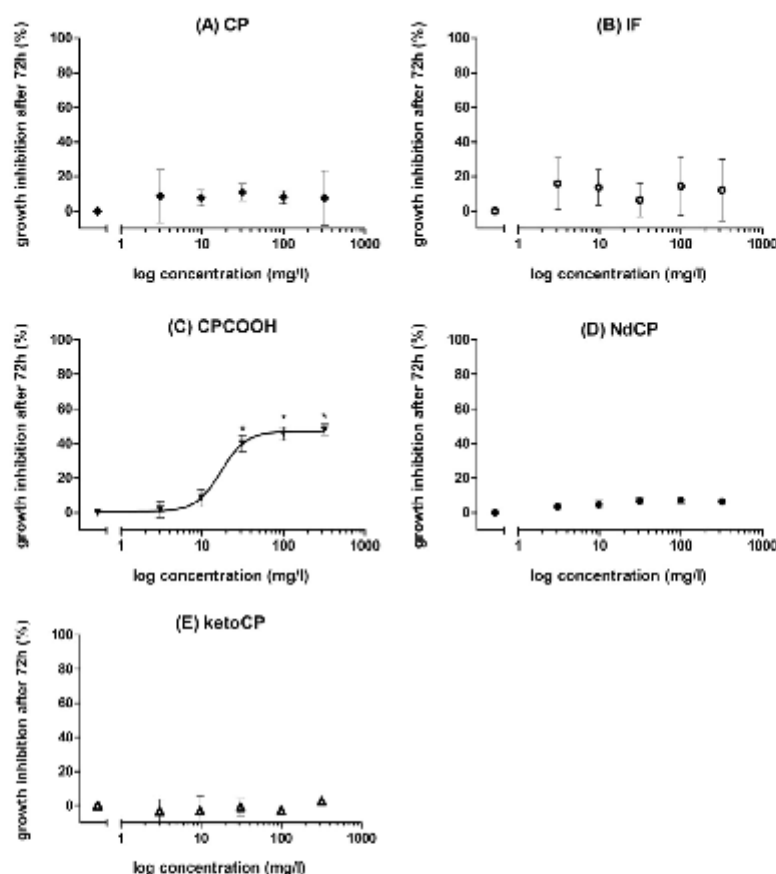


Fig. 1. The effect of the investigated compounds (A–E) on the growth of *S. leopoliensis*. Growth inhibition (as the mean $\pm$ 95% confidence interval) is presented as a function of log concentration after 72 h of exposure. * indicates statistically significant difference in comparison to the control sample.

La Jolla, USA) software. In the case of ecotoxicity bioassays, the measurements from each individual flask (pooled together) were used for the analysis instead of the means of replicates as described in OECD TG 201 (2011). For the Figures presenting the % growth inhibition (Y) vs. log concentration of the tested compound and the EC_x values, a nonlinear regression model was applied, using the following Eqs. (1–3):

$$\log EC_x = \log EC_{50} + \left(\frac{1}{\text{hill slope}} \right) \times \log \left(\frac{FX}{(100 - FX)} \right) \quad (1)$$

$$FX = \frac{(Y - \text{Bottom})}{(\text{Top} - \text{Bottom})} \times 100 \quad (2)$$

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{(\log EC_{50} - X) \times \text{slope}})} \quad (3)$$

where EC_x represents the concentration of the investigated

compound which gives a response of FX of the way between Bottom and Top (e.g., EC_{10} , EC_{20} , EC_{50}), where Bottom and Top are the plateaus in the units of the response (% growth inhibition). In addition, the slope describes the steepness of the family of curves. The important parameters in Prism 5 were set as: constraint for the bottom of the nonlinear curve to "shared value for all datasets". In addition, the outliers were identified by the Prism 5 software (based on ROUT method) and were excluded from further calculations. Non-parametric ANOVA (Kruskal–Wallis test; $\alpha = 0.05$) and Dunn's post-test ($p < 0.05$) were used to determine if any significant differences exist between the controls and the effect of the investigated compound or mixtures of compounds towards the test organisms.

The calculation of CA model was performed using the Eq. (4):

$$\sum_{i=1}^n \frac{C_i}{EC_{x,i}} = 1 \quad (4)$$

where C_i represents the concentration of the i -th compound in a

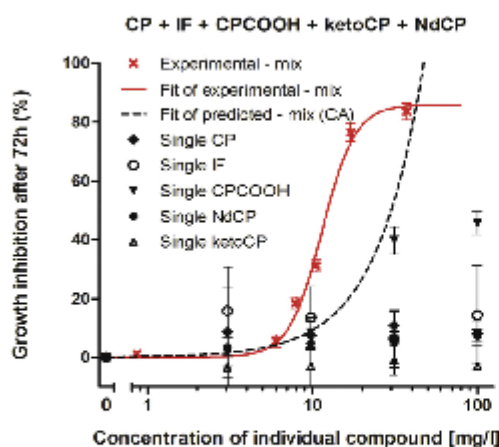


Fig. 2. Growth inhibition (mean $\pm$ 95% confidence interval) based on data from individual compounds (different symbols), experiments with a mixture (mix) of compounds at the same concentration as in case of experiments with individual compounds (full line) and curve of predicted data with CA model (dashed line).

tested mixture that exhibits $x\%$ of the total effect, whereas $EC_{x,i}$ is the concentration of the compound i which provokes the same effect ($x\%$) if applied individually.

The bacterial growth rate (G) for determination of cytotoxic activity was calculated as the ratio of $OD_{600, sample}$ to $OD_{600, control}$ (the blank sample was subtracted in both cases). The calculated growth ratio for a compound/mixture less than 0.75 (i.e. 25% inhibition of biomass growth) was considered as cytotoxic. The β -galactosidase activity (U) in relative units was calculated as the ratio of $OD_{420, sample}$ to $OD_{420, control}$ (the blank sample was subtracted in both cases). The induction ratio (IR) was determined as the ratio of $OD_{420, sample}$ to $OD_{420, control}$ (the blank sample was subtracted in both cases), divided by G . The $IR \geq 2$ was considered as the threshold at which the compound/mixture was considered as genotoxic.

3. Results and discussion

In the present study the ecotoxic and genotoxic effects of the investigated cytostatics, their metabolites/TPs and their mixture were evaluated on primary producers (green algae *P. subcapitata* and cyanobacterium *S. leopoliensis*) and *S. typhimurium* TA1535/pSK1002, respectively.

3.1. Ecotoxicity studies

3.1.1. Individual compounds

Toxicity was assessed in terms of growth inhibition, expressed as EC_{50} and NOEC values (Table 2). Growth inhibition experiments revealed that, within the tested concentration ranges, neither CP or IF inhibited the growth of *P. subcapitata* or *S. leopoliensis* (Fig. 1A, B and SM-3). The observed results for CP on *P. subcapitata* (SM-3) are in the agreement with literature (Grung et al., 2008; Zoukova et al., 2007), whereas to our knowledge the effects of IF towards these species are yet to be reported. Since prokaryotic organisms (cyanobacteria belongs to prokaryotes) are more abundant in biological water treatment than eukaryotic organisms (Wagner and Loy, 2002) and because the alga and cyanobacteria did not differ in the susceptibility towards exposure to CP and IF, all following experiments involving metabolites/TPs, mixtures of compounds and toxicity of the CP and IF after UV irradiation were performed using only *S. leopoliensis*. Growth inhibition of *S. leopoliensis* by investigated human metabolites/TPs is shown in Fig. 1C, D and E. Only CPCOOH significantly inhibited growth of cyanobacteria.

The EU Directive 93/67/EEC (1996) classifies substances according to their EC_{50} values towards aquatic organisms as very toxic ($EC_{50} < 1 \text{ mg L}^{-1}$), toxic ($EC_{50} 1\text{--}10 \text{ mg L}^{-1}$) or harmful ($EC_{50} 10\text{--}100 \text{ mg L}^{-1}$). Substances with EC_{50} above 100 mg L^{-1} are not classified. The EC_{50} value for the metabolite/TP CPCOOH was 17.1 mg/L (Table 2). Therefore, according to the EU Directive 93/67/EEC (1996), CPCOOH is classified as harmful to aquatic organisms. Interestingly, CPCOOH was the only compound that had a significant effect compared to other compounds, despite their similarities in chemical structure and potential mode of action. So far, CPCOOH is the only CP metabolite/TP, which has been detected in hospital wastewater in Spain, whereas environmental concentrations in e.g. surface waters have not been reported yet. Unfortunately, due to the chemical instrumentation used and analysis, which allowed only the confirmation of its presence in the hospital wastewater, the concentration of CPCOOH was not determined in the study. Therefore, any environmental and human risk assessment for CPCOOH is not possible at this stage (Ferrando-Climent et al., 2013).

3.1.2. Mixture of CP, IF and their metabolites/TPs

Exposure to the mixture of CP, IF and their metabolites/TPs revealed higher growth inhibition of *S. leopoliensis* than that predicted by the CA model, which is especially noticeable within $EC_{20\text{--}70}$ effect concentration range (Fig. 2). The experimentally determined EC_{50} was 11.5 mg L^{-1} (95% CI: $10.3\text{--}12.9 \text{ mg L}^{-1}$), whereas the predicted EC_{50} based on the CA model was 21.1 mg L^{-1} . It is also apparent that the dynamics of growth inhibition based on experimental data differ from predicted assumptions for concentrations $> 5 \text{ mg L}^{-1}$, suggesting the potentiation effect of a mixture containing parent compounds and their human metabolites/TPs. A potentiation effect is observed when the toxicity of a mixture of a

Table 2
 EC_{50} , NOEC values and growth inhibition of *S. leopoliensis* for each test compounds; n.c. = not converged (Prism 5).

Compound	EC_{50} and 95% CI (mg L^{-1})	NOEC (mg L^{-1})	Measured maximal growth inhibition in tested concentration range
CP	n.c.	Above 320	0–20%
IF	n.c.	Above 320	0–39%
CPCOOH	EC_{50} : 6.0 (4.4–8.3) EC_{10} : 7.9 (6.0–10.2) EC_{20} : 10.5 (8.5–12.9) EC_{30} : 17.1 (14.4–20.3) EC_{40} : 37.2 (27.8–48.7)	9.8	0–54%
ketoCP	n.c.	Above 320	0–3%
NdCP	n.c.	Above 320	0–7%

non-toxic compound and a toxic compound results in an effect, higher than the determined toxicity of an individual toxic compound (Li et al., 2015). Such phenomenon, shown in Fig. 2, was observed within this experiment, where the sum of a mixture's toxicity, i.e. CP + IF + CPCOOH + ketoCP + NdCP was higher than the toxicity of CPCOOH alone. Although there are different ways of performing such mixture experiments, e.g. by using mass ratio, molar ratio and ratio based on pharmacokinetics, which could lead to different outcomes, we have chosen mass ratio due to the observed effect of only CPCOOH and since all the other work presented in this study is based on mass concentration. Moreover, the EC₅₀ values of individual CPCOOH (17.1 mg L⁻¹) and mixture (11.5 mg L⁻¹) were in the same concentration range, indicating that only mild potentiation effect occurred by the tested mixture. Our results revealed similar "something from nothing" effect, a phenomenon that was already described in literature for e.g. weak estrogenic chemicals (Silva et al., 2002).

3.2. Genotoxicity of individual compounds and the mixture

CP and IF are prodrugs that require cytochrome P450 mediated transformation to nucleophilic intermediates that alkylate DNA

Table 3
The genotoxicity of the investigated compounds and their mixture via the induction of SOS response in *S. typhimurium* TA1535/pSK1002 in the presence (+S9) and in the absence (-S9) of metabolic activation.

Comp.	Conc. (mg L ⁻¹)	- S9		+ S9	
		G ^a ± SD	IR ^b ± SD	G ^a ± SD	IR ^b ± SD
C1 ^c	0	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.03
C2 ^d	0	1.00 ± 0.04	1.00 ± 0.13	1.00 ± 0.05	1.00 ± 0.14
AFB1	5	/	/	0.73 ± 0.13	2.57 ± 0.33
NQNO	1.14	0.91 ± 0.03	3.95 ± 0.11	/	/
CP	3.05	1.09 ± 0.02	1.01 ± 0.04	1.07 ± 0.02	0.90 ± 0.10
	9.7	1.03 ± 0.02	1.00 ± 0.05	1.01 ± 0.03	1.04 ± 0.03
	31.2	1.00 ± 0.02	0.97 ± 0.06	1.03 ± 0.07	0.88 ± 0.21
	100	1.02 ± 0.05	0.95 ± 0.06	0.98 ± 0.03	0.94 ± 0.03
	320	1.01 ± 0.24	1.01 ± 0.04	0.80 ± 0.03	2.72 ± 0.55
IF	3.05	1.01 ± 0.03	1.02 ± 0.07	1.09 ± 0.12	1.04 ± 0.13
	9.7	1.02 ± 0.07	0.95 ± 0.12	1.02 ± 0.05	1.10 ± 0.33
	31.25	1.02 ± 0.07	0.99 ± 0.02	0.97 ± 0.07	1.57 ± 0.59
	100	0.98 ± 0.03	1.03 ± 0.03	0.97 ± 0.04	1.42 ± 0.62
	320	0.98 ± 0.02	1.04 ± 0.07	0.93 ± 0.05	1.33 ± 0.36
CPCOOH	3.05	0.91 ± 0.03	1.09 ± 0.05	1.08 ± 0.08	1.01 ± 0.24
	9.7	0.89 ± 0.04	1.10 ± 0.09	1.02 ± 0.02	1.05 ± 0.18
	31.25	0.87 ± 0.01	1.26 ± 0.01	1.00 ± 0.02	1.08 ± 0.19
	100	0.87 ± 0.01	1.56 ± 0.05	0.94 ± 0.05	1.15 ± 0.24
	320	0.86 ± 0.01	2.75 ± 0.12	0.82 ± 0.04	2.04 ± 0.47
ketoCP	3.05	0.93 ± 0.02	1.17 ± 0.04	1.11 ± 0.06	0.73 ± 0.19
	9.7	0.94 ± 0.02	1.05 ± 0.08	1.10 ± 0.04	0.72 ± 0.33
	31.25	0.93 ± 0.02	1.04 ± 0.06	0.96 ± 0.00	0.96 ± 0.08
	100	0.96 ± 0.01	0.96 ± 0.08	1.01 ± 0.02	0.78 ± 0.05
	320	0.94 ± 0.06	1.04 ± 0.15	0.93 ± 0.04	0.89 ± 0.18
NdCP	3.05	0.96 ± 0.04	1.08 ± 0.03	1.02 ± 0.04	1.08 ± 0.31
	9.7	0.95 ± 0.04	1.17 ± 0.05	1.02 ± 0.06	0.96 ± 0.33
	31.25	0.92 ± 0.04	1.23 ± 0.06	1.07 ± 0.03	1.03 ± 0.13
	100	0.94 ± 0.01	1.13 ± 0.02	1.11 ± 0.04	0.87 ± 0.25
	320	0.91 ± 0.02	1.12 ± 0.03	1.07 ± 0.04	1.03 ± 0.32
mixture ^e	6.0	1.05 ± 0.04	1.04 ± 0.04	1.01 ± 0.08	1.54 ± 0.54
	7.9	1.07 ± 0.05	1.17 ± 0.09	0.98 ± 0.05	1.75 ± 0.13
	10.5	1.08 ± 0.04	1.15 ± 0.06	1.05 ± 0.06	1.72 ± 0.21
	17.1	1.00 ± 0.02	1.23 ± 0.11	1.03 ± 0.04	1.70 ± 0.57
	37.2	0.99 ± 0.03	1.35 ± 0.08	1.00 ± 0.07	2.18 ± 0.49

Note a: G = bacterial growth rate; G < 0.75 is considered as toxic for bacteria.

Note b: IR = induction ratio; IR > 2 is considered as genotoxic response.

Note c: control used for the calculation of genotoxicity of the metabolites/TPs (NBCP, CPCOOH, ketoCP).

Note d: control used for calculation of the genotoxicity of the CP, IF and mixture.

Note e: the indicated concentrations are referred to the concentration of each of each of the individual compound in the mixture.

molecule (Sladek, 1988). Therefore genotoxic properties of CP, IF, their metabolites/TPs and the mixture were assessed using SOS/umuC assay (Reifferscheid et al., 1991; Schmid et al., 1997; Yasunaga et al., 2006) in the absence (-S9) and presence (+S9) of metabolic activation. The results are presented in Table 3. Within the selected concentration range, none of the tested compounds/mixture showed cytotoxic effects (G values were >0.75; Table 3). Of the two parent compounds, only CP exhibited significant genotoxic activity in the presence of S9 at the highest concentration tested (IR = 2.72 ± 0.55), which indicates that metabolic activation led to the formation of genotoxic metabolites. This is in accordance with the published studies addressing CP genotoxicity using Ames test, where CP was mutagenic only in the presence of S9 metabolic activation (Benedict et al., 1977; Hales, 1982; Winckler et al., 1984; Yasunaga et al., 2006). On the contrary to our results, Benedict et al. (1977) reported mutagenic activity also for IF in the presence of S9, however compared to CP it was significantly less potent. Of the three investigated metabolites/TPs, only CPCOOH exhibited significant genotoxic activity in the absence (2.75 ± 0.12) and the presence (2.04 ± 0.47) of metabolic activation (Table 3). This partially agrees with the study by Winckler et al. (1984), who investigated mutagenic activities of CP and its main metabolites (including ketoCP and CPCOOH) with the Ames test. The authors report higher genotoxic potential of CPCOOH and similar genotoxic potential for ketoCP, if compared to CP, regardless of the metabolic activation. In our study, higher genotoxic potential was observed when CPCOOH was tested in the absence of metabolic activation (2.75 vs. 2.04; Table 3), whereas Winckler et al. (1984) reported that all CP metabolites tested in the Ames assay, including CPCOOH, were activated by S9 mix to a higher mutagenic potential.

The finding that CPCOOH was active also without metabolic activation in the SOS/umuC test may explain the ecotoxic activity of CPCOOH towards *S. leopoliensis*. Cyanobacteria do not express cytochrome P450 enzymes. Consequently CP and IF were not transformed to nucleophilic intermediates that react with DNA and suppress cell division, which rendered cyanobacteria unsuspensible to the exposure to the parent compounds.

The mixture of the two parent compounds and their three metabolites at the concentrations tested without metabolic activation revealed no significant genotoxic activity, which is in agreement with the negative results of individual compounds. Only CPCOOH exerted genotoxic effects in the absence of S9; however at much higher concentrations. On the other hand in the presence of the metabolic activation the mixture revealed significant genotoxic activity at the highest concentration tested (the concentration of each compound in the mixture was 37.2 mg L⁻¹). This result can be correlated with the outcomes obtained with individual compounds, where CP and CPCOOH had genotoxic activity. However, the concentrations of individual CP and CPCOOH with IR factor >2 were 320 mg L⁻¹, while the highest concentration of each individual compound in the mixture was 37.2 mg L⁻¹, hence, nearly 10-fold lower. This suggests that in the metabolically activated mixture the inactive components were either transformed to genotoxic metabolites or they potentiated genotoxicity of CP and CPCOOH.

3.3. Degradation and ecotoxicity of CP and IF during UV treatment

This study also investigated the ecotoxicity of CP and IF mixture during UV irradiation. Prior to UV irradiation of CP and IF, to determine whether or not the degradation products exert a different toxicity from that of the parent compounds, the toxicities of both compounds individually and in a mixture were compared. Since there were no statistical differences in growth inhibition when testing compounds either individually or as a mixture (SM-4), both compounds were UV-irradiated simultaneously. In

addition, in the control experiment (48 h, no UV irradiation) degradation of CP and IF was negligible and there was no significant change in ecotoxicity (SM-5).

The results of the degradation study of CP and IF mixture showed that both compounds were degraded during UV irradiation, resulting in $69.4 \pm 3.6\%$ and $62.9 \pm 2.3\%$ final removal of CP and IF, respectively (Fig. 3).

When exposed to UV irradiation, CP and IF concentration decreased exponentially with time (0–48 h) and followed pseudo-first-order kinetics. The degradation rate constants, half-lives ($t_{1/2}$) and the R^2 of the degradation curves for CP were 0.024 h^{-1} , 28.8 h and 0.975, respectively. Similarly, for IF, the degradation rate constants, $t_{1/2}$ and the R^2 were 0.020 h^{-1} , 34.66 h and 0.937, respectively.

The DOC analysis of UV-irradiated samples revealed no significant decrease in DOC over the 48 h. This suggests that UV degradation failed to mineralize both compounds to CO_2 and H_2O . Instead, it was assumed that only partial degradation was achieved, resulting in the formation of TPs. The GC/MS analysis revealed that no investigated human metabolites/TPs within this study were formed upon UV irradiation experiment or their presence was below LODs (SM-6). Similar recent studies by Lutterbeck et al. (2015) and Lai et al. (2015) report the identification of CP and IF TPs during similar photo-assisted advanced oxidation processes, namely UV/ H_2O_2 and UV/ TiO_2 , respectively, with the accompanied toxicity evaluation of the treated samples. Both studies proposed TPs formation pathways, where among all identified TPs, ketoCP and NdCP are reported, whereas CPCOOH was not detected/identified as a TP. Due to the similarity of these treatments with the UV irradiation performed within this study, we assume that ketoCP and NdCP were formed also during UV irradiation, but were below the detection limit of the method.

The ecotoxicity evaluation of the UV irradiated CP and IF mixture samples towards *S. leopoliensis* showed no growth inhibition (Fig. 3). This agrees with the findings of a recent study by Lutterbeck et al. (2015), who also report no significant toxicity of treated samples containing CP towards *Vibrio fischeri*, where CP removals were up to 100%. Similarly, the study by Lai et al. (2015) reported no final toxicity (after the initial increase) towards *V. fischeri* of the samples containing either CP or IF treated with UV/ TiO_2 . This is in agreement with the results of this study regarding

the toxicity of individual compounds, where parent compounds as well as metabolites/TPs ketoCP and NdCP were not toxic towards the test organism. In a study where ozonation was used to degrade CP and IF, the authors report high removal of both compounds (up to 100% at pH = 11), accompanied by a significant increase in toxicity towards *V. fischeri* (Lin et al., 2015). This study did not identify the TPs that were formed during ozonation. Since it was shown that CPCOOH is capable of growth inhibition of the test organism in the present study, it is possible that this TP was formed during the ozonation treatment and contributed to the overall toxicity as Lin et al. (2015) reported. However, further studies are necessary to confirm these assumptions.

4. Conclusions

Within the present study, we investigated the ecotoxicity and genotoxicity of CP, IF, their metabolites/TPs as individual compounds and as mixture. With the exception of human metabolite/TP, CPCOOH, which revealed significant ecotoxic activity towards *S. leopoliensis*, all other compounds, namely CP, IF, ketoCP and NdCP, were non-toxic at the investigated concentrations. In accordance with the EU Directive 93/67/EEC (1996) CPCOOH can be classified as harmful to aquatic organisms. These findings underline the importance of ecotoxicity evaluation, not only for the parent compounds, but also for their structurally related human metabolites/TPs. Furthermore, the toxicity of investigated parent compounds and metabolites/TPs as a mixture suggests potentiation ecotoxic effects. The CA model predicted lower ecotoxicity of the mixture than observed within the performed experiments, which shows the importance of toxicity assessment of the mixture. The genotoxicity evaluation of the tested compounds and their mixtures with the SOS/umuC assay confirmed genotoxic activity of CP in the presence of the metabolic activation, whereas CPCOOH was active also without metabolic activation. Considering the mechanism of toxicity of CP and IF the latter finding indicate that the metabolite CPCOOH can directly interact with DNA and consequently in *S. leopoliensis* only CPCOOH caused growth inhibition. For the environmental risk assessment this is particularly important finding which indicates that for the phytoplankton this metabolite is more hazardous than parent compounds. Finally, despite significant degradation of CP and IF, the UV irradiated samples were not toxic towards investigated organism suggesting that unknown TPs formed during UV irradiation are non-ecotoxic and therefore further structural elucidation of these species is of minor importance.

The findings of the present study indicate that to carry out a proper environmental risk assessment further studies, especially on the occurrence of CPCOOH in the environment, are needed. Current pesticides regulations for risk assessment regarding soil already take into account metabolites/TPs, since certain TPs are more toxic than the parent compounds (European Food Safety Authority, 2014). Based on the results of this study, the same guidance for evaluating risk should be established for the cytostatic and other pharmaceutical residues that might occur in the aquatic environment.

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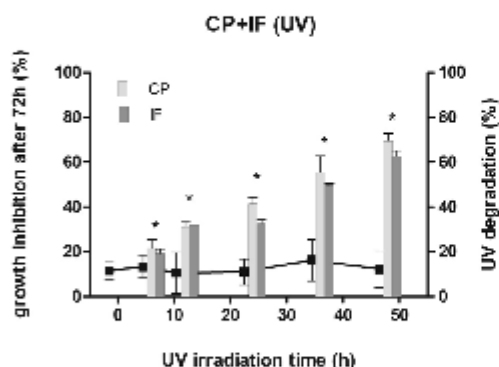


Fig. 3. Growth inhibition (black line with ■, representing the mean $\pm$ 95% confidence interval) and degradation of CP and IF (mean $\pm$ SD, both at 10 mg L^{-1}) during UV treatment. * Indicates statistically significant difference of CP and IF degradation at determined sampling time compared to control (0 h).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2015.12.017>.

References

- Benedict, W.F., Baker, M.S., Hanson, L., Choi, E., Ames, B.N., 1977. Mutagenicity of cancer chemotherapeutic agents in the Salmonella/microsome test. *Cancer Res.* 37, 2209–2213.
- Besse, J.-P., Latou, J.-F., Garric, J., 2012. Anticancer drugs in surface waters: what can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? *Environ. Int.* 39, 73–86.
- Brain, R.A., Johnson, D.J., Richards, S.M., Hanson, M.L., Sanderson, H., Lam, M.W., Young, C., Mabury, S.A., Sibley, P.K., Solomon, K.R., 2004. Microcosm evaluation of the effects of an eight pharmaceutical mixture to the aquatic macrophytes *Lemna gibba* and *Myriophyllum sibiricum*. *Aquat. Toxicol.* 70, 23–40.
- Brezovšek, P., Eleršek, T., Filipič, M., 2013. The effects of selected cytostatics and their mixtures on aquatic primary producers. *Toxicol. Lett.* 221, Supplement, S96.
- Brezovšek, P., Eleršek, T., Filipič, M., 2014. Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Synechococcus leopoliensis*. *Water Res.* 52, 168–177.
- Buerge, U.J., Buser, H.-R., Poiger, T., Müller, M.D., 2006. Occurrence and fate of the cytostatic drugs cyclophosphamide and ifosfamide in wastewater and surface waters. *Environ. Sci. Technol.* 40, 7242–7250.
- Chen, Z., Fu, G., Heckes, P., Westerhoff, P., 2008. Physicochemical treatment of three chemotherapy drugs: irinotecan, taxotane, and cyclophosphamide. *J. Adv. Oxid. Technol.* 11, 254–260.
- Commission of the European Communities, 1996. Technical Guidance Document in Support of Commission Directive 93/67/EEC on Risk Assessment for New Notified Substances and Commission Regulation (EC) No 1488/94 on Risk Assessment for Existing Substances. Part II: Environmental Risk Assessment. Office for Official Publications of the European Communities, Luxembourg.
- Cesen, M., Kosjek, T., Laimou-Geraniou, M., Kompare, B., Širok, B., Lambropoulou, D., Heath, E., 2015. Occurrence of cyclophosphamide and ifosfamide in aqueous environment and their removal by biological and abiotic wastewater treatment processes. *Sci. Total Environ.* 527–528, 465–473.
- Delgado, L.F., Fauce-Marquis, V., Pfohl-Leszkowicz, A., Dosande, C., Marion, B., Scherette, S., Albal, C., 2011. Cytotoxicity of micropollutants removal in a crossflow membrane bioreactor. *Bioresour. Technol.* 102, 4395–4401.
- Delgado, L.F., Scherette, S., Gonzalez, C., Albal, C., 2010. Effect of cytostatic drugs on microbial behaviour in membrane bioreactor system. *Bioresour. Technol.* 101, 527–536.
- Eitel, A., Scherette, S., Kümmerer, K., 2000. Handling Cytostatic Drugs: a Practical Guide. Bristol-Myers Squibb (2000).
- Eleršek, T., 2012. The advantages of flow cytometry in comparison to fluorimetric measurement in algal toxicity test. *Acta Biol. Slov.* 55 (2), 3–11.
- European Food Safety Authority, 2014. EFSA Guidance Document for evaluating laboratory and field dissipation studies to obtain DegT50 values of active substances of plant protection products and transformation products of these active substances in soil. EFSA J. 12, 3662.
- Faust, M., Altenburger, R., Backhaus, T., Banck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vigli, M., Grimme, L.H., 2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. *Aquat. Toxicol.* 63, 43–63.
- Fernández, L.A., Hernández, C., Baralier, M., Véliz, E., López, A., Ledea, O., Padrón, S., 2010. Cyclophosphamide degradation by advanced oxidation processes. *Water Environ. J.* 24, 174–180.
- Fernando-Climent, L., Rodríguez-Mozaz, S., Barceló, D., 2013. Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. *Anal. Bioanal. Chem.* 405, 5937–5952.
- Geis, S.W., Fleming, K.L., Korthals, E.T., Searle, G., Reynolds, L., Kemer, D.A., 2000. Modifications to the algal growth inhibition test for use as a regulatory assay. *Environ. Toxicol. Chem.* 19, 36–41.
- Grung, M., Källqvist, T., Sakshaug, S., Skurtveit, S., Thomas, K.V., 2008. Environmental assessment of Norwegian priority pharmaceuticals based on the EMEA guideline. *Ecotoxicol. Environ. Sci.* 71, 328–340.
- Gómez-Canela, C., Cortés-Francisco, N., Oliva, X., Pujol, C., Ventura, F., Lacorte, S., Calbach, J., 2012. Occurrence of cyclophosphamide and epirubicin in wastewaters by direct injection analysis–liquid chromatography–high-resolution mass spectrometry. *Environ. Sci. Pollut. Res.* 19, 3210–3218.
- Hales, B.F., 1982. Comparison of the mutagenicity and testogenicity of cyclophosphamide and its active metabolites, 4-hydroxycyclophosphamide, phosphoramide mustard, and acrolein. *Cancer Res.* 42, 3016–3021.
- Hansel, S., Castegnaro, M., Sportouch, M.H., Méo, M.D., Millhew, J.C., Lager, M., Duménil, G., 1996. Chemical degradation of wastes of antineoplastic agents: cyclophosphamide, ifosfamide and melphalan. *Int. Archives Occup. Environ. Health* 69, 109–114.
- Kittmeyer, T., Götz, H.-J., Jursch, M., Liders, U., 1998. Trace enrichment, chromatographic separation and biodegradation of cytostatic compounds in surface water. *Fresenius' J. Anal. Chem.* 361, 185–191.
- Kim, I., Yamashita, N., Tanaka, H., 2009a. Performance of UV and UV/H₂O₂ processes for the removal of pharmaceuticals detected in secondary effluent of a sewage treatment plant in Japan. *J. Hazard. Mater.* 166, 1134–1140.
- Kim, I., Yamashita, N., Tanaka, H., 2009b. Photodegradation of pharmaceuticals and personal care products during UV and UV/H₂O₂ treatments. *Chemosphere* 77, 518–525.
- Kováčová, L., Siegrist, H., Singer, H., Witter, A., McDowell, C.S., 2012. Hospital wastewater treatment by membrane bioreactor: performance and efficiency for organic micropollutant elimination. *Environ. Sci. Technol.* 46, 1536–1545.
- Köhler, C., Venditti, S., Igo, E., Klepšewski, K., Benetto, E., Cornelissen, A., 2012. Elimination of pharmaceutical residues in biologically pre-treated hospital wastewater using advanced UV irradiation technology: a comparative assessment. *J. Hazard. Mater.* 239–240, 70–77.
- Kümmerer, K., Al-Ahmad, A., Beitsam, B., Wiestler, M., 2000. Biodegradability of antineoplastic compounds in screening tests: influence of glucosidation and of stereochemistry. *Chemosphere* 40, 767–773.
- Kümmerer, K., Steger-Hartmann, T., Meyer, M., 1997. Biodegradability of the antitumour agent ifosfamide and its occurrence in hospital effluents and communal sewage. *Water Res.* 31, 2705–2710.
- Lai, W.W.-P., Lin, H.H.-H., Lin, A.Y.-C., 2015. TiO₂ photocatalytic degradation and transformation of oxazaphosphorine drugs in an aqueous environment. *J. Hazard. Mater.* 287, 133–141.
- Lenz, K.M.S., Weissenbacher, N., Mader, R.M., Krenn, P., Hann, S., Koellensperger, G., Uhl, M., Knaumüller, S., Reif, F., Bursch, W., Furtmüller, M., 2007. Monitoring, Removal and Risk Assessment of Cytostatic Drugs in Hospital Wastewater, 56, pp. 141–149.
- Lester, Y., Avisar D. Fau - Gozlan, I., Gozlan I. Fau - Mamane, H., and Mamane, H., Removal of pharmaceuticals using combination of UV/H₂O₂/O₃ advanced oxidation process.
- Lester, Y., Avisar D. Fau - Gozlan, I., Gozlan I. Fau - Mamane, H., Mamane, H., 2011. Removal of Pharmaceuticals Using Combination of UV/H₂O₂/O₃ Advanced Oxidation Process.
- Li, L., Fernández-Cruz, M.J., Connolly, M., Conde, E., Fernández, M., Schuster, M., Navas, J.M., 2015. The potentiation effect makes the difference: non-toxic concentrations of ZnO nanoparticles enhance Cu nanoparticle toxicity in vitro. *Sci. Total Environ.* 505, 253–260.
- Lin, A.Y.-C., Lin, Y.-C., Lee, W.-N., 2014. Prevalence and sunlight photolysis of controlled and chemotherapeutic drugs in aqueous environments. *Environ. Pollut.* 187, 170–181.
- Lin, A.Y., Hsueh, J.H., Hong, P.K., 2015. Removal of antineoplastic drugs cyclophosphamide, ifosfamide, and 5-fluorouracil and a vasodilator drug pentoxifylline from wastewaters by ozonation. *Environ. Sci. Pollut. Res.* 22, 508–515.
- Loewe, S., 1927. Die Mischcharzei – Versuch einer allgemeinen Pharmakologie der Arzneimittelkombinationen. *Klin. Wochenschr.* 6, 1077–1085.
- Loewe, S., Mulschnek, H., 1926. Über kombinationswirkungen. I. Mitteilung: Hilfsmittel der fragestellung Schmelzdeb. *Arch. Exp. Pathol. Pharmacol.* 114, 313–326.
- Lutterbeck, C.A., Machado, E.J., Kümmerer, K., 2015. Photodegradation of the antineoplastic cyclophosphamide: a comparative study of the efficiencies of UV/H₂O₂, UV/Fe²⁺/H₂O₂ and UV/TiO₂ processes. *Chemosphere* 120, 538–546.
- Moldovan, Z., 2006. Occurrences of pharmaceutical and personal care products as micropollutants in rivers from Romania. *Chemosphere* 64, 1808–1817.
- Negreira, N., de Alda, M.L., Barceló, D., 2014. Cytostatic drugs and metabolites in municipal and hospital wastewaters in Spain: filtration, occurrence, and environmental risk. *Sci. Total Environ.* 497–498, 68–77.
- Negreira, N., López de Alda, M., Barceló, D., 2013a. On-line solid phase extraction–liquid chromatography–tandem mass spectrometry for the determination of 17 cytostatics and metabolites in waste, surface and ground water samples. *J. Chromatogr. A* 1280, 64–74.
- Negreira, N., Mastrolanni, N., López de Alda, M., Barceló, D., 2013b. Multianalyte determination of 24 cytostatics and metabolites by liquid chromatography–electrospray–tandem mass spectrometry and study of their stability and optimum storage conditions in aqueous solution. *Talanta* 116, 290–298.
- OECD TG 201, 2011. Text No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. OECD Publishing.
- Parella, A., Kundi, M., Lavorgna, M., Cefisculo, E., Russo, C., Isidori, M., 2014. Toxicity of exposure to binary mixtures of four anti-neoplastic drugs in *Daphnia magna* and *Ceriodaphnia dubia*. *Aquat. Toxicol.* 157, 41–46.
- Pfleger, T., McFarlane, C., Sherman, R., Volk, A.N.D.G., 1990. Short-term Bioassay for Whole Plant Toxicity. U.S. Environmental Protection Agency, Washington, D.C., EPA/600/D-90/080 (NTIS P890246489).
- Reifferscheid, G., Heil, J., Oda, Y., Zahn, R.K., 1991. A microplate version of the SOS/umu-test for rapid detection of genotoxins and genotoxic potentials of environmental samples. *Mutat. Research/Environmental Mutagen. Relat. Subj.* 253, 215–222.
- Schmid, C., Reifferscheid, G., Zahn, R.K., Bachmann, M., 1997. Increase of sensitivity and validity of the SOS/umu-test after replacement of the β -galactosidase reporter gene with luciferase. *Mutat. Research/Genetic Toxicol. Environ. Mutagen.* 394, 9–16.
- Silva, E., Rajapakse, N., Kortenamp, A., 2002. Something from “nothing”—eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ. Sci. Technol.* 36, 1751–1756.
- Sladek, N.E., 1988. Metabolism oxazaphosphorines. *Pharmacol. Ther.* 37, 301–355.
- Steger-Hartmann, T., Kümmerer, K., Hartmann, A., 1997. Biological degradation of

3.4. Ecotoxicity and Genotoxicity of Cyclophosphamide, Ifosfamide, Their Metabolites/Transformation Products and Their Mixtures 87

- cyclophosphamide and its occurrence in sewage water. *Ecotoxicol. Environ. Saf.* 36, 174–179.
- Steger-Hartmann, T., Kümmerer, K., Schecker, J., 1996. Trace analysis of the anti-neoplastics ifosfamide and cyclophosphamide in sewage water by two-step solid-phase extraction and gas chromatography-mass spectrometry. *J. Chromatogr. A* 726, 179–184.
- Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.* 32, 3245–3260.
- Toolaram, A.P., Kümmerer, K., Schneider, M., 2014. Environmental risk assessment of anti-cancer drugs and their transformation products: a focus on their genotoxicity characterization-state of knowledge and short comings. *Mutat. Res. Reviews Mutat. Res.* 760, 18–35.
- Valcárcel, Y., González-Alonso, S., Rodríguez-Gil, J.L., Gil, A., Catalá, M., 2011. Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. *Chemosphere* 84, 1336–1348.
- Wagner, M., Loy, A., 2002. Bacterial community composition and function in sewage treatment systems. *Curr. Opin. Biotechnol.* 13, 218–227.
- Winckler, K., Obe, G., Madle, S., Nau, H., 1984. Mutagenic activities of cyclophosphamide (NSC-26271) and its main metabolites in *Salmonella typhimurium*, human peripheral lymphocytes and Chinese hamster ovary cells. *Mutat. Res. Research/Fundamental Mol. Mech. Mutagen.* 129, 47–55.
- Wols, B.A., Hofman-Castis, C.H.M., Harmsen, D.J.H., Beerendonk, E.F., 2013. Degradation of 40 selected pharmaceuticals by UV/H₂O₂. *Water Res.* 47, 5876–5888.
- Yasunaga, K., Kiyonari, A., Nakagawa, M., Yoshikawa, K., 2006. Investigation into the ability of the *Salmonella* umu test to detect DNA damage using antitumor drugs. *Toxicol. Vitro* 20, 712–728.
- Yin, J., Shao, B., Zhang, J., Li, K., 2010. A preliminary study on the occurrence of cytostatic drugs in hospital effluents in Beijing, China. *Bull. Environ. Contam. Toxicol.* 84, 39–45.
- Zounkova, R., Kovalova, I., Blaha, I., Ditt, W., 2010. Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites. *Chemosphere* 81, 253–260.
- Zounkova, R., Odrasik, P., Dolezalova, L., Hilscherova, K., Masalik, B., Blaha, I., 2007. Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals. *Environ. Toxicol. Chem.* 26, 2208–2214.
- Zuccato, E., Castiglioni, S., Fanelli, R., 2005. Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment. *J. Hazard. Mater.* 122, 205–209.

3.5 Cytotoxicity and Genotoxicity of Anticancer Drug Residues and Their Mixtures in Experimental Model With Zebrafish Liver Cells

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Hospital effluents present a major source of cytostatic residues into the environment and the occurrence of these drugs in hospital effluents can serve as a starting point to monitor their fate in the environment and to individualise the magnitude of putative pollution problems. The concentrations of cytostatic residues in effluents depend on consumption amounts, patients pharmacokinetic and dilution factor. Usually cytostatic residues occur in the environment at low doses, however as complex mixtures with possible unwanted synergistic effects.

In the present study, we demonstrated that two widely used cytostatics CP and IF induced DNA strand breaks (comet assay) and genomic stability (CBMN) in exposed zebrafish liver (ZFL) cells, however at relatively high concentrations that are not relevant for the aquatic contamination. DNA strand breaks showed to be a more sensitive endpoint than the micronuclei formation with lower observed effect concentrations.

The complex mixture that consisted of four cytostatics (CP, IF, CDDP and 5-FU) at their maximal detected concentrations in the effluents of the Oncological Ward in Slovenia increased the formation of DNA strand breaks in exposed zebrafish liver (ZFL) cells. The mixture induced the formation of DNA strand breaks at concentrations of individual compounds that were lower than those that were effective when tested as individual compounds. Contrary to the individual drugs, the mixture did not induce the micronuclei formation.

The results of the present study indicate that cytostatic residues when they occur in a mixture may pose a threat to aquatic organisms even at low environmentally relevant concentrations and contribute to the accumulating evidence that it is not always possible to predict adverse effects of complex mixtures based on the toxicological data for individual compounds.

The PhD candidate performed the MTS (3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)(cytotoxicity), comet and CBMN assay with cyclophosphamide (CP) and ifosfamide (IF). He also supervised student Barbara Modic who performed the experiments with the mixtures. He analysed raw data and drafted the manuscript.



Cytotoxicity and genotoxicity of anticancer drug residues and their mixtures in experimental model with zebrafish liver cells



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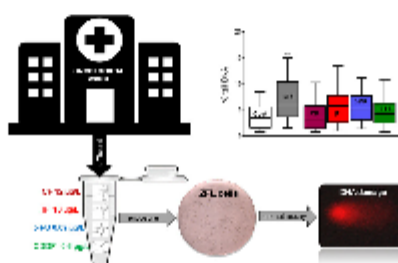
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HIGHLIGHTS

- Cyclophosphamide and ifosfamide induce genotoxic effects at high concentrations in ZFL cells.
- Mixture of anticancer drugs induces DNA damage at environmentally relevant concentrations.
- DNA damaging potential of the mixture was higher than that of single drugs.
- Mixtures of anticancer drugs may pose threat to aquatic organisms.

GRAPHICAL ABSTRACT



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ABSTRACT

Anticancer drugs enter aquatic environment predominantly via hospital and municipal wastewater effluents where they may, due to their genotoxic potential, cause adverse environmental effects even at very low doses. In this study we evaluated cytotoxic and genotoxic potential of two widely used anticancer drugs, cyclophosphamide (CP) and ifosfamide (IF) as individual compounds and in a complex mixture together with 5-fluorouracil (5-FU) and cisplatin (CDDP) because these four drugs have been frequently detected in an oncological ward effluents. As an experimental model we used zebrafish liver cell (ZFL) line. The cytotoxicity was determined with the MTS assay and genotoxicity with the comet assay and cytokinesis block micronucleus (CBMN) assay to measure the formation of DNA strand breaks and genomic instability, respectively. CP and IF exerted low cytotoxicity towards ZFL cells. Both compounds induced DNA strand breaks and genomic instability, however at relatively high concentrations that are not relevant for the contamination of aquatic environment. The mixture of CP, IF, 5-FU and CDDP was tested at maximal detected concentrations of each drug as determined in the effluents from the oncological ward. The mixture was not cytotoxic and did not induce genomic instability, but it induced significant increase in the formation of DNA strand breaks at concentrations of individual compounds that were several orders of magnitude lower from those that were effective when tested as individual compounds. The results indicate that such mixtures of anticancer drugs may pose a threat to aquatic organisms at environmentally relevant concentrations and contribute to the accumulating evidence that it is not always possible to predict adverse effects of complex mixtures based on the toxicological data for individual compounds.

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1. Introduction

Anticancer drugs (also called cytostatic or anti-neoplastic drugs) are a broad group of compounds with potent mechanisms of action mainly used for cancer treatment. Most of them prevent uncontrolled proliferation of cancer cells via their interactions with DNA function and cell signalling (McKnight, 2003). However, the effects of anticancer drugs are not selective enough, and in addition to tumour cells, these drugs affect normal growing cells and tissues, which during the chemotherapy often cause undesirable side effects.

The consumption of anticancer drugs is globally increasing; on one hand due to the increase in the incidence of cancer diseases in human population and on the other due to better early diagnostics and treatment of cancer diseases (Besse et al., 2012; Kummerer et al., 2014). After administration to patients the residues of anticancer drugs are excreted as unmetabolised active compound together with their metabolites and enter aquatic environment via hospital and municipal wastewater effluents (Ferrando-Climent et al., 2014; Kosjek and Heath, 2011; Negreiros et al., 2014; Yin et al., 2010). In the environment the anticancer drug residues are generally present at low (sub- to few ng/L) concentrations (Buerge et al., 2006; Isidori et al., 2016; Kosjek and Heath, 2011; Moldovan, 2006; Negreiros et al., 2014; Valcarcel et al., 2011; Yin et al., 2010; Zhang et al., 2013). However, due to their mechanisms of action most of these drugs are genotoxic, carcinogenic, mutagenic and/or teratogenic and can cause adverse effects in aquatic organisms, especially upon chronic exposure (Kummerer et al., 2014; Toolaram et al., 2014).

The major source of the residues of anticancer drugs are hospital wastewaters that are usually discharged to the sewage system without any treatment prior to the arrival at the municipal wastewater treatment plants (WWTPs) (Cesen et al., 2015; Ferk et al., 2009; Verlicchi et al., 2012; Zhang et al., 2013). Municipal wastewaters receive a substantial contribution of excreted anticancer drugs also as the result of outpatient treatment (Ferrando-Climent et al., 2014). Numerous studies demonstrated that many of these compounds are resistant to biodegradation and are thus not efficiently removed by conventional municipal WWTPs (Ferrando-Climent et al., 2014; Martin et al., 2014; Orías and Perrodin, 2013; Zhang et al., 2013). Thus, the likelihood that anticancer drug residues remain active after release from WWTPs, and reach surface waters is high (Besse et al., 2012; Johnson et al., 2013; Rowney et al., 2009).

Cyclophosphamide (CP) and its structural analogue ifosfamide (IF) belong to the group of alkylating agents and are among the oldest anticancer drugs in clinical practice that are still widely used in cancer chemotherapy (Zhang et al., 2006). Cyclophosphamide is used also for treatment of certain life-threatening events in autoimmune and immune-mediated diseases (Ahlmann et al., 2016). Both compounds are prodrugs that require metabolic activation by specific liver CYP enzymes (CYP3A4, CYP2B6 and CYP2C9) to phosphoramidate mustard and acrolein that interact with DNA and proteins resulting in the formation of adducts and cross-links that prevent DNA replication resulting in subsequent cell death (Emadi et al., 2009; Giraudo et al., 2010). In treatment of various cancers CP and IF are used as a single agents, but more frequently they are used in combination with other anticancer agents to obtain synergistic or additive therapeutic effect (Zhang et al., 2006). Due to the wide use of the two drugs in clinical practice their residues, belong to the most often detected anticancer drugs in hospital wastewaters (Cesen et al., 2016; Gomez-Canela et al., 2014; Isidori et al., 2016; Kim and Tanaka, 2010; Kummerer et al., 1997; Steger-Hartmann et al., 1997; Steger-Hartmann et al., 1996). Moreover, CP and IF have limited biodegradability and are released from WWTPs to the environment in unchanged concentrations (Buerge et al., 2006; Kilmeyer et al., 2013; Kummerer et al., 1997; Steger-Hartmann et al., 1997). Therefore, it is not surprising that the residues of the two drugs have been detected also in surface waters (Buerge et al., 2006; Valcarcel et al., 2011; Zuccato et al., 2000).

The literature overview revealed only few ecotoxicological studies of CP and IF. In the growth inhibition test with the bacteria *Pseudomonas putida* and alga *Pseudokirchneriella subcapitata* the no observed effect concentrations (NOEC) for CP were >1000 and ≥250 mg/L, respectively. In *Daphnia magna* acute immobilization assay the NOEC for CP was >1000 mg/L (Zoumkova et al., 2007) and for IF 162 mg/L, whereas in the *D. magna* reproduction (chronic) assay the NOEC for CP was 56 mg/L (Grung et al., 2008). In zebrafish (*Danio rerio*) embryos exposed for 3 days CP and IF induced lethal effects were observed at concentrations ≥2200 mg/L and ≥840 mg/L, respectively, whereas teratogenic effects were observed at lower concentrations (261 mg/L) (Weigt et al., 2011).

Both drugs are bacterial mutagens and induce gene mutations and chromosome aberrations in different mammalian cells in vitro (Anderson et al., 1995; Benedict et al., 1977; Ishidate et al., 1988; Jembro et al., 2007; Le Hegarat et al., 2010; Toolaram et al., 2014). In vivo CP and IF induce chromosomal damage and micronuclei formation in rats, mice and Chinese hamsters (Anderson et al., 1995; Toolaram et al., 2014). Cyclophosphamide has also been demonstrated to induce DNA strand breaks and micronuclei formation in several fish species (Ayllon and Garcia-Vazquez, 2001; Grisolia et al., 2009; Pandrangi et al., 1995; Rodriguez-Cea et al., 2003) and eel in vivo (Santos and Pacheco, 1995).

5-fluorouracil (5-FU) and cisplatin (CDDP) are another two widely used anticancer drugs that are often detected in hospital wastewaters (Mahnik et al., 2004; Lenz et al., 2007). Both compounds are genotoxic; 5-FU inhibits thymidylate synthase (TS) and induces misincorporation of fluoronucleotides (Meyers et al., 2003), whereas CDDP interacts directly with DNA causing the alkylation of DNA and DNA-DNA and DNA-protein cross-links (Siddik, 2003). Several recent ecotoxicological studies have shown that the two compounds induce reproductive and genotoxic effects also in non-target aquatic organisms. In crustaceans, 5-FU and CDDP induced DNA strand breaks and inhibited reproduction at concentrations in the range of microgram per litre (NOEC for 5-FU 2 µg/L and for CDDP 1 µg/L) (Parrella et al., 2014b; Parrella et al., 2015). Inhibition of reproduction was also observed in algae and cyanobacteria, however, at higher concentrations (NOEC for 5-FU 0.01 mg/L and CDDP 0.1 mg/L) (Brezovsek et al., 2014). In a two generation toxicity study with zebrafish 5-FU induced histopathological changes in liver and kidney, along with induction of DNA strand breaks and micronuclei (MNI) formation that were observed at concentration as low as 10 ng/L (Kovacs et al., 2015). In fresh water mussels *Unio pictorum* and *Unio tumidus* 5-fluorouracil induced DNA strand breaks were observed at concentrations in the range of milligrams per litre (Gadic et al., 2014). In higher plants (*Allium* and *Tradescantia*) 5-FU and CDDP induced micronuclei (MNI) formation was also observed at relatively high concentrations (Misik et al., 2014).

The residues of anticancer drugs are not released into the environment as single compounds, but rather as complex mixtures of parent compounds and their metabolites. Although their concentrations in the environment are low, in mixtures they may affect the organisms at much lower concentrations than exposure to a single compound. Recently, several studies have been focused on exploring the combined toxic and genotoxic effects of different anticancer drugs, mostly as binary mixtures in various aquatic organisms (Brezovsek et al., 2014; Cesen et al., 2016; Elerssek et al., 2016; Kundi et al., 2016; Misik et al., 2015; Parrella et al., 2014a) and in cell based models in vitro (Cesen et al., 2016; Gajski et al., 2016a; Mater et al., 2014). Different interactions; antagonistic, synergistic and potentiating, between anticancer drugs present in the mixtures were observed, depending on the test organism, selected anticancer drug and the concentrations of individual drug in the mixture.

The aim of this study was to evaluate cytotoxic and genotoxic potential of CP and IF in the experimental model with zebrafish liver (ZFL) cell line that has been recently reported to be highly sensitive for detection of toxic effects of anticancer drugs (Gajski et al., 2016b). In addition ZFL cells retain cyp1A activity (Colodi et al., 1994) and express cyp3a65, an

ortolog of mammalian cyp3a4, which catalyzes the majority of known drug-metabolizing reactions (Eide et al., 2014) and should be able to detect genotoxic activity of indirect acting genotoxins. The cytotoxicity was determined with the MTS assay and genotoxicity with two complementary assays: the comet assay and cytokinesis block micronucleus (CBMN) assay that measure the formation of DNA strand breaks and genomic instability, respectively. Furthermore, we evaluated cytotoxic and genotoxic potential of the complex synthetic mixture composed of four anticancer drugs that are frequently found in hospital effluents namely CP, IF, 5-fluorouracil (5-FU) and cisplatin (CDDP) at their maximal detected concentrations as determined in the effluents from the Oncological ward in Ljubljana (Cesen et al., 2015; Kosjek et al., 2013; Vidmar et al., 2015).

2. Materials and methods

2.1. Chemicals

The anticancer drugs cyclophosphamide (CP; MW 261.03; CAS 50-18-0) and ifosfamide (IF; MW 261.03; CAS 3778-73-2) were from Sigma Aldrich (Hong Kong, China), and 5-fluorouracil (5-FU; MW 130.08 g/mol; CAS 51-21-8), cisplatin (CDDP; MW 300.05 g/mol; CAS 15663-27-1) were from Sigma (St Louis, USA). Stock solutions were prepared in DMSO: CP and IF at 25 g/L (95.8 mM); CDDP at 10 g/L (33.3 mM) and 5-FU at 10 g/L (76.9 mM), aliquoted and stored at -20°C (Fig. 1).

Acridine orange (AO), bleomycin, cytochalasin-B, dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), low melting point (LMP) agarose, normal melting point (NMP) agarose, were from Sigma (St Louis, USA); penicillin/streptomycin and phosphate buffered saline (PBS) were from PAA Laboratories (Dartmouth, USA); trypsin was from BD-Difco (Le Port-De-Claix Cedex, France); Leibovitz L-15 medium and foetal bovine serum were from American Type Culture Collection (Manassas, VA, USA); Dulbecco's modified Eagle's medium and Ham F-12 medium were from Gibco (Waltham, MA, USA); HEPES and epidermal growth factor (EGF) were from Invitrogen (Paisley, UK). All other chemical reagents were of the purest grade available and all solutions were made using Milli-Q water.

2.2. Cell culture

ZFL cells derived from normal liver of a dult zebrafish (Ghosh et al., 1994) were obtained from American Type Culture Collection (ATCC number: CRL-2634). They were cultured under humidified air atmosphere at 28°C in a medium composed of 50% Leibovitz L-15, 35% DMEM, and 15% Ham F-12, supplemented with 15 mM HEPES, 0.15 g/L NaHCO_3 , 0.01 mg/mL insulin, 50 ng/mL EGF, 0.1% penicillin/streptomycin, and 5% heat inactivated FBS.

2.3. Concentrations of the tested anticancer drugs for cytotoxicity and genotoxicity determination

The cytotoxicity of CP and IF was tested within the concentration range from 0.3–300 mg/L and the genotoxicity was determined at concentrations from 10 to 300 mg/L.

The complex mixture of CP, IF, 5-FU and CDDP was tested at concentrations of each of the individual anticancer drug that were determined in the oncological ward effluents (MIX 1) and at 10-fold higher concentrations (MIX 10) (Table 1). In each experiment, also individual compounds at the same concentration as were present in the mixture, negative (solvent and growth medium) and positive [etoposide (ET) and benzo(a)pyrene (BaP)] controls were tested.

2.4. Cytotoxicity testing

The viability of ZFL cells after the exposure to the anticancer drugs and their mixtures was determined with MTS tetrazolium reduction assay (Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, USA) according to the manufacturer's instructions, with minor modifications. Prior to the treatment, the cells were seeded onto 96-well microplates (Corning Costar Corporation, New York, USA) at a density of 8000 cells/well and incubated for 24 h to attach. The growth medium was then replaced by fresh medium containing appropriate concentrations of individual compounds or mixtures and incubated for 72 h. At the end of the exposure, 40 μL of freshly prepared MTS:PMS solution (20:1) was added directly to the 200 μL of medium in the culture wells and incubated for additional 3 h (28°C , humidified air atmosphere). Finally, the absorbance was measured at wavelength of 490 nm using a Microplate Reader (Synergy MX, BioTek, Winooski, VT, USA). Cell viability was determined by comparing the OD (optical density) of the wells containing the cells treated with anticancer drugs with those of unexposed cells and presented as % of cell viability $\pm$ SD. Two independent experiments were performed, each time with five replicates per treatment point.

2.5. Genotoxicity

2.5.1. Comet assay

Comet assay was performed as described by Singh et al. (1988) with minor modifications (Gajski et al., 2016b). The cells were seeded onto 12-well tissue culture treated plates (Corning Costar Corporation, New York, USA) at a density of 100,000 cell/well and incubated for 24 h to attach. The growth medium was then replaced by fresh medium containing appropriate concentrations of individual compounds or mixtures and incubated for 72 h. After the treatment ZFL cells were trypsinized, collected and centrifuged. 30 μL of cell suspension was mixed with 70 μL 1% LMP agarose and added to fully frosted slides pre-coated with 80 μL of 1% NMP agarose, followed by lyses of the cells (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h at 4°C . Then DNA was denaturated in electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH 13) for 20 min at 4°C and the slides were electrophoresed for 20 min at 25 V (at 1 V/cm). Finally, the nuclei were stained with ethidium bromide (10 $\mu\text{g/mL}$) and images of 50 randomly selected nuclei per experimental point were analysed using a fluorescence microscope (Nikon, Eclipse 800) at 400 $\times$ magnification and the image analysis software (Comet Assay IV, Perceptive Instruments Ltd, UK). Three independent experiments were performed.

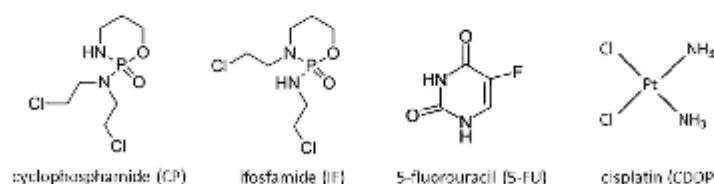


Fig. 1. The structural formulas of the studied anticancer drugs.

295

M. Novak et al. / Science of the Total Environment 601–602 (2017) 293–300

Table 1
Concentrations of CP, IF, 5-FU and CDDP in the tested mixtures.

Compound	Concentration in the mixture µg/L	
	MIX 1	MIX 10
CP	12	120
IF	10	100
5-FU	0.09	0.9
CDDP	0.6	6

2.5.2. Cytokinesis-block micronucleus (CBMN) assay

The CBMN cytome assay was performed according to Fenech (2006) with minor modifications (Gajski et al., 2016b). The cells (800,000 cell/plate) were seeded onto 25 cm² culture plates (Corning Costar Corporation, New York, USA) and incubated for 24 h to attach. The growth medium was then replaced by fresh medium containing appropriate concentrations of individual compounds or mixtures and incubated for 72 h. The treatment medium was then replaced with fresh medium containing cytochalasin B (2 µg/mL) and cells were incubated for additional 48 h at 28 °C. Afterwards, floating and adherent cells were collected by trypsinization, centrifuged, washed with 1× PBS and incubated for 5 min in cold hypotonic solution (75 mM KCl). The cells were then fixed with methanol/acetic acid (3/1) (v/v) and formaldehyde. The fixed cells were placed on a microscope slide, air dried, randomized and coded. The slides were stained with AO (20 µg/mL) and analysed under the fluorescence microscope (Eclipse 800, Nikon, Japan) at 400× magnification. A total of 2000 binucleated (BNC) cells (1000 BNC per replicate) with preserved cytoplasm were manually scored per experimental point. The number of micronuclei (MNI; expressed as the number of micronucleated (MNed) cells), nuclear buds (NBUDs) and nucleoplasmic bridges (NPBs) were determined according to standard criteria (Fenech, 2000). The nuclear division index (NDI) was estimated by scoring 500 cells with one to four nuclei. The NDI was calculated using the formula $[M1 + 2M2 + 3(M3 + M4)]/500$, where M1, M2, M3 and M4 represent the number of cells with one to four nuclei, respectively (Kirsch-Volders et al., 2003). The experiments were independently repeated twice.

2.6. Statistical analysis

Graph Pad Prism 5 (Graph Pad Software, La Jolla, USA) was used for the statistical analyses. Dose-dependent response for the samples was assessed with non-linear regression. For the MTS assay Student's *t*-test was used to compare the viability of exposed cells to that of unexposed and solvent exposed. For the comet assay data, statistical significance between the treated groups and control was determined by one-way analysis of variance (ANOVA, Kruskal-Wallis) and Dunn's multiple comparison test. The significance of CBMN assay parameters was tested using chi-square test.

3. Results

3.1. Cytotoxicity

The viability of ZFL cells, determined with the MTS assay, was reduced after 72 h exposure to CP or IF in a dose dependent manner; however, even at the highest tested concentration (300 mg/L), it remained >70% compared to untreated cells (Fig. 2A). The mixture of CP, IF, 5-FU and CDDP at concentrations determined in hospital effluents (MIX1) as well as individual anticancer drug at the same concentration as in the mixture after 72 h exposure did not significantly affect the viability of ZFL cells. At 10-fold higher concentrations the cell viability was slightly lower; however, it did not decrease for >30% compared to control cells (Fig. 2B). Therefore, these concentrations were used also in genotoxicity tests. No differences have been observed between solvent control and growth medium control (data not shown).

3.2. Genotoxicity

Genotoxic potential of CP, IF and the mixtures of CP, IF, 5-FU and CDDP in ZFL cells was determined with the comet assay and cytokinesis block micronucleus (CBMN) assay after 72 h of exposure.

3.2.1. Comet assay

Cyclophosphamide and IF induced statistically significant increase in the formation of DNA strand breaks at concentrations ≥ 37.5 mg/L; however, no clear dose response has been observed (Fig. 3A).

The mixture of CP, IF, 5-FU and CDDP significantly increased the formation of DNA strand breaks at both applied concentrations (MIX1 and MIX10) in a dose dependent manner, whereas the individual compounds at the same concentrations as present in the mixtures did not induce increased formation of DNA strand breaks (Fig. 3B).

3.2.2. Cytokinesis block micronucleus (CBMN) assay

In the CBMN assay, CP induced statistically significant increase in the MNI frequency at 150 and 300 mg/L. The number of induced MNI was 1.6- and 3.0-fold higher than in the control cells, respectively (Fig. 4).

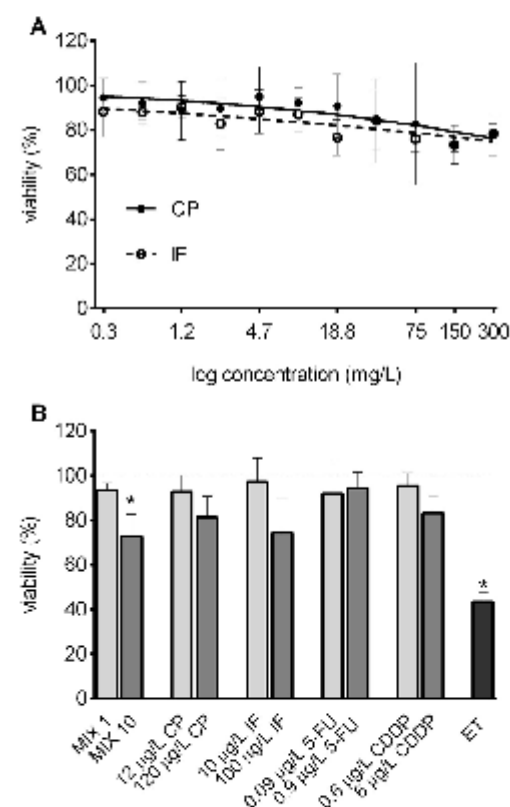


Fig. 2. The effect of cyclophosphamide (CP) and ifosfamid (IF) (A) and the complex mixture of CP, IF, 5-fluorouracil (5-FU) and cisplatin (CDDP) and individual compounds at the same concentrations as in the mixture (B) on the viability of zebrafish liver (ZFL) cells. The viability of the cells was determined with the MTS assay after 72 h exposure to the test samples. Two independent experiments, each with five replicates, were performed. The data are presented as mean values $\pm$ SD. * $p < 0.05$, significant difference between non-treated control and treated cells (Student *t*-test).

Ifosfamide was less active; statistically significant 1.5-fold increase in the frequency of MNed was observed only at the highest tested concentration (Fig. 4). At the applied concentrations, none of the tested anticancer drugs significantly affected the number of NBUDs, NPBs nor nuclear division index (NDI) (data not shown).

The mixtures of anticancer drugs and the individual anticancer drugs at the same concentrations as in the mixtures did not induce increase in the frequency of MNed, NBUD nor in NPB, and did not affect NDI (Supplementary Table 1S).

4. Discussion

The occurrence of the residues of anticancer drugs in the aquatic environment is considered to be of particular concern because these drugs may, due to their genotoxic potential, cause adverse environmental effects even at very low doses (Kummerer et al., 2014; Toolaram et al., 2014). In this study, we demonstrated that in ZFL cells CP and IF,

induced the formation of DNA strand breaks and genomic instability; however, at relatively high concentrations that are not relevant for the aquatic environment. On the other hand, we demonstrated that the complex mixture of four most abundant anticancer drugs (CP, IF, 5-FU and CDDP) in the effluents of Oncological ward in Slovenia at their maximal detected concentrations increased the formation of DNA strand breaks. The mixture induced the formation of DNA strand breaks at concentrations of individual compounds that were lower from those that were effective when tested as individual compounds.

The genotoxicity of CP, has been extensively studied and the studies demonstrated that in bacteria and in mammalian cells that are metabolically incompetent genotoxic effects were observed only when tested in the presence of the metabolic activation (Anderson et al., 1995; Cesen et al., 2016; Hartmann et al., 1995; Westerink et al., 2011), whereas in human hepatic cell lines known to retain metabolic activity such as HepG2 and HepaRG cells CP induced genotoxic effects without the addition of exogenous metabolic activation (Le Hegarat et al., 2010; Westerink et al., 2011). In ZFL cells both pro-drugs induced DNA strand breaks and micronuclei formation indicating that the cells metabolically activated both drugs. The lowest effective concentration (LOEC) for DNA strand break formation was for both compounds 37.5 mg/L, while the LOEC values for the induction of MNi formation were 150 and 300 mg/L for CP and IF, respectively. The sensitivity of ZFL cells to detect genotoxicity of CP is comparable to that of human hepatic cell lines HepG2 (Cao et al., 2007; Westerink et al., 2011) and HepaRG (Le Hegarat et al., 2010).

Hospital effluents are considered as the main source of the release anticancer drugs into the environment (Yin et al., 2010), with the highest expected and measured concentrations of these drugs among all effluents. Therefore, the occurrence of anticancer drugs in hospital effluents can serve as a starting point to monitor their fate in the environment and to individualise the magnitude of putative pollution problems (Mahnke et al., 2007). The composition and concentrations of anticancer drug residues in hospital effluents may differ across countries and mainly depend on their consumption amounts, usage patterns, excretion rates, removal rates and local factors (Toolaram et al., 2014). Predicted and measured concentrations of anticancer drugs residues in the aquatic environment are generally much lower than those expected to cause adverse effects in aquatic organisms (Besse et al., 2012; Brezovsek et al., 2014; Buerge et al., 2006; Kummerer et al., 2014; Parrella et al., 2014b;

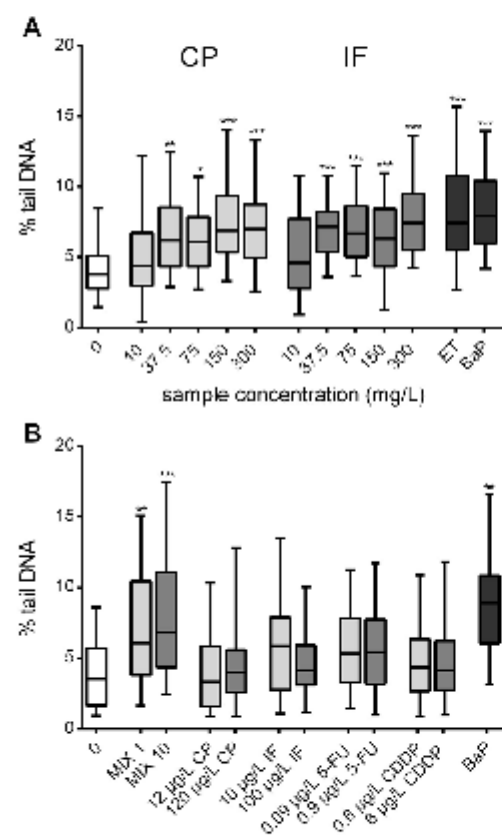


Fig. 3. DNA strand break formation induced by cyclophosphamide (CP) and ifosfamide (IF) (A) and the complex mixtures of CP, IF, 5-fluorouracil (5-FU) and cisplatin (CDDP) and individual compounds at the same concentrations as in the mixtures (B). The ZFL cells were exposed to the test samples for 72 h. The formation of DNA strand breaks was assessed with the comet assay, and is expressed as percentages of DNA in the comet tails. Fifty cells were analysed per experimental point in each of three independent experimental cultures. Positive controls were 0.1 µg/mL etoposide (ET) and 50 µM benzo(a)pyrene (BaP). * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$, significant difference between control and treated cells (1-way ANOVA, Dunn's post-test).

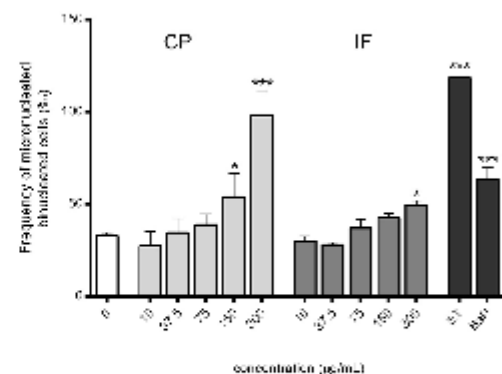


Fig. 4. Induction of micronuclei in zebrafish liver (ZFL) cells, after 72 h of exposure to cyclophosphamide (CP) and ifosfamide (IF) evaluated by cytokinesis-block micronucleus (CBMN) assay. The frequencies of micronucleated (MNed) cells were determined in 1000 binucleated ZFL cells per replicate per experimental point. Etoposide (ET; 0.1 µg/L) and benzo(a)pyrene (BaP; 10 µM) were used as positive controls. The significant difference in the number of MNed cells between treated and control cells was determined with χ^2 test * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$. Data on the figure are presented as mean values of two independent experiments $\pm$ SD.

Verlicchi et al., 2012). However, the residues of anticancer drugs occur in the environment as complex mixtures with possible unwanted synergistic effects between the drugs. Numerous studies showed that assessment of the risk that is based on ecotoxicological data for individual substances can lead to underestimation of the real environmental impact of various pollutants (Hagenbuch and Pinckney, 2012; Magnusson et al., 2010; Pomati et al., 2008; Silva et al., 2002; Sumpter et al., 2006). This can be particularly relevant for the exposure to mixtures of anticancer drug residues as in many cancer treatment regimens anticancer drugs are used in combinations to exploit their synergistic effects and achieve improved efficiency and better therapeutic effect. Moreover, these drugs interact with DNA and safe threshold level cannot be defined.

Here we evaluated cytotoxic and genotoxic activity of a complex mixture composed of CP, IF, 5-FU and CDDP at their maximal detected concentrations (12, 10, 0.09 and 0.6 µg/L, respectively) in the samples of effluents from an Oncological ward in Slovenia (Cesen et al., 2015; Kosjek et al., 2013; Vidmar et al., 2015). Although the applied concentrations are not an exact match to what has been found at one sampling time, the concentrations and composition of the complex mixture represent environmentally relevant situation. These four anticancer drugs also belong to the most abundant anticancer drugs determined in hospital effluents (Booker et al., 2014).

The mixture of the four drugs was not cytotoxic to ZFL cells and did not induce MNI formation, but it induced significant increase in DNA strand break formation in a dose dependent manner. As expected none of the individual compounds at the same concentrations as presented in the mixtures caused increased DNA strand break formation, as the LOEC values for individual compounds are several orders of magnitude higher from those in the mixtures. In our previous study we demonstrated that for the induction of DNA strand breaks the LOEC values for 5-FU and CDDP were 0.01 mg/L and 0.1 mg/L, respectively (Gajski et al., 2016b), while the LOEC value for CP and IF as demonstrated in this study is 37.5 mg/L. Similarly to our study, Mater et al. (2014) observed induction of DNA strand breaks in HepG2 exposed to complex mixture of three drugs used in chemotherapy (CP, ciprofloxacin and tamoxifen), while individual compounds at the same concentrations as present in the mixture did not induce significant increase in DNA strand breaks. Also Gajski et al. (2016a) demonstrated that the mixture of three anticancer drugs (5-FU, etoposide and imatinib mesylate) in isolated human peripheral lymphocytes induced genomic instability at concentrations of individual compounds that were several orders of magnitude lower from those that were effective when tested as individual compounds. Kundi et al. (2016) reported a synergistic effect of a binary mixture composed of 5-FU and CDDP on the induction of DNA damage in *Daphnia magna*. In the mixture, the effective concentrations of the two drugs were >10-fold lower from the effective concentrations of the individual drugs.

On the other hand, in ZFL cells the mixture did not induce chromosomal damage. This can be explained by the fact, that in ZFL cells also individual compounds, except CDDP, induced MNI formation at much higher concentrations than DNA strand breaks (Gajski et al., 2016b). However, comet assay and CMBN assay detect different types of DNA lesions with different sensitivities. Comet assay enables detection of primary DNA lesions such as alkali labile sites, single- and double strand breaks as well as DNA repair intermediates (Azqueta and Collins, 2013). These lesions are usually formed soon after the exposure and may be repaired by cellular DNA repair machinery. CMBN assay detects chromosomal damage that arises from primary DNA damage during DNA replication and cell division and is irreversible (Fenech, 2006). The positive response in the comet assay indicates that the mixture induced transient primary DNA lesions that were repaired and did not lead to the formation chromosomal damage that would be detected with the CMBN assay.

Altogether this study demonstrated that the residues of CP, IF, 5-FU and CDDP, that are all known to be genotoxic, when in a mixture, caused

in ZFL cells increased DNA strand break formation at concentrations relevant for environmental exposure. Considering that in the aquatic environment organisms are exposed chronically to complex mixtures of anticancer drug residues the consequences of adverse effects for fish as well as other aquatic organisms cannot be excluded. The assumption is supported by studies that reported DNA strand break formation and genomic instability as well as epizootic neoplasms in sentinel aquatic organisms from sites polluted by different genotoxic pollutants (Bolognesi and Hayashi, 2011; Frenzilli et al., 2009; Martins and Costa, 2015).

5. Conclusions

In fish-derived ZFL cells CP and IF induced DNA strand break formation and genomic instability at relatively high, environmentally irrelevant concentrations, whereas the mixture of four anticancer drugs composed of CP, IF, 5-FU and CDDP induced an increase in DNA strand break formation at low, for environmental contamination relevant concentrations at which individual compounds are inactive. This indicates that adverse effects in exposed aquatic organisms cannot be excluded. The results contribute to the accumulating evidence that the maximum pollutant concentration levels permitted in a water body, which are established based on the toxicological data for individual compounds, are not always sufficiently low to be protective against combined effects of mixtures of toxic pollutants. Future research should, in addition to exploring toxic and genotoxic potential of mixtures of wider range of relevant pharmaceuticals and other pollutants, focus also on exploring and understanding the consequences of continuous exposure to such mixtures over the life span of an organism.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2017.05.115>.

Conflict of interests

None declared.

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References

- Altman, M., Hempel, G., et al., 2016. Cancer Chemother. Pharmacol. 78, 651–671.
- Anderson, D., Bishopp, J.B., Garner, R.C., Ostrosky-Wegman, P., Selby, P.B., 1995. Cyclophosphamide: review of its mutagenicity for an assessment of potential germ cell risks. *Mutat. Res. Fundam. Mol. Mech. Mutagen.* 330, 115–181.
- Aylton, F., Garcia-Vazquez, E., 2001. Micronuclei and other nuclear lesions as genotoxicity indicators in rainbow trout *Oncorhynchus mykiss*. *Ecotoxicol. Environ. Saf.* 49, 221–225.
- Azqueta, A., Collins, A.R., 2013. The essential comet assay: a comprehensive guide to measuring DNA damage and repair. *Arch. Toxicol.* 87, 940–968.
- Benedict, W.F., Baker, M.S., Haroun, L., Choi, E., Ames, B.N., 1977. Mutagenicity of cancer chemotherapeutic agents in salmonella-microsome test. *Cancer Res.* 37, 2209–2213.
- Besse, J.P., Latour, J.F., Garic, J., 2012. Anticancer drugs in surface waters: what can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? *Environ. Int.* 39, 73–85.
- Bolognesi, C., Hayashi, M., 2011. Micronucleus assay in aquatic animals. *Mutagenesis* 26, 205–213.
- Booker, V., Hakkil, C., Llewellyn, N., Johnson, A., Williams, R., 2014. Prioritising anticancer drugs for environmental monitoring and risk assessment purposes. *Sci. Total Environ.* 473–474, 159–170.
- Brezovsek, P., Elensik, T., Filipic, M., 2014. Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchnerella subcapitata* and the cyanobacterium *Synechococcus leopoldensis*. *Water Res.* 52, 168–177.
- Buerge, U.J., Buser, H.R., Poiger, T., Müller, M.D., 2006. Occurrence and fate of the cytotoxic drugs cyclophosphamide and ifosfamide in wastewater and surface waters. *Environ. Sci. Technol.* 40, 7242–7250.
- Cao, J., Jiang, L.P., Liu, Y., Yang, G., Yao, X.F., Zhong, L.F., 2007. Curcumin-induced genotoxicity and antigenotoxicity in HepG2 cells. *Toxicol.* 49, 1219–1222.

- Cesen, M., Kojek, T., Laimou-Geraniou, M., Kompan, B., Sirok, B., Lambropoulos, D., Heath, E., 2015. Occurrence of cyclophosphamide and ifosfamide in aqueous environment and their removal by biological and abiotic wastewater treatment processes. *Sci. Total Environ.* 527–528, 465–473.
- Cesen, M., Elerssek, T., Novak, M., Zegura, B., Kojek, T., Filipic, M., Heath, E., 2016. Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites, transformation products and their mixtures. *Environ. Pollut.* 210, 192–201.
- Colloff, P., Miranda, C.L., Zhao, X., Babbler, D.R., Barnes, D.W., 1994. Induction of zebrafish (*Brachydanio rerio*) p450 in vivo and in cell culture. *Xenobiotica* 24, 487–493.
- Eide, M., Ruten, M., Mule, R., Jensen, K.H.M., Goksoyr, A., 2014. A characterization of the ZFL cell line and primary hepatocytes as *in vitro* liver cell models for the zebrafish (*Danio rerio*). *Aquat. Toxicol.* 147, 7–17.
- Elerssek, T., Milavc, S., Komsec, M., Brezovsek, P., Negreia, N., Zonja, B., Lopez de Alda, M., Barcelo, D., Heath, E., Ščančar, J., Filipic, M., 2016. Toxicity of the mixture of selected antineoplastic drugs against aquatic primary producers. *Environ. Sci. Pollut. Res.* 15, 14780–14790.
- Emadi, A., Jones, R.J., Brodsky, R.A., 2009. Cyclophosphamide and cancer: golden anniversary. *Nat. Rev. Clin. Oncol.* 6, 638–647.
- Fenech, M., 2000. The *in vitro* micronucleus technique. *Mutat. Res. Fundam. Mol. Mech. Mutagen.* 455, 81–95.
- Fenech, M., 2005. Cytokinesis-block micronucleus assay evolves into a “cytochrome” assay of chromosomal instability, mitotic dysfunction and cell death. *Mutat. Res.* 600, 58–65.
- Ferk, F., Misik, M., Gummert, T., Majer, B., Fuchschacker, M., Buchmann, C., Vital, M., Uhl, M., Lenz, K., Grillich, B., Paterfall, W., Neresenyan, A., Knasmüller, S., 2009. Genotoxic effects of wastewater from an oncological ward. *Mutat. Res.* 672, 69–75.
- Fernando-Clement, I., Rodriguez-Mozaz, S., Barcelo, D., 2014. Incidence of anticancer drugs in an aquatic urban system: from hospital effluents through urban wastewater to natural environment. *Environ. Pollut.* 193, 216–223.
- Frenzel, G., Nijbo, M., Lyons, B.P., 2009. The comet assay for the evaluation of genotoxic impact in aquatic environments. *Mutat. Res. Rev. Mutat. Res.* 681, 80–92.
- Gacic, Z., Kolarevic, S., Sunjig, K., Kracun-Kolarevic, M., Pasmovic, M., Knezevic-Vukovic, J., Vukovic-Gacic, B., 2014. The impact of *in vitro* and *in vivo* exposure to base analogue 5-FU on the level of DNA damage in haematocytes of freshwater mussels *Unio pictorum* and *Unio lamifus*. *Environ. Pollut.* 191, 145–150.
- Gajski, G., Gerić, M., Domijan, A.-M., Garaj-Vrhovac, V., 2016a. Combined cytogenotoxic activity of a selected antineoplastic drug mixture in human circulating blood cells. *Chemosphere* 165, 520–538.
- Gajski, G., Gerić, M., Zegura, B., Novak, M., Nanić, J., Bajraktarevic, D., Garaj-Vrhovac, V., Filipic, M., 2016b. Genotoxic potential of selected cytostatic drugs in human and zebrafish cells. *Environ. Sci. Pollut. Res.* 23, 14739–14750.
- Ghosh, C., Zhou, Y.L., Golladi, P., 1994. Derivation and characterization of a zebrafish liver cell line. *Cell Biol. Toxicol.* 10, 167–176.
- Gislad, B., Hebert, G., Deroussent, A., Veal, G.J., Vassal, G., Paci, A., 2010. Oxazaphosphorine: new therapeutic strategies for an old class of drugs. *Expert Opin. Drug Metab. Toxicol.* 6, 919–938.
- Gomez-Canela, C., Ventura, F., Calvo, J., Lacorte, S., 2014. Occurrence of cytostatic compounds in hospital effluents and wastewater, determined by liquid chromatography coupled to high-resolution mass spectrometry. *Anal. Bioanal. Chem.* 405, 3801–3814.
- Grisolia, C.K., Oliveira, R., Domingues, I., Oliveira-Filho, E.C., Monerat, R.G., Soares, A.M., 2009. Genotoxic evaluation of different delta-endotoxins from *bacillus thuringiensis* on zebrafish adults and development in early life stages. *Mutat. Res.* 672, 119–123.
- Guang, M., Kallqvist, T., Salhaug, S., Sturtvik, S., Thomas, K.V., 2008. Environmental assessment of Norwegian priority pharmaceuticals is based on the EMEA guideline. *Ecotoxicol. Environ. Saf.* 71, 328–340.
- Hagenbuch, L.M., Pincney, J.L., 2012. Toxic effect of the combined antibiotics ciprofloxacin, linezolid, and tylosin on two species of marine diatoms. *Water Res.* 46, 5028–5036.
- Hartmann, A., Herkommer, K., Gluck, M., Speit, G., 1995. DNA-damaging effect of cyclophosphamide on human blood cells *in vivo* and *in vitro* studied with the single-cell gel test (comet assay). *Environ. Mol. Mutagen.* 25, 180–187.
- Ishidate Jr., M., Harino, M.C., Sofuni, T., 1988. A comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell cultures. *Mutat. Res.* 195, 151–213.
- Isidori, M., Lavorgna, M., Russo, C., Kundli, M., Zegura, B., Novak, M., Filipic, M., Misik, M., Knasmüller, S., Lopez de Alda, M., Barcelo, D., Zonja, B., Cesen, M., Ščančar, J., Kojek, T., Heath, E., 2016. Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain. *Environ. Pollut.* 219, 275–287.
- Jermol, S., Rocha, P.S., Ketter, S., Stutlarek, D., Farber, H., Jones, P.D., Giesy, J.P., Holbert, H., Engwall, M., 2007. Perfluorooctane sulfonate increases the genotoxicity of cyclophosphamide in the micronucleus assay with V79 cells. Further proof of alterations in cell membrane properties caused by PFOS. *Environ. Sci. Pollut. Res.* 14, 85–87.
- Johnson, A.C., Oldenkamp, R., Dumont, E., Sumpter, J.P., 2013. Predicting concentrations of the cytotoxic drugs cyclophosphamide, carboplatin, 5-fluorouracil, and capecitabine throughout the sewage effluents and surface waters of Europe. *Environ. Toxicol. Chem.* 32, 1954–1961.
- Köffmeyer, T.K., Turk, J., Hahn, M., Stuetz, H., Hadtstein, C., Heinemann, A., Eickmann, U., 2013. Application and assessment of a regular environmental monitoring of the antineoplastic drug contamination level in pharmacies – the MEVAP project. *Ann. Occup. Hyg.* 57, 444–455.
- Kim, I., Tanaka, H., 2010. Use of ozone-based processes for the removal of pharmaceuticals detected in a wastewater treatment plant. *Water Environ. Res.* 82, 294–301.
- Kirsch-Volders, M., Sofani, T., Aardema, M., Albertini, S., Eastmond, D., Fenech, M., Ishidate, M., Kirschner, S., Loge, E., Mosta, T., Norppa, H., Surralles, J., Wakata, A., 2003. Report from the *in vitro* micronucleus assay working group. *Mutat. Res.* 540, 153–163.
- Kojek, T., Heath, E., 2011. Occurrence, fate and determination of cytostatic pharmaceuticals in the environment. *Toxicol. Trends Anal. Chem.* 30, 1055–1087.
- Kojek, T., Perlo, S., Zigon, D., Heath, E., 2013. Fluorouracil in the environment: a analysis, occurrence, degradation and transformation. *J. Chromatogr. A* 1290, 62–72.
- Kovacs, R., Cseni, Z., Bakos, K., Urbanyi, B., Horvath, A., Garaj-Vrhovac, V., Gajski, G., Gerić, M., Negreia, N., Lopez de Alda, M., Barcelo, D., Heath, E., Kojek, T., Zegura, B., Novak, M., Zajc, I., Baelier, S., Rotter, A., Ramsak, T., Filipic, M., 2015. Assessment of toxicity and genotoxicity of low doses of 5-fluorouracil in zebrafish (*Danio rerio*) two-generation study. *Water Res.* 77, 201–212.
- Kummerer, K., Steger-Hartmann, T., Meyer, M., 1997. Biodegradability of the anti-tumour agent ifosfamide and its occurrence in hospital effluents and communal sewage. *Water Res.* 31, 2705–2710.
- Kummerer, K., Hain, A., Schuster, A., Hein, A., Ebert, I., 2014. Antineoplastic compounds in the environment: substances of special concern. *Environ. Sci. Pollut. Res.* 23, 14791–14804.
- Kundi, M., Parnella, A., Lavorgna, M., Griscuolo, E., Russo, C., Isidori, M., 2016. Prediction and assessment of ecogenotoxicity of antineoplastic drugs in binary mixtures. *Environ. Sci. Pollut. Res.* 23, 14771–14779.
- Le Heganat, L., Dumont, J., Jasse, R., Huet, S., Lancelot, R., Mounot, A., Paul, J.M., Gugen-Guillouzo, C., Guillozo, A., Fessard, V., 2010. Assessment of the genotoxic potential of indirect chemical mutagens in HepaRG cells by the comet and the cytokinesis-block micronucleus assays. *Mutagenesis* 25, 555–560.
- Lenz, K., Mahnik, S.N., Weissenbacher, N., Mader, R.M., Krenn, P., Hain, S., Koellensperger, G., Uhl, M., Knasmüller, S., Ferk, F., Bursh, W., Fuchschacker, M., 2007. Monitoring, removal and risk assessment of cytostatic drugs in hospital wastewater. *Water Sci. Technol.* 56, 141–148.
- Magnusson, M., Heilmann, K., Quayle, P., Negri, A.P., 2010. Additive toxicity of herbicide mixtures and comparative sensitivity of tropical benthic microalgae. *Mar. Pollut. Bull.* 60, 1978–1987.
- Mahnik, S.N., Rixová, B., Fuchschacker, M., Mader, R.M., 2004. Determination of 5-fluorouracil in hospital effluents. *Anal. Bioanal. Chem.* 380, 31–35.
- Mahnik, S.N., Lenz, K., Weissenbacher, N., Mader, R.M., Fuchschacker, M., 2007. Fate of 5-fluorouracil, doxorubicin, epirubicin, and daunorubicin in hospital wastewater and their elimination by activated sludge and treatment in a membrane-bio-reactor system. *Chemosphere* 66, 30–37.
- Martin, J., Camacho-Munoz, D., Santos, J.L., Aguilera, I., Alonso, E., 2014. Occurrence and ecotoxicological risk assessment of 14 cytostatic drugs in wastewater. *Water Air Soil Pollut.* 225, 210.
- Martins, M., Costa, P.M., 2015. The comet assay in environmental risk assessment of marine pollutants: applications, assets and handicaps of surveying genotoxicity in non-model organisms. *Mutagenesis* 30, 89–106.
- Mate, N., Geret, F., Castillo, I., Falcón-Marquis, V., Abasi, C., Pfohl-Leschowicz, A., 2014. *In vitro* tests aiding ecotoxicological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water. *Environ. Int.* 63, 191–200.
- McKnight, J.A., 2003. Principles of chemotherapy. *Clin. Tech. Small Anim. Pract.* 18, 67–72.
- Meyers, M., Huang, A., Wagner, M.W., Brumming, A.J., Veigl, M.L., Sedwick, W.D., Boothman, D.A., 2003. A role for DNA mismatch repair in sensing and responding to fluoropyrimidine damage. *Oncogene* 22, 7376–7388.
- Misik, M., Pichter, C., Rainer, R., Filipic, M., Neresenyan, A., Knasmüller, S., 2014. Acute toxic and genotoxic activities of widely used cytostatic drugs in higher plants: possible impact on the environment. *Environ. Res.* 135, 196–203.
- Misik, M., Filipic, M., Neresenyan, A., Misikova, K., Knasmüller, S., Kundli, M., 2015. Analyses of combined effects of cytostatic drugs on micronucleus formation in the *Tradescantia*. *Environ. Sci. Pollut. Res.* 15, 14762–14770.
- Moldovan, Z., 2006. Occurrences of pharmaceutical and personal care products as micropollutants in rivers from Romania. *Chemosphere* 64, 1808–1817.
- Negreia, N., de Alda, M.L., Barcelo, D., 2014. Cytostatic drugs and metabolites in municipal and hospital wastewaters in Spain: filtration, occurrence, and environmental risk. *Sci. Total Environ.* 487, 68–77.
- Olias, F., Perodin, Y., 2013. Characterisation of the ecotoxicity of hospital effluents: a review. *Sci. Total Environ.* 464, 250–276.
- Pandurangi, R., Petras, M., Ralph, S., Vitoz, M., 1995. Alkaline single cell gel (comet) assay and genotoxicity monitoring using bullheads and carp. *Environ. Mol. Mutagen.* 26, 345–356.
- Parnella, A., Kundli, M., Lavorgna, M., Griscuolo, E., Russo, C., Isidori, M., 2014a. Toxicity of exposure to binary mixtures of four anti-neoplastic drugs in *Daphnia magna* and *Ceriodaphnia dubia*. *Aquat. Toxicol.* 157, 41–46.
- Parnella, A., Lavorgna, M., Griscuolo, E., Russo, C., Fumano, V., Isidori, M., 2014b. Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans. *Chemosphere* 115, 59–65.
- Parnella, A., Lavorgna, M., Griscuolo, E., Russo, C., Isidori, M., 2015. Ecogenotoxicity of six anticancer drugs using comet assay in daphnids. *J. Hazard. Mater.* 295, 573–580.
- Pomati, F., Orlandi, C., Cricchi, M., Luciani, F., Zucaro, E., 2008. Effects and interactions in an environmentally relevant mixture of pharmaceuticals. *Toxicol. Sci.* 102, 129–137.
- Rodriguez-Cea, A., Aylon, F., Garcia-Vazquez, E., 2003. Micronucleus test in freshwater fish species: an evaluation of its sensitivity for application in field surveys. *Ecotoxicol. Environ. Saf.* 56, 442–448.
- Rowney, N.C., Johnson, A.C., Williams, R.J., 2009. Cytotoxic drugs in drinking water: a prediction and risk assessment exercise for the Thames catchment in the United Kingdom. *Environ. Toxicol. Chem.* 28, 2733–2743.
- Santos, M.A., Pacheco, M., 1995. Mutagenicity of cyclophosphamide and left mill effluent and sediment on the cell *Anguilla anguilla* L. *Sci. Total Environ.* 171, 127–130.
- Siddik, Z.H., 2008. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 22, 7265–7279.
- Silva, E., Rajapakse, N., Kortenkamp, A., 2002. Something from “nothing” – eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ. Sci. Technol.* 36, 1751–1756.
- Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175, 184–191.
- Steger-Hartmann, T., Kummerer, K., Schaefer, J., 1996. Trace analysis of the antineoplastic ifosfamide and cyclophosphamide in sewage water by two-step solid-phase extraction and gas chromatography mass spectrometry. *J. Chromatogr. A* 726, 179–184.

- Steger-Hartmann, T., Kummerer, K., Hartmann, A., 1997. Biological degradation of cyclophosphamide and its occurrence in sewage water. *Ecotoxicol. Environ. Saf.* 36, 174–179.
- Sumpter, J.P., Johnson, A.C., Williams, R.J., Kortenkamp, A., Scholze, M., 2006. Modeling effects of mixtures of endocrine disrupting chemicals at the river catchment scale. *Environ. Sci. Technol.* 40, 5478–5489.
- Taoussan, A.P., Kummerer, K., Schneider, M., 2014. Environmental risk assessment of anti-cancer drugs and their transformation products: a focus on their genotoxicity characterization-state of knowledge and short comings. *Mutat. Res. Rev. Mutat. Res.* 760, 18–35.
- Valcarlos, V., Gonzalez Alonso, S., Rodriguez-Gil, J.L., Gil, A., Catala, M., 2011. Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. *Chemosphere* 84, 1336–1348.
- Vedlichi, P., Al Aulidy, M., Galletti, A., Petrovic, M., Barcelo, D., 2012. Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. *Sci. Total Environ.* 430, 109–118.
- Vidmar, J., Martincic, A., Milacic, R., Scancar, J., 2015. Speciation of cisplatin in environmental water samples by hydrophilic interaction liquid chromatography coupled to inductively coupled plasma mass spectrometry. *Talanta* 138, 1–7.
- Weigt, S., Huebler, N., Strecker, R., Baurbeck, T., Broschard, T.H., 2011. Zebrafish (*Danio rerio*) embryos as a model for testing potentogens. *Toxicology* 281, 25–36.
- Westonik, W.M.A., Schlaris, T.J.J., Hotbach, G.J., Schoonen, W.G.E.J., 2011. Development and validation of a high-content screening in vitro micronucleus assay in CHO-K1 and HepG2 cells. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 724, 7–21.
- Yin, J., Shao, B., Zhang, J., Li, K., 2010. A preliminary study on the occurrence of cytostatic drugs in hospital effluents in Beijing, China. *Bull. Environ. Contam. Toxicol.* 84, 39–45.
- Zhang, H., Tian, Q., Zhou, S.F., 2006. Clinical pharmacology of cyclophosphamide and ifosfamide. *Curr. Drug Ther.* 1, 55–84.
- Zhang, J., Chang, V.W., Gianni, A., Wang, J.Y., 2013. Removal of cytostatic drugs from aquatic environment: a review. *Sci. Total Environ.* 445–446, 281–298.
- Zoukova, R., Odrasik, P., Dolezalova, L., Hilschenova, K., Masalek, B., Blaha, L., 2007. Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals. *Environ. Toxicol. Chem.* 26, 2208–2214.
- Zucato, E., Calamari, D., Natangelo, M., Fanelli, R., 2000. Presence of therapeutic drugs in the environment. *Lancet* 355, 1780–1790.

3.6 Chemical and Toxicological Characterisation of Anticancer Drugs in Hospital and Municipal Wastewaters From Slovenia and Spain

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In the present study samples of hospital effluents and influents/-effluents from waste water treatment plants (WWTPs) from Slovenia and Spain in two different sampling campaigns (January and June) were analyzed for the presence of twenty-two selected cytostatics, their metabolites and transformation products. Acute and chronic toxicity tests were performed on the crustacean *Ceriodaphnia dubia*, genotoxicity with micronucleus (MN) assay with *Tradescantia* and *Allium cepa* and *in vitro* comet assay with zebrafish (*Danio rerio*) liver cell line (ZFL cells).

The results showed widespread presence of cytostatic residues in various wastewaters with higher measured concentrations in Slovenian wastewaters. The most frequently detected cytostatics are platinum-based compounds and among individual drugs erlotinib, cyclophosphamide, tamoxifen and methotrexate.

The effluent samples were toxic to *C. dubia* after acute exposure, reduced reproduction after chronic exposure, increased MN frequencies in *Tradescantia* and induced DNA strand breaks in ZFL cells. These results demonstrated that effluents could be harmful to aquatic organisms. A statistical correlation between toxicity data and the presence of some of the identified drugs has been found. However, observed toxicity did not correlate with the total burden of the samples and the residues of cytostatics indicating, that other unidentified pollutants contributed to these differences.

Basically, the effluents from the WWTPs investigated should be diluted by more than 90% to obtain no observed toxic effects as shown by no observed effect concentration values. The possible presence of different pollutants in complex mixtures makes it difficult to comprehend the actual ecological risk posed by the targeted compounds. Thus, a chemical characterisation closely linked to an eco/genotoxicological approach, as used in this study, should be applied for better understanding of the environmental toxic/genotoxic impact of xenobiotic mixtures.

The PhD candidate performed the MTS and comet assay with ZFL cells and analysed data. He contributed in preparing the manuscript.



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Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain^{☆,☆☆}



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ABSTRACT

Anticancer drugs are continuously released into hospital and urban wastewaters, where they, most commonly, undergo conventional treatment in wastewater treatment plants (WWTPs). Wastewaters contain complex mixtures of substances including parent compounds, their metabolites and transformation products (TPs). In this study, samples of hospital effluents and WWTP influents and effluents from Slovenia and Spain were analyzed for twenty-two selected anticancer drugs, their metabolites and transformation products. Acute and chronic toxicity tests were performed on the crustacean *Ceriodaphnia dubia*, genotoxicity was determined with *Tradescantia* and *Allium cepa* micronucleus (MN) assays and *in vitro* comet assay in zebrafish (*Danio rerio*) liver cell line (ZFL cells). Sixty of the two hundred-twenty determinations revealed detectable levels of anticancer drug residues. Among the targeted compounds, platinum based were most frequently detected (90%). Furthermore, erlotinib was detected in 80%, cyclophosphamide and tamoxifen in 70% and methotrexate in 60% of the samples. Seven of ten samples were toxic to *C. dubia* after acute exposure, whereas after chronic exposure all samples reduced reproduction of *C. dubia* at high sample dilutions. *Allium cepa* proved insensitive to the potential genotoxicity of the tested samples, while in *Tradescantia* increased MN frequencies were induced by a hospital effluent and WWTP influents. In ZFL comet assay all but one sample induced a significant increase of DNA strand breaks. Correlations of chemotherapeutics or their TPs were detected for all bioassays except for *Allium cepa* genotoxicity test, however for each test the highest correlations were found for different substances indicating differential sensitivities of the test organisms.

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1. Introduction

The presence of anticancer drugs in the aquatic environment has prompted significant interest concerning their potential adverse ecological effects. After administration to patients the drugs are excreted through faeces and urine as mixtures of unchanged parent compounds and their metabolites and can enter the aquatic environment predominantly via treated and untreated

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hospital and municipal wastewaters. These excreted mixtures of parent compounds and metabolites may undergo further abiotic and/or biotic transformation, either during wastewater treatment or in the environment. Recent scientific interest has focused especially on occurrence and fate of anticancer drugs, their metabolites and transformation products (TPs) in aquatic systems (Kosjek et al., 2013; Martín et al., 2014; Negreira et al., 2014a; Česen et al., 2015, 2016a).

Anticancer drug residues occur in the aquatic environment at sub-ng L⁻¹ levels (Xie, 2012), which are too low to pose an immediate threat to aquatic organisms, but can cause long-term delayed toxic effects since they interfere directly or indirectly with DNA. Recently, Brezovšek et al. (2014) showed that 5-fluorouracil and cisplatin cause chronic effects in green algae (*Pseudokirchneriella subcapitata*), resulting in growth inhibition at concentrations equivalent to those found in hospital effluents. Chronic exposure to these drugs was also shown to inhibit reproduction in crustaceans (Parrella et al., 2014a). At chronic exposure, 5-fluorouracil produced in zebrafish histopathological changes, and genotoxic effects at environmental concentrations (Kovács et al., 2015). It is crucial, therefore, to consider the possible toxic/genotoxic effects in organisms exposed over their life span to the continuous presence and possible accumulation of not just the parent anticancer drugs, but also their metabolites and transformation products (Todiaram et al., 2014; Česen et al., 2016b).

Hospital wastewaters are a major source of anticancer drugs. Usually these waters are not treated at source, but are discharged directly into the sewerage system, finally arriving at a wastewater treatment plant (WWTP) (Perk et al., 2009; Verlicchi et al., 2012; Zhang et al., 2013; Česen et al., 2015). In addition, urban wastewaters receive a substantial contribution of excreted anticancer drugs as the result of outpatient treatment (Ferrando-Clement et al., 2013). Studies reveal that conventional treatments do not achieve high removal efficiencies for these compounds, which are in many cases resistant to biodegradation (Zhang et al., 2013; Ferrando-Clement et al., 2014; Martín et al., 2014; Orias and Perrodin, 2014). Thus, the likelihood of pharmaceuticals and their residues remaining active after release from WWTPs, and reaching surface waters is high (Rowney et al., 2009; Besse et al., 2012; Johnson et al., 2013).

The aims of this study were to evaluate, in two sampling campaigns, the occurrence of twenty-two selected anticancer drug residues including their metabolites and transformation products (from this point onwards collectively named as TPs) in hospital effluents (a Slovenian oncological clinic and a Spanish general hospital) and municipal WWTP influents and effluents from the same two countries that differ in terms of water resources, WWTP technology and water reuse. Moreover, to investigate the relationship between the occurrence of anticancer drugs in wastewater samples and their possible biological and ecological effects, a multispecies toxicological evaluation was performed on some indicators, followed by a correlation analysis. Chemical characterisation was performed using chromatography coupled to mass spectrometry (GC-MS and LC-MS/MS), while the toxicological evaluation was performed using the following test systems: acute and chronic aquatic toxicity tests in the crustacean *Ceriodaphnia dubia*, a very sensitive primary consumer of the freshwater aquatic chain; micronucleus (MN) assays in *Tradescantia* and *Allium cepa* as representatives of higher plants and an *in vitro* cytotoxicity test and a comet assay for genotoxicity using zebrafish (*Danio rerio*) liver cell line (ZFL cells) as a model for vertebrates. These bioindicators are extensively used to investigate the whole toxicity of chemicals in the environment. These organisms are sensitive to a wide range of aquatic contaminants and allow to address the biological effects of chemicals on different organizational structures.

2. Materials and methods

2.1. Chemicals and standards

The following compounds were determined in the wastewater samples: cisplatin (ds-Pt, as total Pt), 5-fluorouracil (5-FU, CAS 51-21-8), cyclophosphamide (CP, CAS 50-18-0), ifosfamide (IF, CAS 3778-73-2), keto-cyclophosphamide (keto-CP, CAS 27046-19-1), 2-dechloroethylifosfamide or *N*-dechloroethylcyclophosphamide (*N*-decl-CP, CAS 36761-83-8), carboxy-cyclophosphamide (carboxy-CP, CAS 22788-18-7), capecitabine (CAP, CAS 154361-50-9), doxorubicin (DOX, CAS 23214-92-8), erlotinib (ERL, CAS 183321-74-6), etoposide (ETP, CAS 33419-42-0), gemcitabine (GEM, CAS 95058-81-4), imatinib mesylate (IMA, CAS 220127-57-1), irinotecan (IRI, CAS 97682-44-5), methotrexate (MET, CAS 59-05-2), hydroxymethotrexate (OH-MET, CAS 5939-37-7), paclitaxel (PAC, CAS 33069-62-4), 6(α)-hydroxypaclitaxel (OH-PAC, CAS 153212-75-0), tamoxifen (TAM, CAS 10540-29-1), endoxifen or 4-hydroxy-*N*-desmethyl-tamoxifen (OH-D-TAM, CAS 112093-28-4), (Z)-4-hydroxytamoxifen (OH-TAM, CAS 68047-06-3) and temozolomide (TMZ, CAS 85622-93-1). Limits of detection (LOD) and quantification (LOQ) are shown in Table 1.

2.1.1. Cyclophosphamide, ifosfamide and their TPs

Cyclophosphamide (99%) and IF (99%) were purchased from Sigma Aldrich (Hong Kong, China). Carboxy-CP, 4-keto-CP, *N*-decl-CP and deuterated cyclophosphamide (CP-d₆; CAS 951173-63-0) used as internal standard for CP and IF analysis were obtained from Niomech - IIT GmbH (Bielefeld, Germany). The deuterated ibuprofen (IB-d₆, CAS 121662-14-4), obtained from CDN Isotopes (Quebec, Canada), was used as internal standard for the analysis of TPs. The derivatizing agent trifluoroacetic anhydride (TFAA, 99%) was purchased from Fluka (Buchs, Switzerland) and *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (MTBSTFA with 1% TBDMCS, 95%) was purchased from Sigma Aldrich (Steinheim, Germany). All solvents were of analytical grade purity.

2.1.2. Fluorouracil

Fluorouracil (CAS 51-21-8; ≥99%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Internal standard 5-FU-d₆ (CAS 90344-84-6; 98%) was purchased from LGC Standards GmbH (Wesel, Germany) while 5-Chlorouracil (5-ClU, CAS 1820-81-1; 98%) was obtained from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). The agent used for derivatization, MTBSTFA was purchased from Acros Organics (Geel, Belgium). All solvents were of analytical grade purity.

2.1.3. Pt

Merck stock Pt solution (1000 µg Pt mL⁻¹ in 8% hydrochloric acid; HCl) was diluted daily with water for the preparation of fresh calibration standard solutions that were used for the determination of the total concentrations of Pt in the samples. All chemicals were of analytical reagent grade and acids of suprapure quality (Merck, Darmstadt, Germany). All water used was of ultrapure quality (18.2 MΩ cm, Direct-Q 5 Ultrapure water system, Millipore Watertown, MA, USA).

2.1.4. Multi-target analysis of 15 anticancer drugs and TPs

Capecitabine, DOX hydrochloride, OH-D-TAM, ERL hydrochloride, ETP, GEM hydrochloride, IRI hydrochloride trihydrate, OH-MET, OH-PAC, OH-TAM, IMA mesylate, MET, TAM citrate and TMZ were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and PAC was supplied by Aldrich (Milwaukee, WI, USA) at the highest available purity (>99%). The isotopically labeled

Table 1

Abbreviations for substances analyzed in wastewater samples (H from hospital, Wi WWTP influent, We WWTP effluent) and the limits of detection (LOD) and quantification (LOQ).

Abbreviation	Substance	LOD (ng/L)		LOQ (ng/L)	
		We	H/Wi	We	H/Wi
5-FU	5-fluorouracil	0.5	0.5	1.6	1.6
CAP	capecitabine	0.5	0.7	3.5	5.0
Carboxy-CP	carboxy-cyclophosphamide	23	23	78	78
CP	cyclophosphamide	23	23	7.7	7.7
DOC	docetaxel	0.7	0.8	2.4	2.5
ERL	erlotinib	0.9	0.5	3.0	1.7
ETP	etoposide	12	20	40	65
GEM	gemcitabine	0.7	0.7	9.3	9.3
IF	ifosfamide	4.8	4.8	16	16
IMA	imatinib	36	54	120	180
IRI	irinotecan	0.4	1.4	1.2	4.5
Keto-CP	keto-cyclophosphamide	13	13	44	44
MET	methotrexate	0.5	0.6	1.8	2.0
N-decl-CP	2-dechloroethylcyclophosphamide or N-dechloroethylcyclophosphamide endosulfen or	2.0	2.0	6.7	6.7
OH-D-TAM	4-hydroxy-N-desmethyl-tamoxifen	1.5	1.5	5.0	5.0
OH-MET	hydroxymethotrexate	1.3	1.6	4.3	5.2
OH-PAC	(α)-hydroxypaclitaxel	1.1	1.1	3.6	3.6
OH-TAM	(Z)-4-hydroxytamoxifen	0.3	0.7	1.1	5.0
PAC	paclitaxel	1.2	1.3	4.0	4.4
Pt	platinum	1.0	1.0	2.0	2.0
TAM	tamoxifen	0.9	1.0	3.0	3.4
TMZ	temozolomide	1.0	1.1	9.3	9.3

standards capecitabine- d_{11} , gemcitabine- $^{13}C^{15}N_2$ hydrochloride, erlotinib- d_8 hydrochloride, etoposide- d_3 , 7-hydroxymethotrexate- d_3 , 4-hydroxy-ethyl-tamoxifen- d_5 , 4-hydroxy-N-desmethyl-tamoxifen- d_5 , 6 α -hydroxypaclitaxel- d_5 , irinotecan- d_{10} hydrochloride, methotrexate-methyl- d_3 , N-desmethyl-imatinib- d_8 , paclitaxel- d_3 and temozolomide- d_3 were purchased from Santa Cruz Biotechnology.

2.2. Sampling

For the purpose of the present study, ten wastewater samples were collected in total, five in Ljubljana (Slovenia) and five in Barcelona (Spain). Two types of samples were collected in January 2014 in Ljubljana (L) and Barcelona (B), namely, hospital effluents (H) and wastewater treatment plant influents (Wi) (Table 2). The next sampling campaign, performed in both countries in June 2014, included three sites: H, Wi, and wastewater treatment plant effluents (We, Table 2). The coding and sampling details are presented in Table 2. The Slovenian hospital under investigation is a medium-sized specialised oncological clinic with 290 beds, 13,000 admissions and 110,000 outpatients per year, with a hydraulic load

of 50–60 m³/day. The Spanish hospital is a large general hospital including cancer treatment ward with 850 beds, 41,000 admissions and 465,000 outpatients per year, with a hydraulic load of 400 m³/day. Water treatment technologies receiving wastewater from hospitals in Slovenia and Spain included mechanical and conventional biological treatment with suspended biomass. The Slovenian wastewater treatment plant is designed for 360,000 population equivalents (PE) with an average load/inflow of 80,000 m³ wastewater entering the WWTP per day. The sludge retention time is 7 days, while the hydraulic retention time is 21 h. The average biomass concentration in the biological tank is 3.2 g L⁻¹. The Spanish wastewater treatment plant is designed for 1,700,000 PE with an average load/inflow of 234,000 m³ of wastewater entering the WWTP on a daily basis. The sludge retention time is 15–20 days, while the hydraulic retention time is 8–12 h. The average biomass concentration in the biological tank is 3.5–4.0 g L⁻¹. All collected samples (between 5 and 10 L) were filtered (0.5 μ m glass fibre filters) and frozen immediately after collection. Shipment took place 2 days after sample collection. All samples were received at final destination frozen and were either defrosted and analyzed or stored frozen for no longer than 2 months prior to analysis, period

Table 2

Coding of collected wastewater samples within sampling campaigns in Slovenia and Spain in January and June 2014.

Code	Country	City	Wastewater type	Type of sampling	Date	T (°C)	Flow (m ³ /day)ow
L-1H	Slovenia	Ljubljana	Hospital	Gmb ^a	16.1.2014	n.a.	
L-1Wi	Slovenia	Ljubljana	WWTP influent	24-h time proportional	29.1.2014	14.0	82,500
L-2H	Slovenia	Ljubljana	Hospital	Gmb ^b	4.6.2014	n.a.	
L-2Wi	Slovenia	Ljubljana	WWTP influent	24-h time proportional	2.6.2014	19.6	59,400
L-2We	Slovenia	Ljubljana	WWTP effluent	24-h time proportional	3.6.2014	21	59,700
B-1H	Spain	Barcelona	Hospital	Gmb ^b	21.1.2014	12.4	334
B-1Wi	Spain	Barcelona	WWTP influent	24-h time proportional	21.1.2014	18.0	177,262
B-2H	Spain	Barcelona	Hospital	Gmb ^b	3.6.2014	23.1	341
B-2Wi	Spain	Barcelona	WWTP influent	24-h time proportional	3.6.2014	24.0	235,670
B-2We	Spain	Barcelona	WWTP effluent	24-h time proportional	3.6.2014	24.0	235,670

n.a. not available.

^a Snap samples collected at approximate 5 min intervals between 9:30 and 10:30.

^b Snap samples collected at approximate 5 min intervals between 12:00 and 13:00.

^c www collected in collection tank (flow not applicable).

during which most compounds are stable (Negreira et al., 2014b).

2.3. Chemical analysis

All the samples were filtered through 0.45 μm -filters. The Slovene laboratory used Sartorius Stedim Biotech, Göttingen, Germany and the Spanish laboratory used Whatman (Fairfield, Connecticut, USA) prior to chemical analyses.

2.3.1. Cyclophosphamide, ifosfamide and TPs

Cyclophosphamide, IF and keto-CP were extracted from 100 mL sample with Oasis HLB (60 mg, 3 cc) cartridges (Waters, Massachusetts, USA). Cartridges were conditioned with ethyl acetate, methanol and water. The sorbent was dried before elution with ethyl acetate. Carboxy-CP and N-decl-CP were extracted with Isolute ENV + (100 mg, 3 cc) cartridges. Conditioning and sorbent drying were performed as for CP, IF and keto-CP, while 5% acetic acid/ethyl acetate was used for elution. CP and IF were derivatized with TFAA and TPs with MTBSTFA with 1% TBDMCS. The details are described elsewhere (Cesen et al., 2015, 2016a).

2.3.2. Fluorouracil

One hundred millilitre samples were concentrated using SPE with Isolute ENV + cartridges (1 g, 6 mL, Biotage AB, Uppsala, Sweden). The cartridges were conditioned with 6 mL of methanol, equilibrated with 6 mL of deionised water and enriched with 100 mL of wastewater samples (pH = 6). These were then vacuum-dried and compounds of interest eluted with 3 $\times$ 2 mL of methanol. The extracts were dried under nitrogen and dissolved in 150 μL ethyl acetate. Derivatization (1h at 80 $^{\circ}\text{C}$) was performed using 30 μL of MTBSTFA. All details are described elsewhere (Kosjek et al., 2013).

2.3.3. Pt

10 mL samples were acidified with 0.1 mL of HNO_3 (CAS 7697-37-2, Merck, Darmstadt, Germany) per 100 mL of sample for total Pt determination (Vidmar et al., 2015).

2.3.4. Fifteen anticancer drugs and TPs

A previously published multi-residue method was used for determination of fifteen selected cytostatic compounds and metabolites (Negreira et al., 2013). In brief, 5 mL of samples acidified to pH 2 containing a mixture of the isotopically labeled standards at 100 ng L^{-1} were filtered and preconcentrated (5 mL) using on-line SPE with an automated SymbiosisTM Pico system from Spark Holland (Emmen, The Netherlands) with PLRP-s (crosslinked styrene-divinylbenzene polymer, 10 mm $\times$ 2 mm i.d., 15–25 μm particle size) cartridges.

2.4. Instrumental analysis

2.4.1. Cyclophosphamide, ifosfamide and TPs

An HP 6890 series (Hewlett-Packard, Waldbron, Germany) gas chromatograph with a single quadrupole mass selective detector was used (Cesen et al., 2015, 2016a). Separation was performed with two different temperature programmes, one for parent compounds and the other for their TPs. The MS was operated in the EI ionisation mode at 70 eV. The presence of investigated compounds in samples was qualitatively and quantitatively confirmed by retention time and by selective ion monitoring (SIM mode) as described in Cesen et al. (2015, 2016a).

2.4.2. Fluorouracil

An Agilent 450-GC hyphenated with an ion trap 240-MS mass spectrometer was employed to determine 5-FU. The details

regarding instrumental conditions are given in Kosjek et al. (2013). The ion trap MS was operated in electron impact (EI) ionisation mode. To confirm the identity of 5-FU in actual water samples the following criteria were applied: retention time matching; MSMS spectrum match and product ion ratios.

2.4.3. Pt

The content of cis-Pt in all the samples analyzed was determined by measuring total Pt using inductively coupled plasma mass spectrometry (Agilent Technologies 7700x ICP-MS, Tokyo, Japan). The ^{195}Pt isotope was monitored. At optimized instrumental parameters, instrumental LOD and LOQ were 0.005 ng Pt mL^{-1} and 0.017 ng Pt mL^{-1} (3 σ and 10 of the blanks). The linearity of the signal was confirmed from LOD to 10 μg Pt mL^{-1} . Repeatability of the measurements was >3%. The following ICP-MS operating parameters were employed: forward power of 1500 W, plasma gas flow of 15.0 L min^{-1} , carrier gas flow of 0.25 L min^{-1} and a dilution gas flow of 0.92 L min^{-1} .

2.4.4. Fifteen anticancer drugs and TPs

As reported previously by Negreira et al. (2013), on-line SPE was coupled to a 4000QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Siex, Foster City, California, USA). The mass spectrometer was operated in the positive ion mode. Data acquisition was performed in the selected reaction monitoring (SRM) mode. Quantification was performed by stable isotope dilution method. For positive confirmation of the presence of a compound in a sample, the LC retention of the compound in the sample must match that of the standard with a margin of $\pm$ 2%, and its SRM1/SRM2 ratio cannot deviate by > 20–50% (depending on the SRM1/SRM2 value) from the ratio in the standard (Council of the European Communities, 2002).

2.5. Toxicity tests

2.5.1. Acute and chronic toxicity in *Ceriodaphnia dubia*

The *C. dubia* acute toxicity test was performed on neonates hatched from ephippia (MicroBioTest) after 3–4 days of incubation under a light source of 6000 lux at 25 ± 1 $^{\circ}\text{C}$ in synthetic ISO medium (hardness of 250 mg L^{-1} expressed as CaCO_3) according to EPA-600-4-90 procedure (US EPA, 1993). At least four sample dilutions were prepared starting from each raw sample in three replicates. Ten organisms were exposed for 24h to 1.0 mL of sample dilutions. Organisms were incubated in the dark at 25 $^{\circ}\text{C}$ in multiwell plates. The dilution causing 50% lethality after 24h was indicated as LC50.

The *C. dubia* population growth inhibition test (ISO 20665, 2008) was performed over 7 days on neonates (less than 24h old) of at least the third generation of females coming from a healthy mass culture (starting organisms were purchased from Aquatic Research Organisms, Inc., Hampton, NH, USA). Organisms were individually exposed in glass beakers with 25 mL of sample (ten replicates for each sample dilutions). In both, acute and chronic tests, the highest dilution was $\leq$ 90% (v/v) of the raw sample, on account of the 10% of test medium, which is required for a sufficiently high concentration of dissolved oxygen and, in case of chronic test, food supply. Beakers with daphnids were incubated at 25 $^{\circ}\text{C}$ with a 16:8 h light: dark cycle (600 lux). Daily, at the renewal time, the offspring produced by each parent organism, was counted and removed (starting from the fourth day of exposure). The organisms were fed daily on 200 μL of an YCT/algae (1/1) suspension. YCT is a mix of 5 g L^{-1} each of food fish, *Saccharomyces cerevisiae* and Alfalfa (*Medicago sativa*). The algal cells were from cultures of *Pseudokirchneriella subcapitata* (10^8 cells mL^{-1}). The reproductive output of females

exposed to the samples was compared to that of the negative control to calculate the dilution able to determine the median reproduction inhibition effect in 7 days (EC50).

Two independent experiments were performed for each sample. Computations of EC50 values were performed by estimating the number of offspring from each sample by negative binomial regression with a log link. EC50 values were then computed as $\ln(2)$ divided by the slope parameter. No Observed Effect Concentration (NOEC) for each wastewater sample was determined based on the regression equation by calculating the dose that corresponds to the lower 95% confidence limit of the number of offspring in the control sample.

2.5.2. *Micronucleus (MN) assay with Tradescantia*

Tradescantia MN (Trad MN) assays were performed according to the protocol of Ma et al. (1994) with clone #4430. Per concentration, 15 cuttings were exposed to the water samples for 24h followed by a 24h recovery time. After the treatment, the inflorescences were fixed in a mix of ethanol/acetic acid (3:1) for 24h and stored in 70% ethanol. Per experimental point, tetrad preparations of at least five buds were made and stained with 2% acetocarmine (Sigma St. Louis, USA). 1500 early phase tetrads were scored in each experimental group. Tap water was used as a negative control. Maleic hydrazide (MH, Sigma St. Louis, USA) (20 mg L^{-1}) was used in all experimental series as a positive control. The results were analyzed by generalized linear model with Poisson counts and a log link. Comparisons to negative control were done by simple contrasts and p-values were corrected according to Bonferroni-Holm; p-values ≤ 0.05 were considered as significant.

2.5.3. *Micronucleus assay in Allium cepa*

Allium MN assays were performed according to the standard protocol published by Ma et al. (1995). Young onion bulbs (diameter 12–21 mm, Schneeball Weiss, Austrosaat, Vienna, Austria) were placed in 13 mL glass tubes filled with tap water in the dark for 24h. Subsequently, the roots (length = 1 cm) were exposed to test samples and their dilutions in the dark for 24h and then transferred to fresh tap water for an additional 24h. At the end of the recovery period, the roots were fixed in a mix of ethanol and glacial acetic acid (3:1) for 24h and stored in 70% ethanol. Tap water was used in all experiments as a negative control. Maleic hydrazide (MH) 10 mg L^{-1} was used in all experimental series as a positive control.

Micronuclei were scored according to the criteria described by Ma et al. (1995) using 2% acetocarmine for staining. For each experimental point, the MN frequencies were determined in five plants. From each bulb, two slides were made and 500 cells were evaluated per slide (5000 cells per dose) after staining. Furthermore, also the mitotic indices (MIs) were determined in 1000 cells (100 cells/root) per experimental point. The results were analyzed by a generalized linear model with Poisson counts and a log link. Comparisons to negative control were done by simple contrasts and p-values were corrected according to Bonferroni-Holm; p-values ≤ 0.05 were considered as significant.

2.5.4. *In vitro comet assay in zebrafish (Danio rerio) liver cell line*

The zebrafish (*Danio rerio*) liver cell line (ZFL) is derived from adult zebrafish (Chosh et al., 1994) and was obtained from the American Type Culture Collection (ATCC number: CRL-2634). Cells were cultured under a humidified air atmosphere at 28°C in a medium containing 50% Leibovitz L-15 (ATTC), 35% DMEM (Gibco), and 15% Ham F-12 (Gibco), supplemented with 15 mM HEPES (Invitrogen, Paisley, UK), 0.15 g L^{-1} NaHCO_3 , 0.01 mg mL^{-1} insulin, 50 ng mL^{-1} epidermal growth factor (EGF; Invitrogen, Paisley, UK), 0.1% penicillin/streptomycin, and 5% heat inactivated fetal bovine

serum (FBS; ATTC).

Prior to the genotoxicity testing the cytotoxicity of the samples was determined with the MTS (CellTiter 96[®] Aqueous Non-Radioactive Cell Proliferation Assay (Promega, USA) according to the manufacturers' protocol. To ensure sterile conditions the samples were filtered through $0.22 \mu\text{m}$ -filters (Corning Costar Corporation, Corning, NY, USA). ZFL cells (8000 cells/well) were seeded into 96-well microtiter plates (Nunc, Naperville, IL, USA) and incubated 24 h at 28°C to attach. The growth medium was then replaced by fresh medium containing 0, 10, 20 and 30 v/v % of the sample and incubated for a further 72h. Subsequently, the MTS/PMS mixture was added to each well and incubated at 28°C for additional 3h. The optical density (OD) was measured at 490 nm with a microplate spectrophotometer (Synergy MX, BioTek, Winooski, USA). Cell viability was determined by comparing absorbance values of control cells with absorbance values of treated cells. The viability was measured in three independent experiments with five replicates per treatment point. Etoposide ($1 \mu\text{g mL}^{-1}$) was used as a positive control. The statistical significance between treated groups and the control was determined with one-way ANOVA followed by Dunnett's multiple comparison test and $p < 0.05$ was considered as statistically significant.

For the comet assay ZFL cells (50,000 cells/well) were seeded into 12-well microtiter plates (Corning Costar Corporation, Corning, NY, USA) and were incubated at 28°C for 24h to attach. The growth medium was then replaced with fresh medium containing non-cytotoxic concentrations of the samples and incubated for 72 h at 28°C . At the end of the exposure, the comet assay was performed as described by Tice et al. (2000) with minor modifications (Straser et al., 2011). The slides were stained with ethidium bromide (5 mg mL^{-1}) and images of 50 randomly selected nuclei per experimental point were analyzed with image analysis software Comet Assay IV (Perceptive Instruments, UK). The results were obtained from three independent experiments. Benzo[a]pyrene (BaP; $50 \mu\text{M}$) and etoposide (ETP; 100 ng mL^{-1}) were used as positive controls. Statistical significance between the control and the treated groups was determined by one-way analysis of variance (ANOVA; Kruskal–Wallis), and Dunnett's multiple comparison for multiple comparison versus the control. DNA damage was expressed as percentages of tail DNA.

2.6. Analysis of the relationship between concentration of chemicals and eco-toxicity/genotoxicity data

For the statistical analysis, only those substances that were detected in at least 7 of the 10 samples were included. This was the case for CP, ERL, MET, OH-TAM, Pt and TAM.

No correlation analyses were performed with the data from *Allium* MN assay as consistently negative results were obtained with this model.

The concentration-response functions for the *C. dubia* acute and chronic toxicity assays were used to calculate the 10, 20, 30, and 100% wastewater responses to match the concentrations applied in the ZFL comet assay and in the *Tradescantia* MN assays. Results of the chemical analysis were used to calculate the concentrations of chemotherapeutics and TPs in the diluted samples. If the dilution would result in a concentration less than half the LOD, the data were omitted. Spearman correlation coefficients were computed for data from the *C. dubia* chronic and acute toxicity tests and the ZFL comet assay and *Tradescantia* MN assay.

To explore the possible effects of a mixture of substances present in wastewater samples, the data were assessed by multivariate stepwise linear regression of the toxicity test results using the log concentrations of the selected compounds. As p-value for inclusion 5% was chosen and for removal a p-value of 10%.

Table 3

Concentration (average $\pm$ SD of three replicates in ng L^{-1}) of analyzed anticancer drugs, metabolites or transformation products detected in wastewater samples from Slovenia and Spain (January and June 2014). Confident limits are in parentheses.

Substance (ng/L)	L-1H ^a	L-1Wi	L-2H	L-2Wi	L-2We	B-1H	B-1Wi	B-2H	B-2Wi	B-2We
Pr ^b	226 $\pm$ 4	27 $\pm$ 3	352 $\pm$ 8	23 $\pm$ 1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CP	1080 $\pm$ 200	27 $\pm$ 7	22100 $\pm$ 800	19 $\pm$ 3	17 $\pm$ 5	32 $\pm$ 1	6.0 $\pm$ 2.5	<LOQ	<LOQ	<LOQ
IF	48 $\pm$ 10	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Keto-CP	270 $\pm$ 4	<LOQ	1340 $\pm$ 10	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
N-decl-CP	847 $\pm$ 58	<LOQ	5520 $\pm$ 110	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Carboxy-CP	17700 $\pm$ 400	<LOQ	60600 $\pm$ 1000	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5-FU	6.9 $\pm$ 1.0	3.1 $\pm$ 0.4	<LOQ	<LOQ	<LOQ	2.1 $\pm$ 0.3	<LOQ	<LOQ	3.5 $\pm$ 0.5	<LOQ
GEM	<LOQ	61 $\pm$ 1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
MET	19 $\pm$ 2	308 $\pm$ 5	3920 $\pm$ 70	29 $\pm$ 1	<LOQ	29 $\pm$ 7	29 $\pm$ 2	<LOQ	8.3 $\pm$ 1.3	<LOQ
OH-MET	<LOQ	365 $\pm$ 35	490 $\pm$ 49	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IRI	9.2 $\pm$ 2.4	49 $\pm$ 10	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ERL	4.0 $\pm$ 0.2	8.1 $\pm$ 2.2	2.0 $\pm$ 0.1	3.5 $\pm$ 0.4	3.8 $\pm$ 0.3	5.5 $\pm$ 0.3	7.2 $\pm$ 0.6	2.4 $\pm$ 0.1	6.1 $\pm$ 0.2	3.3 $\pm$ 0.1
CAP	<LOQ	<LOQ	106 $\pm$ 6	158 $\pm$ 13	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
OH-D-TAM	<LOQ	66 $\pm$ 25	<LOQ	<LOQ	<LOQ	11 $\pm$ 0	75 $\pm$ 8	<LOQ	<LOQ	14 $\pm$ 0
OH-TAM	<LOQ	35 $\pm$ 14	10 $\pm$ 0	<LOQ	<LOQ	<LOQ	7.7 $\pm$ 0.9	<LOQ	<LOQ	<LOQ
TAM	<LOQ	61 $\pm$ 13	10 $\pm$ 0	11 $\pm$ 1	7.1 $\pm$ 0.4	<LOQ	15 $\pm$ 4	7.4 $\pm$ 0.1	6.7 $\pm$ 0.0	<LOQ

TMZ, DOX, PAC and OH-PAC are not shown in the table since they were <LOQ in all samples.

IMA and ETP, not shown since they were detected only in L-1Wi and L-2H, respectively, above LOD but below LOQ.

The abbreviations of the substances are given in Table 1.

^a For codes of the sample sites see Table 2.

^b Cisplatin and all metabolites were below LOD, therefore only total platinum concentrations are given.

During all laboratory work, safety precautions, using personal protective equipment, were taken and proper disposal procedures for hazardous wastes were followed.

3. Results and discussion

3.1. Results of chemical analysis

Table 3 shows concentrations of analyzed anticancer drugs, metabolites or transformation products detected in wastewater samples from Slovenia and Spain. Even though it is difficult, if not impossible, to compare both hospitals and WWTPs since they differ in size and application of tested compounds (Table 2), we still noticed that concentrations of the measured compounds were in general higher in Slovenian than in Spanish wastewaters in both campaigns and at all sampling points. When examining results for Slovene wastewaters, we notice a decrease of the total concentrations of the studied compounds in the following order $H > Wi > We$, while in Spain, levels were generally similar in all three matrices

(Fig. 1). Higher levels of total concentrations in Slovene hospital wastewater, when compared to WWTP samples, were expected since hospital wastewater, which was collected from an oncological ward, represents only 0.07% of the total load at the corresponding WWTP (Fig. 1). On the contrary, Spanish samples were collected at a general hospital, which is approximately 4-times bigger and includes besides cancer treatment ward also other wards diluting anticancer drug concentrations; accordingly the differences between the detected concentrations of analytes in hospital effluents and WWTP influents were less significant.

There was no systematic difference between detected concentrations of analytes in samples collected in January and June in Ljubljana, Slovenia. This agrees with the fact that hospital WW was sampled at the collection basin and that flow at the corresponding WWTP was similar at both sampling campaigns (Table 2). On the contrary, we did find some differences between the two sampling campaigns in the Spanish samples where, with the exception of 5-FU and TAM, the levels of drugs were lower in summer in both the hospital and the WWTP influent samples despite the flows being in

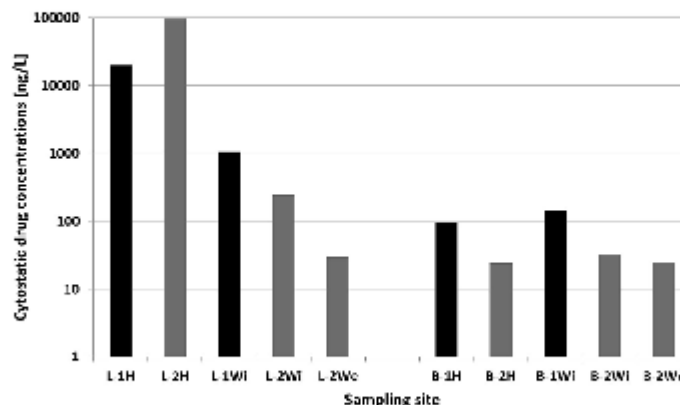


Fig. 1. Cytostatic compounds concentrations in various water samples (January: black bars, June: grey bars, H: hospital wastewater; Wi: WWTP influent; and We: WWTP effluent, L: collected in Ljubljana, Slovenia and B: Barcelona, Spain).

the same range (Table 2). This might be attributed to a higher degradation activity in summer because of the effect of the temperature (12–18 °C in winter vs 23–24 °C).

CP and IF share similar chemical structures and both act as alkylating cytostatic agents. Regardless, CP was detected in all Slovene samples and in two Spanish samples (B-1H and B-1Wi) sampled in January, while IF was above LOD only in Slovene hospital sample (L-1H). This agrees with the fact that CP is administered more often than IF due to less severe side effects after being administered (Česen et al., 2016a). CP, and especially its TPs, were also major contributors to the total load of anticancer drugs in the Slovene hospital effluent (Fig. 2). For example, the sample taken in June at Slovene hospital (L-2H) contained the highest concentration of CP among all analyzed parent compounds (22.0 $\mu\text{g L}^{-1}$). This level was approximately 20-times higher than the one reported in the sample taken in January at the same location (1.08 $\mu\text{g L}^{-1}$). It is difficult to provide an explanation for this difference since many factors can affect the amount of a certain compound, for example hospital water consumption at the time of sampling, sampling method (grab sampling or time/flow proportional sampling), time of sampling, number of hospitalized patients, number of patients receiving chemotherapy with CP and the pharmacokinetics of these patients.

The most abundant TP/metabolite in this study was a CP TP, namely carboxy-CP in L-2H (60.6 $\mu\text{g L}^{-1}$). Moreover, the concentrations of all CP TPs were higher in the hospital sample taken in June (L-2H) and correspond to a higher CP concentration in the same sample if compared to the sample taken in January (L-1H). Regardless, the ratios between the measured concentrations of CP and its TPs are not comparable in samples from both campaigns. The reason for this discrepancy could also be the varying pharmacokinetics of patients receiving the therapy at the time of sampling as all targeted TPs are also known human metabolites (Česen et al., 2016a). With the exception of CP, all the samples taken from WWTPs in Ljubljana and Barcelona contained other CP and IF

residues (carboxy-CP, keto-CP and N-decl-CP) < LODs. The reason is most likely the dilution of hospital wastewater in the wastewater that enters the WWTPs. In addition, among all the detected compounds in WWTP effluents (CP, ERL, TAM and OH-D-TAM), CP was the most abundant (17 ng L^{-1} in L-2We sample). Generally, CP dominated in all Slovene samples including its metabolites in hospital wastewaters, while in Spanish samples, the predominant compounds were MET, ERL and TAM without a clear pattern observed for each matrix.

CAP, which is a prodrug of 5-FU, was found at concentrations above 100 ng L^{-1} in the Slovene hospital and wastewater influent samples L-2H and L-2Wi, respectively, while 5-FU was either not detected or detected at concentrations in the low ng L^{-1} range. This unexpected finding might be explained by the higher biodegradability of 5-FU compared to CAP. In addition, the global trend is towards the prescription of CAP over 5-FU since CAP allows oral administration and has less severe side-effects, while 5-FU is still intravenously administered (Kosjek et al., 2013).

Total platinum was detected in higher concentrations in all samples from Slovenia compared to Spain. The highest concentrations of total platinum were detected in Slovene hospital wastewater (L-1H and L-2H), where a slightly higher concentration was reported for the sample taken in June (352 ng L^{-1}) than in January (226 ng L^{-1}) (Table 3).

Between all tested compounds, only ERL was detected in all the tested samples. In all cases, ERL concentrations were lower in the hospital WWs than in the corresponding WWTP influents and effluents. One reason could be that sources other than hospitals contribute towards the input of ERL in wastewater, i.e. out-patients who receive chemotherapy at the hospital, but excrete cytostatic residues via urine at home. Another explanation is that the difference derives from different sampling approaches. Hospital wastewaters were representative of a short time interval (1h, Table 2), while time-proportional sampling was performed at the corresponding WWTPs (Table 2).

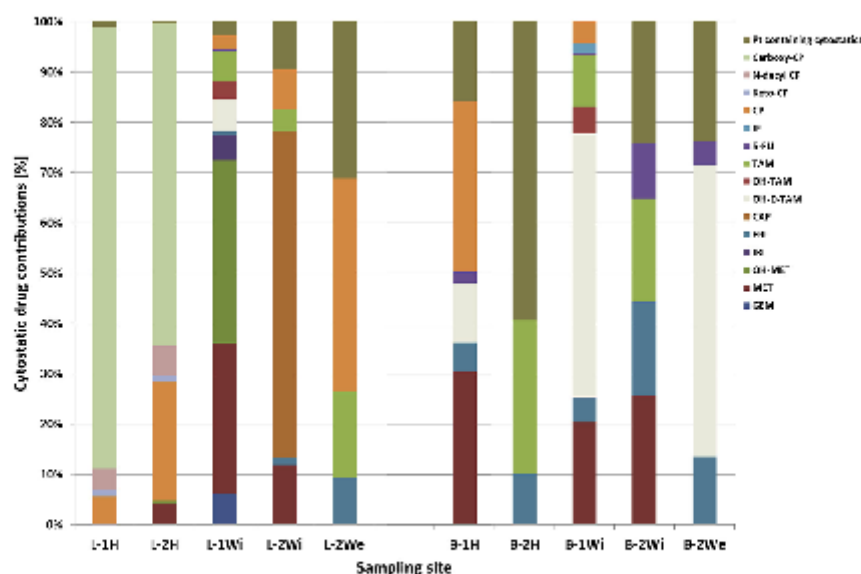


Fig. 2. Relative contribution (%) of total concentration) of the various cytostatic compounds measured in each of the samples analyzed.

In contrast to ERL, several other targeted analytes within this study showed concentrations in the hospital wastewater always higher than in the corresponding WWTP influent, i.e. Pt, IF and the already mentioned CP residues. Overall, 11 out of the 16 compounds detected were at least in one occasion in higher concentrations in the WWTP influent than in the corresponding hospital effluent (Table 3). While for 5-FU, GEM, MET, IRI, TAM and especially CAP the explanation can be the same as for ERL, the TP, namely OH-MET, could be present in higher concentrations in WWTP influent than in hospital effluent due to their transformation from the parent compounds on their way from the hospital to the WWTP (e.g. hydrolysis, biodegradation; Kosjek and Heath, 2011; Kosjek et al., 2015). Similarly, OH-D-TAM was detected at higher concentration in the effluent than in the influent of the Spanish WWTP in June, most likely due to (bio)transformation from TAM during wastewater treatment via benzene hydroxylation and *N*-demethylation, since they are common reaction pathways (Klein et al., 2013). Besides OH-D-TAM and ERL, also CP and TAM were detected in Slovene WWTP effluent at second sampling campaign at concentrations 17 ng L⁻¹ and 7.1 ng L⁻¹, respectively (Table 3). Their calculated removal (8.5% for CP and 35.5% for TAM) was expected as these compounds are known to be poorly biodegradable during the conventional biological treatment (Česen et al., 2015). On the contrary, several compounds were removed during wastewater treatment and their concentrations were <LOD in the WWTP effluent, while they were detected in WWTP influents in June in Slovene samples, i.e. Pt, MET, CAP and, surprisingly, TAM in Spanish samples since this compound is known to be poorly biodegradable (Table 3). Based on the results of this study and others like that conducted by Negreira et al. (2014a), we can conclude that the compounds CP, ERL, TAM and OH-D-TAM are among the most recalcitrant and their occurrence in wastewaters and surface waters should be studied further as well as their potential to be degraded by alternative treatment processes like a advanced oxidation process.

3.2. Results of toxicity tests

3.2.1. Acute and chronic toxicity in *Ceriodaphnia dubia*

When anticancer drugs were tested as single compounds, they induced acute toxic effects in daphnids at concentrations in the order of mg L⁻¹ (Parrella et al., 2014a). These levels were much higher than those found by chemical analysis in the present study (Table 3) where the concentrations of the anticancer residues were generally in the range of ng L⁻¹. Only the concentrations of CP and its TPs were in the order of µg L⁻¹ in Slovenian hospital wastewaters (L-1H and L-2H), although CP occurrence is not of particular concern as it induced acute toxicity in daphnids at concentrations of g L⁻¹ (Sanderson et al., 2003). Of the wastewaters examined, three samples (L-2H, L-2We and B-1H) did not induce acute effect in *C. dubia*, while the LC50 values of other samples ranged from 28.9 to 77.5% (v/v) of the raw sample (Table 4).

The effects of the tested samples on the reproduction of *C. dubia* after 7-day chronic exposure were observed at one-two orders of magnitude higher dilutions than acute toxicity. The most pronounced long-term toxic effect was observed for B-2H, with a median inhibition of reproduction at 1.4% (v/v) of the raw sample and a NOEC value equal to 0.1% (v/v). According to Table 3 and Fig. 2, B-2H toxicity could be due to the presence of TAM, ERL and to a lesser degree to Pt compounds. Indeed, tamoxifen is able to cause chronic toxicity in *C. dubia* at ng L⁻¹ (DellaGreca et al., 2007) and Pt compounds (e.g. Cisplatin) and kinase inhibitors (e.g. Erlotinib or Imatinib) can inhibit daphnid reproduction at µg L⁻¹ (Besse et al., 2012; Parrella et al., 2014a). On the other hand, according to Parrella et al. (2014b), when Pt compounds and kinase inhibitors are in mixtures, they can have additive or synergistic effects in

Table 4
Acute and chronic toxicity of Slovenian and Spanish wastewater samples collected in January and June 2014 in *C. dubia*.

Assay	Effect indicator	L-1H	L-1W	L-2H	L-2We	L-2W	B-1H	B-1W	B-2H	B-2W	B-2We
<i>C. dubia</i> acute	LC50 ^a	64.9 (53.9–70.8)	68.6 (65.9–71.3)	>100	44.3 (40.7–47.8)	>100	>100	64.0 (59.4–68.6)	77.5 (70.9–84.1)	28.9 (25.8–32.0)	51.9 (47.1–56.6)
<i>C. dubia</i> chronic	EC50 ^b	3.7 (2.8–5.3)	3.0 (2.4–4.1)	7.4 (5.2–12.7)	3.6 (2.7–5.4)	7.0 (5.0–11.4)	5.6 (4.0–8.9)	3.5 (2.5–6.0)	1.4 (1.0–2.1)	15.9 (11.2–27.8)	11.7 (8.4–19.2)
	NOEC ^b	2.6	2.1	4.1	1.8	4.7	3.1	2.4	0.1	6.7	6.3

^a For abbreviations of samples sites see Table 2.
^b Median effective concentrations and No Observed Effect Concentration (NOEC) expressed as the percentage (v/v).

C. dubia causing the same effect on reproduction at much lower concentrations than each compound individually tested. Since the same anticancer drugs were found also in the other samples, the observed chronic toxicity could either be due to synergistic effects of these compounds in mixtures or to the presence of other active compounds not identified in this study that might contribute to the overall toxicity of the samples.

3.2.2. Micronucleus assay with *Tradescantia* and *Allium cepa*

Tradescantia MN assay results for June samples are presented in Fig. 3. It can be seen that positive results were obtained with undiluted samples from the Slovenian oncological clinic (L-2H), the corresponding influent from the WWTP (L-2WI) and from the Spanish B-2WI. In the *Allium* MN assay no significant increase of MN frequencies or decrease of mitotic indices were detected (Fig. S1 of supplementary materials, ESI). In previous experiments with *Tradescantia* MN assays, the NOEC values were for CP 1.0 μM (279 $\mu\text{g L}^{-1}$), for Pt compounds 0.1 μM (30 $\mu\text{g L}^{-1}$) and for 5-FU 30 μM (3.9 mg L^{-1}) (Mišik et al., 2014), which are several orders higher than the concentration determined in the positive samples. To our knowledge, no data is available concerning the effects of MET, ERL and GAP that were also detected in the positive samples, in higher plants. It is unlikely that at low doses, which were detected in the wastewater samples, these residues of anticancer drugs induced genotoxic effect. However, previous investigations with *Tradescantia* demonstrated that certain genotoxic agents act synergistically (Gill and Sandhu, 1992; Knasmueller et al., 1992; Ma et al., 1992). Hence, it cannot be excluded that such an effect (i.e. synergism) also accounts for the present findings. Nevertheless, wastewater effluents that are finally released into surface water were not genotoxic and therefore it is unlikely that they represent significant threat for higher plants.

3.2.3. In vitro comet assay in zebrafish (*Danio rerio*) liver cell line

None of the tested wastewater samples at 10, 20, 30% v/v significantly reduced the viability of ZFL cells (Fig. S2). Therefore, the comet assay was performed at the same sample dilutions. All samples caused significant dose dependent increase in DNA damage except L-2We (Fig. 4). The most potent was the sample of oncological clinic wastewater collected in June (L-2H) that induced the highest increase in DNA damage and it was the only sample inducing significant increase in DNA damage at the lowest tested concentration. The sample L-2H had the highest burden with the residues of anticancer drugs (Fig. 1) and showed the highest genotoxic potential also in *Tradescantia* MN assay (Fig. 3). The corresponding WWTP influent (L-2WI) exerted much lower genotoxic

potential and the corresponding effluent (L-2We) was not genotoxic indicating removal or inactivation of genotoxic pollutants. The samples from Spain had similar potential regardless of sampling point. The genotoxicity of January samples was higher than of June samples (Fig. 4). The genotoxic potential of B-2We was comparable to that of the corresponding influent (B-2WI). Previously, it has been shown that under the same exposure conditions as used in the present study in ZFL cells, 5-FU and Pt induced significant increase in DNA damage at concentrations ≥ 10 and $100 \mu\text{g L}^{-1}$, respectively (Gajski et al., 2015), while CP and IF were inactive at concentrations up to 1 mg L^{-1} (personal M. Novak, 16. 7. 2016). These concentrations are several orders higher than the concentrations detected in the samples from the present study. However, potential threat for aquatic organisms cannot be excluded because, based on the results of *in vitro* genotoxicity assays, it is not possible quantitatively predict hazard for exposed organisms.

3.3. Correlation between chemical and toxicological characteristics of the samples

The results of correlation analysis are shown in Tables 5 and 6. In Table 5 the concentrations of chemicals in wastewater samples were correlated to each toxicity/genotoxicity test, while in Table 6 standardized regression coefficients and p-values as well as adjusted multiple correlation coefficients were reported (p-values were: 5% for inclusion and 10% for removal). Nevertheless, because of the restricted number of samples, a more comprehensive multivariate analysis including interaction terms was not possible.

The results of *C. dubia* acute toxicity tests correlated significantly only with the concentrations of ERL (the higher the concentration, the lower the survival), which is also the only chemical that was significant in the stepwise regression analysis (Tables 5 and 6). As reported in different studies, many anticancer drugs belonging to the tyrosine kinase inhibitor family, as ERL, are able to affect the crustacean survival at high concentrations (mg L^{-1} or hundreds of $\mu\text{g L}^{-1}$) (Constantine and Huggett, 2010; Parrella et al., 2014a; Franquet-Griell et al., 2015). Nevertheless, when these chemicals are in mixtures with other anticancer drugs such as Pt compounds and antimetabolites, they are able to cause additive or synergistic toxic effects at lower concentrations (Parrella et al., 2014b). This phenomenon could also occur in wastewaters because organisms, by different pharmacokinetic mechanisms, are able to absorb, accumulate and eliminate drugs. *C. dubia* reproductive toxicity test results correlated well with the levels of all chemicals except for CP; OH-TAM picked up by stepwise regression as the only independent predictor (Table 6). Borgatta et al. (2015) showed that OH-TAM

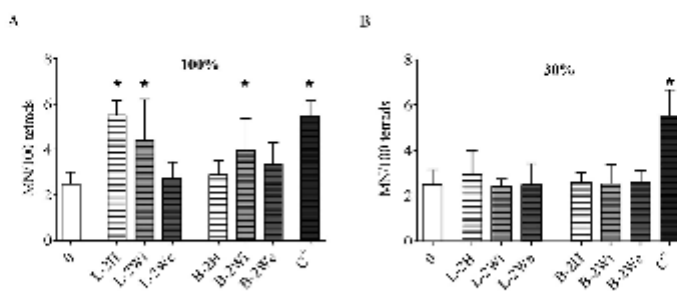


Fig. 3. Results of *Tradescantia* MN assays with undiluted (A, 100%) and with diluted (B, to 30% of original sample) hospital and WWTP wastewater samples collected in June 2014 (for sample codes see Table 2). Bars indicate mean $\pm$ SD, obtained with 5 inflorescences per experimental point. From each inflorescence, 300 tetrads were analyzed. The results were analyzed by generalized linear model with Poisson counts and a log link. Comparisons to negative control were done by simple contrasts and p-values were corrected according to Bonferroni-Holm; *p-values ≤ 0.05 were considered as significant. Tap water was used as negative control and solvent, MH (20 mg L^{-1}) was used as positive control (C*).

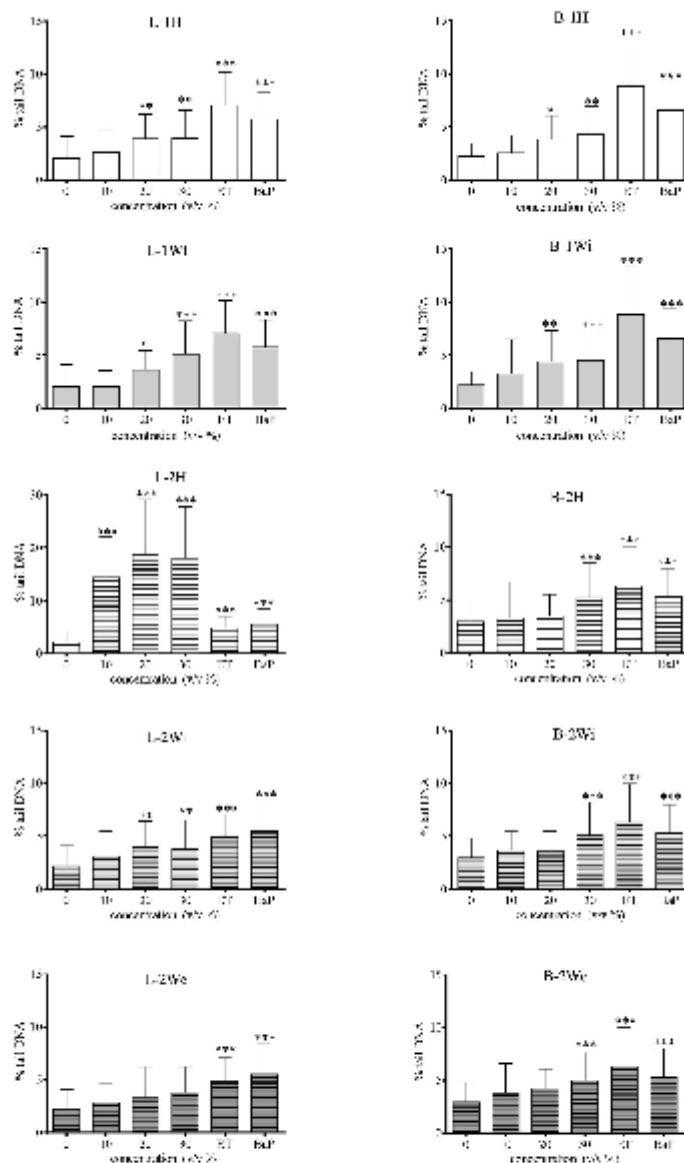


Fig. 4. DNA damage induced by hospital and WWTP wastewater samples (for sample codes see Table 2). The ZFL cells were exposed to 10, 20 or 30% (v/v) of the wastewater sample for 72 h. DNA damage was assessed with the comet assay and is expressed as percent of tail DNA. Fifty nuclei were analyzed per experimental point in each of the three independent experiments. Benzo[a]pyrene (BAP; 50 μ M) and Etoposide (ETP; 100 ng mL⁻¹) were used as the positive controls. Significant difference (1-way ANOVA; Dunnett's Multiple Comparison test) between exposed and the control cells (0) is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

induced toxic effects on non-target aquatic species as the cladoceran crustacean *Daphnia pulex* in a two-generation study starting from units of μ g L⁻¹. These authors hypothesize that the decrease of the daphnids fitness could be due to the interaction of endocrine

disruptors such as OH-TAM with oestrogen-related nuclear receptors in many organisms, including invertebrates (Thomson et al., 2009).

In order to better understand the toxic potential of the samples

Table 5
Spearman's rank correlation coefficients of concentrations of chemicals in wastewater samples with results of toxicity/genotoxicity tests (type of effect indicator in second line). p-values in parentheses.

Substances ^a	<i>C. dubia</i> chronic ^b	<i>C. dubia</i> acute	ZFL comet	Tradescantia MN
ng L ⁻¹	Log offspring	Logit vital	Beta	Log OR
Pt	-0.61 (<0.001)	-0.15 (0.365)	0.42 (0.022)	0.72 (0.008)
CP	-0.31 (0.055)	0.06 (0.720)	0.28 (0.140)	0.33 (0.290)
MET	-0.54 (0.003)	0.16 (0.421)	0.47 (0.031)	0.60 (0.008)
ERL	-0.56 (<0.001)	-0.35 (0.025)	0.17 (0.364)	0.59 (0.045)
OH-TAM	-0.62 (<0.001)	0.10 (0.570)	0.20 (0.359)	0.41 (0.314)
TAM	-0.49 (0.004)	-0.15 (0.409)	0.34 (0.100)	0.79 (0.007)

^a For abbreviations see Table 1.

^b *C. dubia* acute and chronic toxicity test results correlate negatively and ZFL comet and Tradescantia MN assay correlate positively if increasing concentrations are related to increased toxicity.

Table 6
Results of stepwise multivariate linear regression of toxicity/genotoxicity test results on log concentration of anti-cancer drugs and metabolites in wastewater samples. Standardized regression coefficients (Beta) and p-values as well as adjusted multiple correlation coefficients are shown.

Assay	Endpoint	Substance ^a	Beta ^b	p-value	adjR ²
<i>C. dubia</i> acute	Logit vital	ERL	-0.675	<0.001	0.431
<i>C. dubia</i> chronic	Log offspring	OH-TAM	-0.568	0.004	0.292
ZFL comet	Beta	MET	0.896	<0.001	0.808
Tradescantia MN	Log OR	TAM	0.836	0.039	0.625

^a For abbreviations see Table 1.

^b Negative coefficient for *C. dubia* acute and chronic assays indicates increased toxicity with increasing concentration, while for Tradescantia and Zebrafish assays a positive coefficient indicates increased genotoxicity.

investigated, Acute LC50/Chronic EC50 (AC) ratios were calculated. They ranged from 12.3 (L-2Wi) to 22.9 (L-1Wi) for Slovenian wastewaters and from 1.8 (B-2Wi) to 55.4 (B-2H) for Spanish samples. Interestingly, AC ratios were higher than 10 in all instances except for Spanish summer Wi and We with 1.8 and 4.4 values, respectively, showing a more evident lethal than chronic effect without a remarkable occurrence of the anticancer drugs detected (Tables 3 and 4) and finally suggesting that other uninvestigated substances might have affected the observed toxic effects. Another unexpected finding was observed in the case of the Spanish summer sampling characterized by an increase in chronic toxicity from Wi to We with a corresponding increase in the concentration of OH-D-TAM (from < LOD in the influent to 14.4 ng L⁻¹ in the effluent, Table 3).

Increase in MN frequencies in Tradescantia correlated with Pt, ERL and TAM, the latter was also entered in a stepwise regression. Pt compounds and kinase inhibitors can cause genotoxic effects and induce the formation of micronuclei in Tradescantia at concentrations in the order from hundreds of µg L⁻¹ to mg L⁻¹. However, in mixtures, anticancer drugs are able to cause additive or synergistic effects at low concentrations (µg L⁻¹) (Parrella et al., 2014b; Mišić et al., 2015). Ko et al. (2014) showed how in other eukaryotic models tamoxifen could enhance erlotinib-induced cytotoxicity through down-regulating AKT-mediated thymidine phosphorylase expression increasing the genotoxicity of the real samples. Similar results were obtained in the comet assay with ZFL cells, but in this case, significant correlations were obtained only for Pt and MET with MET included in the regression model. Pt compounds are able to cause DNA strand breaks in ZFL after 24 and 72h starting from 100 µg L⁻¹ (Gajski et al., 2015), but it was demonstrated that these drugs in mixture with MET can exert in vitro experimental models a moderate synergism at ng L⁻¹ levels (Chou et al., 1993).

Correlation analyses revealed that only 10–50% of the

differences in toxicity of wastewater samples are explained by the concentration of the substances measured. Indeed, even if MET, ERL and TAM were found at concentrations lower than the other anticancer drugs, they contribute to the results of stepwise multivariate linear regression of toxicity tests. Furthermore, while an additional impact could be exerted by an interaction between these substances, it cannot be excluded that substances in wastewaters not determined in this study contribute to these effects. Indeed, in Spain, Grada-Lor et al. (2012) who monitored the presence of several pharmaceuticals (analgesics and anti-inflammatories, lipid regulators, antibiotics) in urban influent WW and effluent WW from three different WWTPs found that conventional treatment processes do not completely remove these persistent micro-pollutants. Furthermore, in Spanish general hospital wastewaters, different compounds (analgesics and anti-inflammatories, antibiotics, β-blockers, diuretics, iodinated contrast media) were found (ng-mg L⁻¹) that pose a risk to aquatic organisms belonging to different trophic levels (Mendoza et al., 2015). In the Slovenian influent and effluent samples, from different wastewater treatment plants, other emerging contaminants such as Endocrine Disruptor Compounds (EDCs) including estrone, 17β-estradiol and estriol were present in ng L⁻¹ levels as shown by Avberšek et al. (2013). These compounds are able to affect organisms (gonadal abnormalities, reproductive deficiencies, egg and offspring development deficiencies, and vitellogenin induction alterations) at trace concentrations (ng L⁻¹) (Campbell et al., 2006; Isidori et al., 2010; Bistan et al., 2011). Bistan et al. (2011) underline the presence of other EDCs such as nonylphenols and octylphenols in Slovenian surface waters, suggesting that the Slovenian treatment processes are not efficient in removing persistent endocrine disrupting xenobiotics from wastewater. This is not surprising since WWTPs are not designed to remove micro organic pollutants. Considering that hormones and related anti-hormones are used in the endocrine anticancer therapy (Besse et al., 2012) and are discharged in the oncological hospital wastewaters, probably other hormonally active drugs could explain the present findings. The endocrine disruptors, as well as other emerging pollutants, as reported by Kasprzyk-Hordema et al., 2009 and Schug et al., 2011, acting through nonsteroid receptors, transcriptional coactivators and several enzymatic pathways, could represent a risk for different organisms, up to be capable of affecting human population when these xenobiotics are found in wastewaters, especially when they represent the main contributors to river flows involved in the human water recycling and reuse. Also it should be noted that according UNEP (http://www.unep.org/pdf/SickWater_screen.pdf) "A staggering 80–90 per cent of all wastewater generated in developing countries is discharged directly into surface water bodies". Similar data concerning presence of pharmaceutical in these regions are available in work of the Rehman et al. (2015) and indicate elevated health risk for human population in these areas. In this context, our data also underline importance of proper waste water treatment procedures.

4. Conclusions

Hospital wastewater can contain high levels of anticancer drug residues that vary with the therapeutic regimen. In our study, twelve out of the fifteen investigated parent compounds were present in Slovenian and Spanish hospital and municipal wastewaters while six on seven TPAs have also been identified. Interestingly, a statistical correlation between the results of the toxicity tests and the drugs identified was found, so that the aim of this study was reached. Nevertheless, several unknown compounds surely might contribute to the observed effects. The applied eco/genotoxicity tests demonstrated that hospital effluents and WWTP

influent and effluent could be harmful for the aquatic environment at concentrations of ng L^{-1} . It may be desirable that measured concentrations as well as toxicity/genotoxicity results should be considered for the environmental risk assessment in order to encourage the environmental agencies to enforce regulations that these emerging pollutants do not have yet.

Seasonal fluctuations were observed for hospital wastewater regarding both acute and chronic toxicity. However, the toxicity does not correlate with the total burden of the samples and the residues of anticancer drugs indicating that other unidentified pollutants contributed to these differences. Basically, the effluents from the WWTPs investigated should be diluted by more than 90% to obtain no observed toxic effects as shown by NOEC values.

The possible presence of different pollutants in complex mixtures makes it difficult to comprehend the actual ecological risk posed by the targeted compounds. Thus, a chemical characterisation closely linked to an eco/genotoxicological approach as used in this study, can lead to a better understanding of the environmental toxic/genotoxic potential of xenobiotic mixtures.

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Appendix A. Supplementary data

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References

- Avberšek, M., Zegura, B., Filipič, M., Uranjek-Zevart, N., Heath, E., 2013. Determination of estrogenic potential in wastewaters without sample extraction. *J. Hazard. Mater.* 260, 527–533.
- Besie, J.P., Latour, J.F., Garria, J., 2012. Anticancer drugs in surface waters: what can we say about the occurrence and environmental significance of cytotoxic cytostatic and endocrine therapy drugs? *Environ. Int.* 39, 73–86.
- Bisran, M., Marinsek-Logar, R., Tisler, T., 2011. Detection of estrogenic activity in Slovenian wastewaters by bioassay. *Cent. Eur. J. Biol.* 6, 829–837.
- Borgatta, M., Decosterd, L.A., Waridel, P., Budlin, T., Chevre, N., 2015. The anticancer drug metabolites endoxifen and 4-hydroxy-tamoxifen induce toxic effects on *Daphnia pulex* in a two-generation study. *Sci. Total Environ.* 520, 232–240.
- Brezovsek, P., Elersek, T., Filipic, M., 2014. Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Synechococcus leopoldensis*. *Water Res.* 52, 168–177.
- Campbell, C.G., Borglin, S.E., Green, F.B., Grayson, A., Wozel, E., Stringfellow, W.T., 2006. Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine disrupting compounds in water: a review. *Chemosphere*

- 65, 1265–1280.
- Council of the European Communities, 2002. Commission decision 2002/657/EC. Off. J. Eur. Commun. L221.
- Cesen, M., Kosjek, T., Laimou-Gerasimou, M., Kompare, B., Šink, B., Lambropoulou, D., Heath, E., 2015. Occurrence of cyclophosphamide and ifosfamide in aqueous environment and their removal by biological and abiotic wastewater treatment processes. *Sci. Total Environ.* 527–528, 465–473.
- Cesen, M., Kosjek, T., Buseti, F., Kompare, B., Heath, E., 2016a. Human metabolites and transformation products of cyclophosphamide and ifosfamide: analysis, occurrence and formation during abiotic treatments. *Environ. Sci. Pollut. Res. Int.* 1–15.
- Cesen, M., Elersek, T., Novak, M., Zegura, B., Kosjek, T., Filipic, M., Heath, E., 2016b. Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites/ transformation products and their mixtures. *Environ. Pollut.* 210, 192–201.
- Chou, T.C., Tan, Q.H., Sirotnak, F.M., 1993. Quantitation of the synergistic interaction of edaravone and cisplatin in vitro. *Cancer Chemother. Pharmacol.* 31 (4), 259–264.
- Constantine, L.A., Huggert, D.B., 2010. A comparison of the chronic effects of human pharmaceuticals on two cladocerans, *Daphnia magna* and *Ceriodaphnia dubia*. *Chemosphere* 80, 1069–1074.
- Deila Greca, M., Iesce, M.R., Isidori, M., Nardelli, A., Previtera, L., Rubino, M., 2007. Phototransformation products of tamoxifen by sunlight in water. Toxicity of the drug and its derivatives on aquatic organisms. *Chemosphere* 67, 1933–1939.
- Ferk, F., Milik, M., Gaumnitz, T., Majer, B., Fuchthaler, M., Buchmann, C., Vital, M., Uhl, M., Lenz, K., Gollitsch, B., Parzefall, W., Nersegyan, A., Knasmüller, S., 2009. Genotoxic effects of wastewater from an oncological ward. *Mutat. Res.* 672, 69–75.
- Fernando-Climent, L., Rodríguez-Mozaz, S., Barceló, D., 2013. Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. *Anal. Bioanal. Chem.* 405, 5937–5952.
- Fernando-Climent, L., Rodríguez-Mozaz, S., Barceló, D., 2014. Incidence of anticancer drugs in an aquatic urban system: from hospital effluent through urban wastewater to natural environment. *Environ. Pollut.* 193, 236–223.
- Franquet-Griell, H., Gómez-Canela, C., Ventura, F., Lacort, S., 2015. Predicting concentrations of cytostatic drugs in sewage effluents and surface waters of Catalonia (NE Spain). *Environ. Res.* 138, 161–172.
- Gajski, G., Gerić, M., Zegura, B., Novak, M., Nunić, J., Bajektarović, D., Garaj-Vrhovac, V., Filipič, M., 2015. Genotoxic potential of selected cytostatic drugs in human and zebrafish cells. *Environ. Sci. Pollut. Res.* 1–12.
- Ghosh, C., Zhou, Y.L., Collioli, P., 1994. Derivation and characterization of a zebrafish liver cell line. *Cell. Biol. Toxicol.* 10, 167–176.
- Gill, B.S., Sandhu, S.S., 1992. Application of the Tradescantia micronucleus assay for the genetic evaluation of chemical mixtures in soil and aqueous media. *Mutat. Res.* 270, 65–69.
- Gracia-Lor, E., Sancho, J.V., Serrano, R., Hernández, F., 2012. Occurrence and removal of pharmaceuticals in wastewater treatment plants at the Spanish Mediterranean area of Valencia. *Chemosphere* 87, 453–462.
- Isidori, M., Cangiano, M., Parrella, A., 2010. E-screen and Vg-assay for the detection of the estrogenic activity of alkylphenols and trace elements. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 152, 51–56.
- ISO 20665, 2008. Water Quality-determination of Chronic Toxicity to *Ceriodaphnia dubia* in 7 Days-population Growth Inhibition Test. International Organization for Standardization, Geneva, Switzerland.
- Johnson, A.C., Oldenamp, R., Dumont, E., Sumpter, J.P., 2013. Predicting concentrations of the cytostatic drugs cyclophosphamide, carboplatin, 5-fluorouracil, and capecitabine throughout the sewage effluents and surface waters of Europe. *Environ. Tox. Chem.* 32, 1954–1961.
- Kasprzyk-Horderna, B., Dinsdale, R.M., Guwy, A.J., 2009. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. *Water Res.* 43, 363–380.
- Klein, D.J., Thorn, C.F., Desta, Z., Flockhart, D.A., Altman, R.B., Klein, T.E., 2013. PharmGKB summary: tamoxifen pathway, pharmacokinetics. *Pharmacogenet. Genomics* 23 (11), 643–647.
- Knasmüller, S., Kim, T.W., Ma, T.H., 1992. Synergistic effect between tannic acid and X-rays detected by the Tradescantia-micronucleus assay. *Mutat. Res.* 270, 31–37.
- Ko, J.C., Chiu, H.C., Syu, J.J., Jian, Y.J., Chen, C.Y., Jian, Y.T., Huang, Y.J., Wu, T.Y., Lin, Y.W., 2014. Tamoxifen enhances erlotinib-induced cytotoxicity through down-regulating AKT-mediated thymidine phosphorylase expression in human non-small-cell lung cancer cells. *Biochem. Pharmacol.* 88, 119–127.
- Kosjek, T., Heath, E., 2011. Occurrence, fate and determination of cytostatic pharmaceuticals in the environment. *TrAC Trends Anal. Chem.* 30, 1065–1087.
- Kosjek, T., Perko, S., Zigon, D., Heath, E., 2013. Fluorouracil in the environment: analysis, occurrence, degradation and transformation. *J. Chromatogr. A* 1290, 62–72.
- Kosjek, T., Negreia, N., de Alda, M.L., Barceló, D., 2015. Aerobic activated sludge transformation of methotrexate: Identification of biotransformation products. *Chemosphere* 119 (Suppl. 542–550).
- Kovacs, R., Csenki, Z., Bakos, K., Urbani, B., Horvath, A., Garaj-Vrhovac, V., Gajski, G., Gerić, M., Negreia, N., Lopez de Alda, M., Barceló, D., Heath, E., Kosjek, T., Zegura, B., Novak, M., Zajc, I., Baebler, S., Rotter, A., Ramjak, Z., Filipič, M., 2015. Assessment of toxicity and genotoxicity of low doses of 5-fluorouracil in

- zebrafish (*Danio rerio*) two-generation study. *Water Res.* 7, 201–212.
- Ma, T.H., Cabrera, G.L., Chen, R., Gill, B.S., Sandhu, S.S., Vandenberg, A.L., Salamone, M.F., 1994. *Tradescantia* micronucleus bioassay. *Mutat. Res.* 310, 221–230.
- Ma, T.H., Xu, Z., Xu, C., McConnell, H., Rabago, E.V., Amelot, G.A., Zhang, H., 1995. The improved Allium/Vicia root tip micronucleus assay for clastogenicity of environmental pollutants. *Mutat. Res.* 334, 185–195.
- Martín, J., Camacho-Muñoz, D., Santos, J.L., Aparicio, I., Alonso, E., 2014. Occurrence and ecotoxicological risk assessment of 14 cytostatic drugs in wastewater. *Water Air Soil Pollut.* 225, 1–10.
- Mendoza, A., Aceña, J., Pérez, S., López de Alda, M., Barceló, D., Gil, A., Valcárcel, Y., 2015. Pharmaceuticals and iodinated contrast media in a hospital wastewater: a case study to analyse their presence and characterise their environmental risk and hazard. *Environ. Res.* 140, 225–234.
- Millit, M., Pichler, C., Rainer, B., Filipić, M., Nersisyan, A., Knasmueller, S., 2014. Acute toxic and genotoxic activities of widely used cytostatic drugs in higher plants: possible impact on the environment. *Environ. Res.* 135, 196–203.
- Millit, M., Filipić, M., Nersisyan, A., Milčević, K., Knasmueller, S., Kundl, M., 2016. Analyses of combined effects of cytostatic drugs on micronucleus formation in the *Tradescantia*. *Environ. Sci. Pollut. Res.* 23 (15), 14762–14770.
- Negreira, N., López de Alda, M., Barceló, D., 2013. On-line solid phase extraction-liquid chromatography-tandem mass spectrometry for the determination of 17 cytostatics and metabolites in waste, surface and ground water samples. *J. Chrom. A* 1280, 64–74.
- Negreira, N., López de Alda, M., Barceló, D., 2014a. Cytostatic drugs and metabolites in municipal and hospital wastewaters in Spain: filtration, occurrence, and environmental risk. *Sci. Total Environ.* 497–498, 68–77.
- Negreira, N., López de Alda, M., Barceló, D., 2014b. Study of the stability of 26 cytostatic drugs and metabolites in wastewater under different conditions. *Sci. Total Environ.* 482–483, 389–398.
- Orias, F., Perrodin, Y., 2014. Pharmaceuticals in hospital wastewater: their ecotoxicity and contribution to environmental hazard of the effluent. *Chemosphere* 115, 31–39.
- Parrella, A., Lavorgna, M., Criscuolo, E., Russo, C., Fiumano, V., Isidori, M., 2014a. Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans. *Chemosphere* 115, 59–66.
- Parrella, A., Kundl, M., Lavorgna, M., Criscuolo, E., Russo, C., Isidori, M., 2014b. Toxicity of exposure to binary mixtures of four anti-neoplastic drugs in *Daphnia magna* and *Ceriodaphnia dubia*. *Aquat. Toxicol.* 157, 41–46.
- Rehman, M.S., Rashid, N., Ashfaq, M., Saif, A., Ahmad, N., Han, J.L., 2015. Global risk of pharmaceutical contamination from highly populated developing countries. *Chemosphere* 138, 1045–1055.
- Rowney, N.C., Johnson, A.C., Williams, R.J., 2009. Cytotoxic drugs in drinking water: a prediction and risk assessment exercise for the Thames catchment in the United Kingdom. *Environ. Tox. Chem.* 28, 2733–2743.
- Sanderson, H., Johnson, D.J., Wilson, C.J., Brain, R.A., Solomon, K.R., 2003. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. *Toxicol. Lett.* 144, 383–395.
- Schug, T.T., Janesick, A., Blumberg, B., Heindel, J.J., 2011. Endocrine disrupting chemicals and disease susceptibility. *J. Steroid Biochem. Mol. Biol.* 127 (3–5), 204–215.
- Straser, A., Filipić, M., Žegura, B., 2011. Genotoxic effects of the cyanobacterial hepatotoxin cylindrospermopsin in the HepG2 cell line. *Arch. Toxicol.* 85 (12), 1617–1626.
- Thomson, S.A., Baldwin, W.S., Wang, Y.H., Kwon, G., Leblanc, G.A., 2009. Annotation, phylogenetics, and expression of the nuclear receptors in *Daphnia pulex*. *BMC Genomics* 28 (10), 500.
- Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kibayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen* 35 (3), 206–221.
- Toolaram, A.P., Kimmmer, K., Schneider, M., 2014. Environmental risk assessment of anticancer drugs and their transformation products: a focus on their genotoxicity characterization-state of knowledge and shortcomings. *Mutat. Res.* 760, 18–35.
- US EPA, 1993. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, fourth ed. US Environmental Protection Agency, Washington DC. EPA-600-4-90.
- Verlicchi, P., Al Aulady, M., Galletti, A., Petrovic, M., Barceló, D., 2012. Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. *Sci. Total Environ.* 430, 109–118.
- Vidmar, J., Martincic, A., Milas, R., Štancar, J., 2015. Speciation of cisplatin in environmental water samples by hydrophilic interaction liquid chromatography coupled to inductively coupled plasma mass spectrometry. *Talanta* 138, 1–7.
- Xie, H., 2012. Occurrence, ecotoxicology, and treatment of anticancer agents as water contaminants. *J. Environ. Anal. Toxicol.* <http://dx.doi.org/10.1017/2151-0525.52-002.52>.
- Zhang, J., Chang, V.W.C., Gianni, A., Wang, J.Y., 2013. Removal of cytostatic drugs from aquatic environment: a review. *Sci. Total Environ.* 445–446, 281–298.

3.7 Assessment of Toxicity and Genotoxicity of Low Doses of 5-Fluorouracil in Zebrafish (*Danio Rerio*) Two-Generation Study

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Chronic exposure to genotoxic contaminants might cause in environmental organisms gamete loss, decreased reproductive success, embryonic mortality, abnormal development, and changes in genetic diversity. Therefore, in the present paper long-term effects of environmentally relevant concentrations of 5-fluorouracil (5-FU) through two subsequent zebrafish generations has been studied.

5-FU did not affect classical toxicological endpoints such as survival, growth and reproduction of the fish. On the other hand, 5-FU induced histopathological changes in the liver and kidney, along with genotoxic effects. Increases in DNA damage determined using the comet assay were significant in the liver and blood cells, but not in the gills and gonads. In erythrocytes, a significant, dose-dependent increase in the frequency of micronuclei was observed at all 5-FU concentrations. Whole genome transcriptomic analysis of liver samples of F1 generation zebrafish exposed to 0.01 µg/L and 1 µg/L 5-FU revealed dose-dependent increases in the number of differentially expressed genes, including up-regulation of several DNA-damage-responsive genes and oncogenes (i.e., *jun*, *myc*).

Our study demonstrated for the first time that chronic two-generation exposure of zebrafish to 5-FU at concentrations that are relevant for environmental exposure impairs their DNA integrity and induced massive whole-transcriptome changes. Although chronic exposure to 5-FU at these low concentrations did not affect the reproduction of the exposed zebrafish, it cannot be excluded that 5-FU can lead to degenerative changes, including cancers, which over long-term exposure of several generations might affect fish populations. The results suggest that for the prediction of the adverse effects of genotoxic cytostatic drugs to aquatic vertebrate, long-term, multiple generation studies that include detection of genotoxic effect and transcriptomic changes are warranted. However, to enable environmental risk assessment, more data on the occurrence of cytostatic drugs in surface waters are needed.

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Assessment of toxicity and genotoxicity of low doses of 5-fluorouracil in zebrafish (*Danio rerio*) two-generation study

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ABSTRACT

Residues of anti-neoplastic drugs represent new and emerging pollutants in aquatic environments. Many of these drugs are genotoxic, and it has been postulated that they can cause adverse effects in aquatic ecosystems. 5-Fluorouracil (5-FU) is one of the most extensively used anti-neoplastic drugs in cancer therapy, and this article describes the results of the first investigation using a two-generation toxicity study design with zebrafish (*Danio rerio*). Exposure of zebrafish to 5-FU (0.01, 1.0 and 100 µg/L) was initiated with adult zebrafish (F0 generation) and continued through the hatchlings and adults of the F1 generation, and the hatchlings of the F2 generation, to day 33 post-fertilisation. The exposure did not affect survival, growth and reproduction of the zebrafish; however, histopathological changes were observed in the liver and kidney, along with genotoxic effects, at all 5-FU concentrations. Increases in DNA damage determined using the comet assay were significant in the liver and blood cells, but not in the gills and gonads. In erythrocytes, a significant, dose-dependent increase in frequency of micronuclei was observed at all 5-FU concentrations. Whole genome transcriptomic analysis of liver samples of F1 generation zebrafish exposed to 0.01 µg/L and 1 µg/L 5-FU revealed dose-dependent increases in the number of differentially expressed genes, including up-regulation of several DNA-damage-

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responsive genes and oncogenes (i.e., *jun*, *myc*). Although this chronic exposure to environmentally relevant concentrations of 5-FU did not affect the reproduction of the exposed zebrafish, it cannot be excluded that 5-FU can lead to degenerative changes, including cancers, which over long-term exposure of several generations might affect fish populations. The data from this study contribute to a better understanding of the potential consequences of chronic exposure of fish to low concentrations of anti-neoplastic drugs, and they demonstrate that further studies into multi-generation toxicity are needed.

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1. Introduction

Potential risks associated with the release of pharmaceuticals into the aquatic environment are an important issue for environmental regulators and for the pharmaceutical industry. Pharmaceuticals predominantly enter the aquatic environment via the effluent from hospital and wastewater treatment plants and as landfill leachates, and to a minor extent in discharge from the pharmaceutical industry. Due to the ubiquitous presence of pharmaceuticals in the environment that has arisen from their continual input into the aquatic compartment, they are considered as 'pseudo'-persistent pollutants (Hernando et al., 2006). The concentrations of the residues of pharmaceuticals in the environment are relatively low compared to other pollutants, which has led to the belief that these compounds have no environmental impact on living organisms. Also, with ecotoxicological data often based on acute toxicity at high concentrations, these have been considered to be far greater than the actual concentrations in the environment. However, based on their therapeutic functions and mechanisms of action, certain groups of pharmaceuticals are suspected to represent risks for non-target organisms, even at concentration of a few nanogrammes per litre, particularly under conditions of chronic exposure (Johnson et al., 2008).

One such group of pharmaceuticals is the cytostatic anticancer drugs. Their main purpose is to prevent uncontrolled proliferation of cancer cells, through inhibition of cell growth or through inducing the death of cancer cells via interactions with DNA function and cell signalling. Due to their mechanisms of action, many cytostatics are classified as mutagenic, carcinogenic and/or toxic to reproductive systems, and it can be assumed that they can elicit these effects in exposed non-target aquatic organisms (Johnson et al., 2008; Lenz et al., 2007).

The fluoropyrimidine 5-fluorouracil (5-FU) is an antineoplastic that exerts its anticancer effects through inhibition of DNA synthesis and replication, by inhibition of thymidylate synthase, and by incorporation of 5-FU metabolites into RNA and DNA (Longley et al., 2003). 5-FU was introduced as a chemotherapeutic agent more than 50 years ago, and is still the cornerstone of most of the currently applied regimens for the treatment of patients with cancers of the gastrointestinal tract, breast, and head and neck (van Kuilenburg and Maring, 2013). Therefore, it is not surprising that 5-FU is one of the most consumed of the cytostatic drugs (Besse et al., 2012). The

predicted environmental concentrations have been for 5-FU in different EU countries calculated to be from 2.5 ng/L to 8 ng/L (Straub, 2010; Besse et al., 2012; Johnson et al., 2008).

Ecotoxicity and environmental occurrence of 5-FU have been extensively studied over the last 10 years or so. In reproduction tests with the alga *Pseudokirchneriella subcapitata* and the cyanobacteria *Anabaena flos-aquae*, 5-FU was shown to be highly toxic (half maximal effective concentration [EC₅₀], ~0.12 mg/L and ~0.024 mg/L, respectively) (Brezovsek et al., 2014; Straub, 2010). Much lower toxicity of 5-FU was reported for an acute toxicity assay with a crustacean (EC₅₀, 21 mg/L), while in a chronic reproduction assay in this crustacean, 5-FU was highly toxic with the 'no observed effect concentration' at ~0.002 mg/L (Parella et al., 2014). In zebrafish (*Danio rerio*), 5-FU was not toxic after acute exposure and showed low toxicity through a chronic 35-day early life-stage toxicity assay (Straub, 2010). Based on these data, and taking into account the predicted environmental concentrations according to the guidelines on the environmental risk assessment of medicinal products for human use (European Medicines Agency, 2006), Straub (2010) concluded that 5-FU does not represent a significant risk to aquatic environments.

However, 5-FU is genotoxic. In mammalian test systems, it has been shown to induce chromosomal damage in vivo (Ohuchida et al., 1992) and in vitro (Lorge et al., 2006; Oka et al., 2006). It is well known that exposure of aquatic organisms to genotoxic compounds can lead to genetic alterations, even at very low concentrations. In aquatic organisms living in water that is polluted with genotoxic contaminants, numerous studies have shown genotoxic effects (i.e., DNA damage, micronuclei, mutations) (Bolognesi and Hayashi, 2011; Frenzilli et al., 2009), and in fish and mussels, tumours have also been reported (Pinkney et al., 2011; Myers et al., 2003). Chronic exposure to genotoxic contaminants in aquatic organisms might cause gamete loss, decreased reproductive success, embryonic mortality, abnormal development, and changes in genetic diversity (Anderson and Wild, 1994; Fassbender and Braunbeck, 2013).

The present study was conducted to determine whether continuous exposure of zebrafish to 5-FU (0.01, 1.0 and 100 µg/L) through two subsequent generations can induce genotoxic effects, changes in gene expression, histopathological changes, and/or have effects on reproduction and fitness. This exposure to 5-FU was initiated with adult zebrafish, and then continued on through the hatchlings and adults of the F1 generation, and the hatchlings of the F2 generation,

to day 33 post-fertilisation. In addition to standard toxicological endpoints, such as the zebrafish survival, growth and reproduction, the induction of DNA damage and micronucleus formation were determined as genotoxicity endpoints. DNA damage was determined at the level of single cells, for the gills, liver, kidneys, gonads and blood cells using the comet assay. This comet assay is a sensitive method for the detection of single-strand (and double-strand) breaks, alkali-labile sites, DNA–DNA/DNA–protein cross-linking, and single-strand breaks associated with incomplete excision repair (Tice et al., 2000). Complementary to the comet assay, the micronucleus assay was applied to determine chromosomal damage in erythrocytes. Micronuclei are formed during cell division and can occur at different times after DNA damage, depending on the cell-cycle kinetics and the mechanisms of induction (Bolognesi and Hayashi, 2011). Both of these assays have broad applicability in aquatic animals and they have been applied in numerous laboratory exposure and environmental monitoring studies (Bolognesi and Hayashi, 2011; Frenzzilli et al., 2009). Furthermore, in the present study, whole genome gene expression profiling was performed on the F1 generation zebrafish liver samples. Changes in gene expression can either be related to adaptive processes or can be used as indicators of toxic effects (Girardot et al., 2004); furthermore, they can indicate harmful impacts of chemicals in cases where classical toxicological endpoints show no obvious adverse effects (Williams et al., 2014).

2. Materials and methods

2.1. Test organism

The AB line of zebrafish (*D. rerio*) originated from the Department of Aquaculture of Szent István University (Hungary). The parent generation was maintained in a recirculation system (ZebTec; Tecniplast Inc.) under a light regime of 14 h light/10 h dark. The parameters of the recirculation system water were: temperature, 25 ± 0.5 °C; pH, 7.4 ± 0.2 ; and conductivity, 525 ± 50 µS. During the study period, the zebrafish were maintained at 24 °C– 26 °C under a light regime of 14 h light/10 h dark. The animal protocol was approved by the Hungarian Animal Welfare Law (XIV-1-001/2303-4/2012).

2.2. Test substance and chemical analyses

5-FU (CAS 51-21-8; Sigma–Aldrich) was maintained as a 100 mg/L stock solution in dimethylsulphoxide (DMSO). The nominal concentrations of the exposure to 5-FU were 0.01 µg/L, 1.0 µg/L and 100 µg/L were obtained by dilution of stock solution in clean recirculation system water. The final concentration of DMSO in all of the test solutions was 1 µL (0.1%). Two control groups were used: the recirculation system water alone (control), and the recirculation system water supplemented with 1 µL DMSO (vehicle control). All of the treatments were carried out in duplicate.

The study was performed under semistatic conditions during the treatments, with complete renewal of the recirculation system water every 3 days. Chemical analyses of samples of the water were performed immediately after the

complete renewal at the end of the second day (48 h of treatment) and the end of the third day (72 h of treatment). Analysis of water samples with nominal concentrations of 1.0 µg/L and 100 µg/L 5-FU was performed using ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) with a Q Exactive hybrid quadrupole–Orbitrap mass spectrometer equipped with an electrospray ionisation source. Analysis of water with the nominal concentration of 0.01 µg/L 5-FU was performed using solid-phase extraction followed by gas chromatography (GC)–MS/MS, using an Agilent 450–GC system linked to an ion-trap 240–MS mass spectrometer. For further details, see Supplementary Materials.

2.3. Exposure conditions during the two-generation toxicity tests

The conditions of exposure of zebrafish to 5-FU and sampling times for the determination of different toxicological parameters are shown in Fig. 1.

2.3.1. The F0 generation

The tests were initiated with a selection of sexually mature, 6-month old, adult zebrafish as the parent (F0) generation. Seven female and 11 male zebrafish were exposed to 5-FU for 2 weeks, and were spawned twice during the two successive weeks. Fertilised eggs from the second spawning were mixed, and 130 embryos were randomly selected for each replicate.

2.3.2. The F1 generation

Developing embryos were exposed in 10-cm Petri dishes until the free-swimming larval stage (5 days post fertilisation [dpf]) and then placed into 1-L polycarbonate tanks containing the relevant treatment water. The larvae were fed twice a day with small-grain dry feed (SDS Ltd.). From 10 dpf, the diet of the larvae was supplemented with *Artemia* nauplii, with the tanks cleaned of unconsumed food every day. After 21 dpf, the larvae were reared in 3-L polycarbonate tanks. Aeration was applied to the water from this stage. From 33 dpf, the exposure continued in 3.5-L tanks with 55 zebrafish larvae per replicate and from the second month of age in 8-L polycarbonate tanks.

The F1 generation zebrafish spawned for the first time at the age of 5 months, and then on a weekly basis. In all of experimental groups, 8–15 females and an equal number of males were randomly selected for spawning. These zebrafish spawned in groups of 2–3 females and the same number of males. The spawning groups were placed into the spawning tanks with males separated from females till the beginning of the spawning. Eggs were collected when all of the zebrafish had finished spawning. Following the fifth spawning, eggs from the zebrafish within the same replicate were mixed, and 100 eggs per replicate were selected for the F2 generation. To determine fecundity, the numbers of spawned eggs and unfertilised eggs were counted, and the numbers of spawned eggs and infertile eggs were normalised to the number of females used for the given spawning event.

At the age of 4 months, some of the F1 zebrafish were used for genotoxicity tests and gene expression analysis (see Sections 2.5 and 2.6, respectively). The rest were continued under the relevant treatment in parallel with the F2 generation. At 7

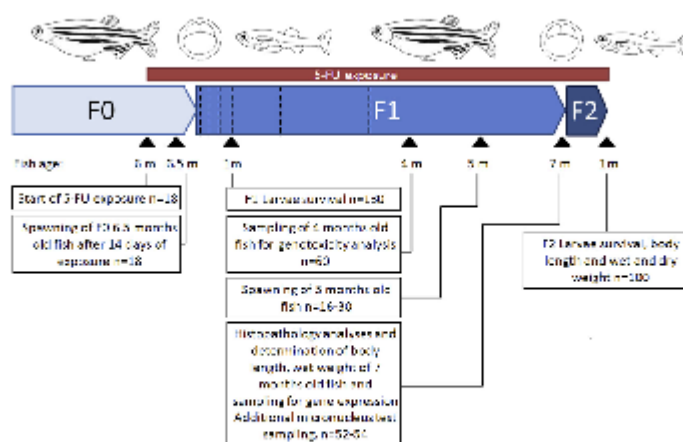


Fig. 1 – Outline of the experimental design of the two-generation exposure study with 5-FU (for details, see Section 2.3. Exposure conditions during the two generation toxicity test). Dotted lines represent fish transfers to larger tanks.

months of age, the F1 generation zebrafish were sacrificed using 0.02% Tricaine (Tricaine methanesulphonate; CAS: 886-86-2, Sigma–Aldrich), for histopathology analyses and determination of standard length and wet weight. Standard length was determined from digital photographs of the zebrafish, using the open-source software ImageJ (<http://imagej.nih.gov/ij/>). The wet weights were measured using laboratory scales with 0.0001 g resolution (Mettler-Toledo AB204-S). Additional sampling of the liver was performed for the micronucleus assay.

2.3.3. The F2 generation

Treatment of the F2 generation was conducted as described above for the F1 generation, and terminated at 33 dpf. Following over-anaesthesia of these zebrafish with 0.02% Tricaine, their standard lengths and wet weights were determined.

2.4. Histopathology analysis

Histopathological changes were determined on 7-month-old F1 zebrafish, for the skin, gill, gut, hepatopancreas, kidney haematopoietic tissue, nephrons, neural tissue and male and female gonads. Whole F1 zebrafish (four per analysis) were fixed in buffered formalin, dehydrated in ascending alcohol concentrations, impregnated with xylol, and embedded in Paraplast at 56 °C. Sections of 3 µm–4 µm thickness were prepared, cleared of paraffin, and stained with haemalaun and eosin for microscopy examination.

2.5. Genotoxicity assays

2.5.1. Comet assay

The comet assay was performed on the gill, liver, gonads and blood cells of adult 4-month-old F1 zebrafish (4 per treatment).

Immediately after these, F1 zebrafish were sacrificed, blood samples and samples of gills, liver and gonads were taken, immersed in chilled homogenisation buffer, and mechanically homogenised, to obtain cell suspensions according to Knežević et al. (2011). The alkaline comet assay was carried out as described by Singh et al. (1988). Briefly, the whole blood or cell suspension was embedded in agarose matrix and the cells were lysed (2.5 M NaCl, 100 mM EDTA_{Na}, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO, pH 10) for at least 3 h at 4 °C. (2.5 M NaCl, 100 mM EDTA_{Na}, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO, pH 10) for 3 h at 4 °C. Then, the slides were placed into alkaline solution (300 mM NaOH, 1 mM EDTA_{Na}, pH 13) C, for 20 min at 4 °C, and then electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralised in 0.4 M Tris buffer (pH 7.5), stained with 10 µg/mL ethidium bromide, and analysed at 250× magnification under an epifluorescence microscope (Zeiss, Göttingen, Germany) connected to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, UK). The tail intensity parameter (% tail DNA) was used to measure the level of DNA damage, and a total of 100 randomly chosen nuclei were examined from each slide.

2.5.2. Micronucleus assay

The induction of micronuclei formation was determined in erythrocytes of blood samples collected from 4-month-old and 7-month-old F1 zebrafish (4 per treatment). Peripheral blood samples were smeared onto slides. The slides were left to air-dry, stained with 5 µM Hoechst 33342 dye, and analysed at 400× under an epifluorescence microscope (Olympus BX-51; Tokyo, Japan), and evaluated for micronuclei that showed blue fluorescence. For the presence of micronuclei a total of 2000 randomly selected cells with complete cytoplasm were examined from each slide. The criteria for micronuclei identification were: (a) smaller than one-third of the main

nuclei; (b) on the same plane of focus as the main nuclei; (c) of the same colour and intensity as the main nuclei; (d) of an oval or round shape; and (e) clearly separated from the main nuclei.

2.6. Toxicogenomic analyses

Gene expression analysis was performed on liver samples isolated from 4-month-old F1 zebrafish exposed to 0.01 µg/L and 1.0 µg/L 5-FU, and vehicle-exposed (0.1% DMSO) liver samples were used as the control. The RNA was isolated by Direct-zol RNA Miniprep (ZymoResearch) from the livers 10 F1 zebrafish per treatment condition from each of the two replicates. The RNA from 3 fish from the same treatment, replicate and gender was pooled, DNase treated (0.2 U/µg RNA DNaseI; Invitrogen). RNA quality and quantity was checked using a Agilent Bioanalyser (RNA 6000 Nano kit, Eukaryote Assay).

Zebrafish V3 4 × 44 Gene Expression Microarrays (4 × 44 K, AMADID 026437; Agilent) were used for the transcriptome profiling. The total RNA was labelled using Low Input Quick-Amp Labelling Kit (one-colour) and the hybridisations and washes were performed using Gene Expression Hybridization Kit, following manufacturer protocols. The hybridised slides were scanned on a DNA microarray scanner, and the signals were extracted, with quality control performed using the Feature Extraction 10.7.3.1 Software (all Agilent Technologies). The data discussed in the present study have been deposited in the NCBI Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE63039.

To validate the microarray data, the expression of eight genes (*myc*, *jun*, *nfe2l3*, *slpr1*, *igfbp1b*, *pak2b*, *gadd45*, *cht*; Supplementary Material, Table S2) was analysed by quantitative PCR, using the RNA of 10 zebrafish per treatment (5 males, 5 females; not all included in the microarray analysis).

2.7. Statistical analysis

Statistical analyses were conducted using the statistical software GraphPad Prism 4.0 (GraphPad, La Jolla, California, USA). Analysis of variance (ANOVA) with Tukey's tests was used to analyze fish lengths and wet weights. ANOVA with Dunnett's tests was used to analyse the fecundity and fertilisation data. Statistical significance was set at $p < 0.05$.

Statistical evaluation of the comet and micronucleus assay data was performed with the free software R (www.R-project.org). For the data from the micronucleus assay, Poisson regression was used to compare the controls and the varying concentrations of 5-FU. Differences in the % tail DNA in the comet assay for the control and 5-FU groups were evaluated statistically using mixed effects modelling for nested experimental designs. Statistical significance was set at $p < 0.05$.

The microarray data analysis was performed in the R environment (version 2.15.1) using the Bioconductor libraries *limma* and *vsr*. *Vsr* normalisation (Huber et al., 2002) was used to eliminate technical differences between arrays. Linear model statistics (Smyth, 2005) was used to find differentially expressed genes ($p < 0.01$) between the control and the samples at both of the concentrations of 5-FU.

Gene set enrichment analysis (Subramanian et al., 2005) was performed to search for the gene ontology slim gene sets that were significantly altered by the 5-FU treatments (for detail description, see Supplementary Materials).

3. Results and discussion

The implications of chronic exposure of aquatic organisms and humans to the residues of anticancer drugs at low concentrations that are relevant to environmental pollution are to a large extent unknown. In the present study, we examined the effects of exposure of zebrafish to 5-FU across F0 to F2 generations, in terms of their survival, growth and reproduction, while also examining histological parameters, induction of genotoxic effects, and changes in whole genome transcription.

3.1. Treatment conditions

The exposure of the zebrafish to 5-FU was performed under semistatic conditions, with complete renewal of the recirculation system water every 3 days. The chemical analyses of the water samples showed that at the time of water renewal, the measured concentrations of 5-FU did not differ from the nominal concentrations of 1.0 µg/L and 100 µg/L 5-FU by more than 20%, while the measured concentration in the samples with the nominal concentration of 0.01 µg/L 5-FU was greater by 40% (Table 1). The measurements of the concentrations of 5-FU in the treatment water at the end of the second and the third days showed significant time-dependent decreases (Table 1). In the samples of the treatment water with an initial nominal concentration of 100 µg/L 5-FU, after 48 h this concentration had declined by 90%, and after 72 h 5-FU was no longer detected. In the samples of the treatment water with the initial nominal concentration 1.0 µg/L 5-FU, this concentration was below the detection limit already after 48 h. In the samples of the treatment water with the nominal

Table 1 – Concentrations of 5-FU in the treatment water within each of the 3 day renewals under the semistatic conditions used.

Nominal 5-FU concentration (µg/L)	Sampling period	Measured 5-FU concentration (µg/L)
100	Start	110.39 ± 0.81
	48 h	11.45 ± 2.85
	72 h	n.d. ^a
1.0	Start	1.15 ± 0.02
	48 h	n.d. ^a
	72 h	n.d. ^a
0.01	Start	0.014 ± 0.002
	48 h	0.006 ± 0.003
	72 h	0.004 ± 0.000

^a Not detected (limit of detection, 0.033 µg/L). Note that the method for chemical analysis of 5-FU at the two higher concentrations is different from that the lowest concentration (see Section 2.2. Test substance and chemical analyses) and that the two methods have different limits of detection.

concentration of 0.01 $\mu\text{g/L}$ 5-FU, this concentration had decreased by 55% after 48 h, and by 68% after 72 h, compared to the measured concentration of 5-FU at the time of water renewal. The exposure during the experiment is shown also as time weighted concentrations, which are at 100 and 1 $\mu\text{g/L}$ lower than nominal concentrations (Table 1). A previous study showed that in HPLC water, 1.0 $\mu\text{g/L}$ 5-FU is relatively stable (Negreiros et al., 2013). However, 5-FU has been shown to be photodegradable, with a half-life of 15 min (Kosjek et al., 2013; Lin et al., 2014). This might in part explain the rapid degradation of the 5-FU during the treatments that were performed under the light regime here (14 h light/10 h dark). Bioaccumulation of the 5-FU in the zebrafish could also be another contributing factor (Ud-Daula et al., 2012). In the environment, fluctuations in toxicant concentrations are expected, and therefore the semistatic exposure conditions used in the present study can be considered to mimic the pulse exposure to 5-FU that can be expected in an aquatic environment. For this reason we used for the presentation of the results the nominal concentrations of 5-FU at the renewals of the treatment water.

3.2. Survival and growth of the F1 and F2 generation zebrafish

No mortalities were observed during the 2-week treatment of the F0 zebrafish. In the F1 zebrafish, during the very early life stages (12 dpf), the mortality in the control and solvent-control groups did not exceed 10%. At 33 dpf, the F1 zebrafish mortality in control and all of the treatment groups ranged between 35.8% $\pm$ 8.1% and 56.2% $\pm$ 3.2%; and was not related to the 5-FU exposure (Table 2).

In the F2 zebrafish at the very early life stage (12 dpf), the mortality in the controls was higher than in the F1 zebrafish. Although the mortality of treatment groups was higher than solvent control group it was neither significant nor dose-dependent. At 33 dpf, the mortality in all of the groups of the F2 zebrafish ranged between 61% and 70%, and remained unrelated to the 5-FU exposure (Table 2). Similar high mortality of zebrafish under semistatic treatment conditions were reported by Fassbender and Braunbeck (2013). They considered that the reason for such high overall mortality is the renewals of the water during this sensitive growth phase. Furthermore, when the larvae were transferred from 1-L to 3-L tanks at 21 dpf they might have experienced mechanical damage, along with difficulties in uniform feeding. Like the F1 generation also in F2 generation no 5-FU exposure related mortality was observed.

Table 3 – Body parameters of F1 and F2 generation of zebrafish exposed to 0.01, 1 and 100 $\mu\text{g/L}$ 5-FU.

Zebrafish generation	Condition (n) ^a	Length (mm)	Wet weight (mg)
F1 (7 months)	Control (32)	23.20 $\pm$ 1.89	395 $\pm$ 32
	DMSO control (32) ^b	22.43 $\pm$ 1.60	394 $\pm$ 39
	0.01 $\mu\text{g/L}$ 5-FU (34)	22.41 $\pm$ 1.85	363 $\pm$ 34*
	1.0 $\mu\text{g/L}$ 5-FU (32)	24.14 $\pm$ 1.73	295 $\pm$ 38*
	100 $\mu\text{g/L}$ 5-FU (34)	23.05 $\pm$ 1.05	225 $\pm$ 44*
F2 (33 dpf)	Control (37)	7.30 $\pm$ 1.08	3.91 $\pm$ 0.12
	DMSO control (78) ^b	6.97 $\pm$ 1.03	4.53 $\pm$ 0.49
	0.01 $\mu\text{g/L}$ 5-FU (74)	7.10 $\pm$ 1.11	4.97 $\pm$ 0.72
	1.0 $\mu\text{g/L}$ 5-FU (60)	7.42 $\pm$ 1.00	4.90 $\pm$ 0.28
	100 $\mu\text{g/L}$ 5-FU (58)	7.36 $\pm$ 0.92	4.58 $\pm$ 0.62

* Significantly different from solvent control $p < 0.05$.

^a n = number of fish.

^b Vehicle control: 0.1% DMSO.

After 7 months, when the exposure of the F1 zebrafish to 5-FU was terminated, no significant differences were observed between the wet weights of the control group and the DMSO control group; however, there were significantly lower wet weights in the 0.01 $\mu\text{g/L}$ 5-FU group compared to the controls ($p \leq 0.05$) (Table 3). A controversial effect was observed at the higher concentrations of 5-FU, as the 1.0 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$ groups showed higher wet weights than the control groups, however the effect was not dose dependent (Table 3). These effects might be related to histopathological changes in the liver and kidney (see Section 3.4). The early life stage of the F2 zebrafish showed no significant differences in the wet weight and standard length between any of the treatment groups (Table 3).

These findings are in line with the data from previous studies where the adverse effects in zebrafish exposed to 5-FU were observed at concentrations of several higher orders of magnitude. In a chronic 35-day early-life-stage toxicity assay with zebrafish, the lowest effective concentration for overall subchronic effects was 100 mg/L 5-FU (Straub, 2010).

3.3. Developmental and reproduction parameters

No significant differences were observed for the fecundity of the female zebrafish, in terms of the number of eggs produced per female, nor for the number of infertile females among the control and treated groups (Table 4). A significant difference was found in the number of infertile eggs per female in the 1.0 $\mu\text{g/L}$ 5-FU group compared to the control during the fifth

Table 2 – Mortality of the F1 and F2 zebrafish under the experimental conditions used.

Condition	F1 zebrafish mortality [% $\pm$ SD]		F2 zebrafish mortality [% $\pm$ SD]	
	12 dpf	33 dpf	12 dpf	33 dpf
Control	3.84 $\pm$ 2.18	35.8 $\pm$ 8.13	10 $\pm$ 7.07	68 $\pm$ 0.0
DMSO control ^a	8.85 $\pm$ 1.63	52.7 $\pm$ 10.32	12.5 $\pm$ 0.71	61 $\pm$ 9.9
0.01 $\mu\text{g/L}$ 5-FU	4.32 $\pm$ 0.54	45 $\pm$ 2.69	19.5 $\pm$ 2.12	68 $\pm$ 8.49
1.0 $\mu\text{g/L}$ 5-FU	17.31 $\pm$ 8.16	56.16 $\pm$ 3.26	16.5 $\pm$ 2.12	70 $\pm$ 2.83
100 $\mu\text{g/L}$ 5-FU	5.77 $\pm$ 2.72	41.5 $\pm$ 0.06	20.5 $\pm$ 12.02	66 $\pm$ 4.24

^a Vehicle control: 0.1% DMSO.

Table 4 – Fecundity and numbers of infertile eggs normalized to the number of zebrafish females treated for 5 months as indicated, over 5 consecutive spawnings^a.

Condition	1st Spawning		2nd Spawning		3rd Spawning		5th Spawning	
	Fecundity (eggs spawned/female)	Infertile eggs (/female)	Fecundity (eggs spawned/female)	Infertile eggs (/female)	Fecundity (eggs spawned/female)	Infertile eggs (/female)	Fecundity (eggs spawned/female)	Infertile eggs (/female)
Control	30.4 ± 27.9	0.60 ± 1.20	21.6 ± 15.3	0.00 ± 0.00	19.5 ± 14.7	0.53 ± 0.58	36.3 ± 14.3	0.05 ± 0.16
DMSO control ^b	13.1 ± 20.1	0.15 ± 0.47	39.3 ± 38.5	0.00 ± 0.00	40.9 ± 16.6	0.98 ± 1.02	46.1 ± 17.1	0.12 ± 0.19
0.01 µg/L 5-FU	20.4 ± 16.6	0.00 ± 0.00	38.4 ± 38.1	0.00 ± 0.00	20.7 ± 18.9	0.08 ± 0.18	35.2 ± 11.6	0.65 ± 0.81
1.0 µg/L 5-FU	12.4 ± 14.5	0.00 ± 0.00	41.7 ± 25.8	1.17 ± 2.41	36.3 ± 20.4	1.19 ± 1.08	36.8 ± 21.6	3.47 ± 2.13*
200 µg/L 5-FU	30.4 ± 26.9	0.00 ± 0.00	35.0 ± 22.5	0.00 ± 0.00	15.2 ± 15.3	0.37 ± 0.51	21.1 ± 14.7	0.83 ± 0.74

^a Data from the 4th spawning are not given as they were significantly lower than those in the preceding and subsequent spawning due to human error.

^b Vehicle control: 0.1% DMSO.

spawning event ($p \leq 0.05$); however, this difference was not observed in the other two 5-FU-treated groups. At present, there are no data available regarding adverse effects of exposure to 5-FU on reproduction in fish models. Studies with the *Caenorhabditis elegans* have shown that 5-FU induces cell-cycle arrest in germ cells, changes in vulva development, reduced egg laying, and delay in reproductive time; however, these occurred at concentrations higher than 0.6 mg/L 5-FU (Kumar et al., 2010).

No morphological alterations were seen for the embryonic development of the F1 and F2 zebrafish, and there were no differences between the control and treatment groups in terms of the duration of embryonic development. In laboratory mammals 5-FU is a well established teratogen (Lau et al., 2000). Also in the embryotoxicity assay with *Pimephales promelas* exposure to 5-FU caused of embryo malformation (lowest effect concentration, 50 mg/L 5-FU) (DeYoung et al., 1996). However, the doses of 5-FU used in these studies were in comparison to the doses used in our study much higher 5-FU causes abnormalities in cell replication and/or cell death by inhibition of thymidylate synthetase that leads to imbalance of the deoxyribonucleotide pool (dNTP) (Lingley et al., 2003). A biologically based dose-response modelling of the relationship between administered dose of 5-FU, dNTP pool perturbations and malformations in rats showed that dNTP pool perturbations occurred at lower doses than developmental malformations (Lau et al., 2000). Therefore, homeostatic mechanism(s) responsible for the maintenance of normal foetal development the lower doses act downstream of dNTP pool perturbation. This explains why in zebrafish exposed to these low doses of 5-FU adverse effects on development did not occur.

3.4. Histopathology analysis

The histological examinations of the 7-month-old F1 zebrafish revealed histological changes to the liver and kidney in these F1 zebrafish treated with 5-FU, while no alterations were observed in the gonads of the F1 zebrafish of either sex, nor in the neural tissues, gills or intestinal tract. The absence of alterations in the reproductive performance of fish corresponds with histopathological observations. Lipidosis and regressive degeneration of the liver was observed in these F1 zebrafish for all of the 5-FU treatment groups, but not in the control and solvent control. Liver atrophy was observed in these F1 zebrafish treated with the highest concentration of 5-FU (Fig. 2, 100 µg/L 5-FU). This supports the observations that wet weights of these F1 fish groups exposed to 1 and 100 µg/L were significantly higher than those of the control groups. Similar histopathological changes in the liver as observed in the present study have been observed in albino rats after i.p. treatment with low (10 mg 5-FU/kg b.w.) and therapeutic (20 mg 5-FU/kg b.w.) doses of 5-FU (El-Sayyad et al., 2009).

Slight hyperplasia of the haematopoietic tissue of the kidney was observed in these F1 zebrafish treated with 0.01 µg/L and 1.0 µg/L 5-FU. In the 100 µg/L 5-FU treatment group, however, atrophy and depletion of the haematopoietic tissues were also apparent, as well as degeneration of the kidney tubular epithelium (tubulonephrosis) (Fig. 2). In zebrafish, the organ of haematopoiesis is the kidney marrow, which is

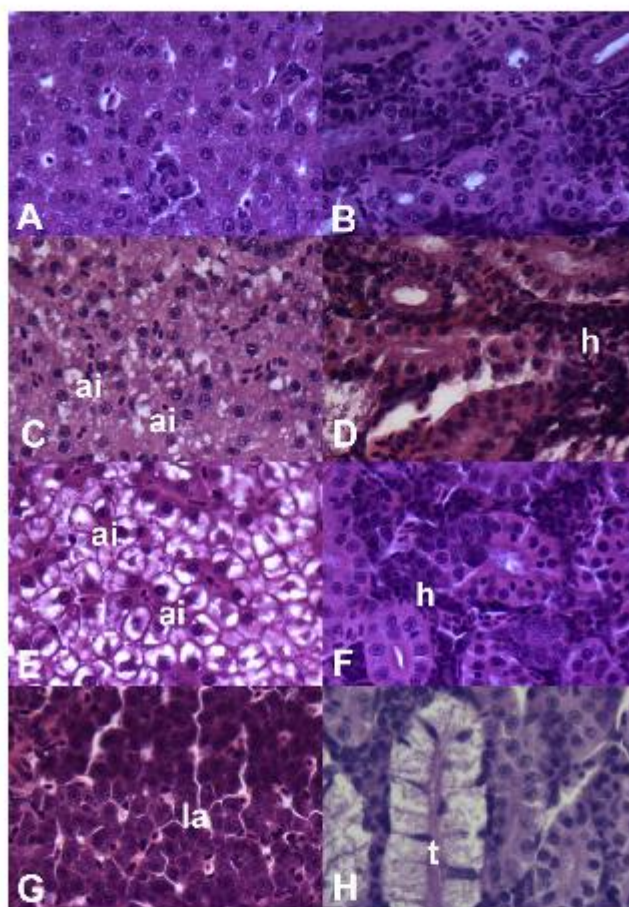


Fig. 2 – Representative micrographs of liver and kidney sections of zebrafish exposed for 7 months to various concentrations of 5-fluorouracil (5-FU). Histological alterations are not seen in the liver (A) or kidney (B) of the zebrafish in the control group. Minor adipose infiltration (lipidosis) (ai) can be seen in the liver of zebrafish exposed to 0.01 µg/L 5-FU (C) and 1.0 µg/L 5-FU (E), accompanied by slight hyperplasia (h) of the haematopoietic tissue in the kidney of the zebrafish exposed to 0.01 µg/L 5-FU (D) and 1.0 µg/L 5-FU (F). Liver atrophy (la) is visible in the zebrafish exposed to 100 µg/L 5-FU (G), along with tubulonephrosis (t) in the kidney (H). Magnification, 40x.

analogous to the mammalian bone marrow (Amatruda and Zon, 1999). Thus our observations in these F1 zebrafish are in line with the well-documented myelotoxicity of 5-FU in humans and rodents (Macdonald, 1999; Schuetz et al., 1984).

3.5. Genotoxic effects

The genotoxic effects of 5-FU during the chronic exposure of these F1 zebrafish were evaluated using the comet assay and the micronucleus assay. With the comet assay, significantly increased DNA damage was observed in liver cells at the

higher two 5-FU concentrations, and in the blood cells at 1.0 µg/L 5-FU (Fig. 3). There was also an increase in DNA damage with 5-FU treatment in the gonad cells; however, this was neither statistically significant nor dose dependent. No increases in DNA damage were observed in the gill cells. In the liver of the 5-FU-treated F1 zebrafish, histopathological changes were also observed, and it cannot be excluded that the DNA damage observed was also the consequence of unspecific cytotoxic effects (Vasquez, 2012).

In the erythrocytes from these F1 zebrafish, a significant dose-dependent increase in micronuclei formation was

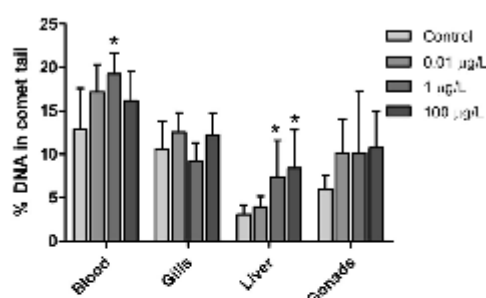


Fig. 3 – 5-FU-induced DNA damage in blood, gill, liver and gonad cells of 4-month-old F1 zebrafish. The zebrafish were treated with 5-FU (0, 0.01, 1.0, 100 µg/L) as described in the Materials and methods. DNA damage was assessed with the alkaline comet assay, and is expressed as percentages of tail DNA. Data are means $\pm$ SD from 4 individual zebrafish, with 100 cells analysed per concentration. Differences in % tail DNA between exposed and control groups (DMSO 0.1%) were statistically evaluated using R. The level of statistical significance was set at $p < 0.05$.

detected at all of the concentrations of 5-FU (Fig. 4). To confirm this, a second blood sampling was performed when the F1 zebrafish were 7 months old. The increase of micronuclei frequency over the level in the control group was at the lower two concentrations comparable in these F1 zebrafish after 4 months and 7 months of 5-FU exposure (~3 fold and 5 fold, respectively). At the highest tested concentration the increase of micronuclei frequency over the level in the control group was after 7 months higher than after 4 months of 5-FU treatment (~11 fold and 6 fold, respectively).

5-FU-induced micronuclei formation has been consistently shown in *in-vivo* studies with rodents after 5-FU i.p. administration (Choudhury et al., 2001; Zhou et al., 2013) and

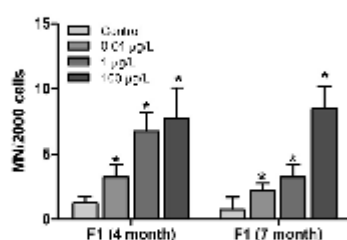


Fig. 4 – 5-FU-induced micronuclei (MN) in zebrafish erythrocytes. Zebrafish were exposed to 5-FU (0, 0.01, 1.0, 100 µg/L) as described in the Materials and methods. The micronuclei were determined in the F1 zebrafish treated with 5-FU for 4 months and 7 months. Data are means $\pm$ SD from four individuals. Significant difference (R) between treated and control (0.1% DMSO) zebrafish is indicated by * ($P < 0.05$).

after cutaneous 5-FU application (Zuniga-Gonzalez et al., 2003), and in fish after intra-abdominal 5-FU administration (2.5 mg/kg b.w.) (Grisolia and Cordeiro, 2000). However, these studies were short-term with a single administration of high-dose 5-FU, which is not directly relevant for the present environmental exposure scenario. Here, we show that chronic exposure to very low concentrations of 5-FU that are relevant to environmental exposure can cause chromosomal damage.

3.6. Toxicogenomic analyses

Toxicogenomics determines global transcriptomic responses to chemical exposure, and can predict the effects and provide information on the mechanisms of action. The whole genome gene expression analyses of the liver samples of the F1 zebrafish in response to chronic exposure to 0.01 µg/L and 1.0 µg/L 5-FU showed clear dose-dependent increases in the number of differentially expressed genes. At the lower 5-FU concentration, 1879 genes were differentially expressed, while at the higher concentration, 4608 genes were differentially expressed (Fig. 5). Approximately 20% of the genes (1041) were differentially expressed regardless of the 5-FU concentration. For these genes, no dose-dependent responses were seen, as their changes in expression were similar at both of the 5-FU concentrations analysed (data not shown). Our validation of the microarray gene expression data by analysis of the expression of eight biologically relevant genes (*myca*, *jun*, *nfkb1b*, *sipr1*, *igfbp1b*, *pak2b*, *gadd45* and *ctb*) by quantitative PCR showed good correlation (correlation coefficient, 0.91), (Supplementary Material, Table S3).

The functional analysis of the transcriptome signature revealed that the targets of chronic exposure to 5-FU are processes that are involved in primary metabolism, cell division and signalling, and immune and developmental processes (Supplementary Material, Table S4). Up-regulation of genes involved in cell death and down-regulation of protein synthesis observed at both of the 5-FU concentrations tested can be linked to the primary effects of this cytostatic agent.

It has been shown before that induction of DNA damage in zebrafish embryos is reflected in the expression of genes involved in response to DNA damage and DNA repair, which was dependent of the dose and time of the inducers (Reinardy

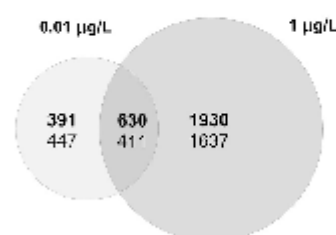


Fig. 5 – The number of unique and common differentially expressed genes in liver samples of F1 zebrafish exposed to 0.01 and 1 µg/L of 5-FU versus the vehicle control (0.1% DMSO) ($p < 0.01$; no false discovery rate; bold, up-regulated; regular, down-regulated).

et al., 2013). Among the genes that are specifically involved in responses to DNA damage, there was deregulation of the expression of the tumour suppressor gene p53 and its downstream regulated genes: cyclin-dependent kinase inhibitors pak 2a and pak 2b, the growth arrest and DNA damage inducible gene gadd45 ab, the pro-apoptotic and anti-apoptotic genes bax and bcl2, respectively, and the DNA repair gene xrc5 (Supplementary Material, Table S5). The observed up-regulation of the expression of genes involved in responses to DNA damage and repair can be linked with the dose-dependent increase in DNA damage that we observed for the liver of these 5-FU-treated zebrafish.

Cancer development is frequently associated with mutations, and consequently, deregulation of the expression of oncogenes (Loeb et al., 2008). We observed up-regulation of several oncogenes following the 5-FU treatments here (oncogenes: myc, jun, ets2, maf; Supplementary Material, Table S5). Myc genes are key regulators of cell proliferation, and their deregulation contributes to the genesis of most human tumours (Adhikary and Eilers, 2005; Meyer and Penn, 2008). The mechanisms of oncogenic activation of MYC generally involve chromosomal translocations and amplifications that result in its elevated expression (Meyer and Penn, 2008). The observed up-regulation of the expression of the myc oncogene and other oncogenes can therefore be linked to the induction of micronuclei detected in the peripheral erythrocytes of these 5-FU-treated zebrafish.

3.7. Significance of the observed genotoxic effect and transcriptomic changes in the context of ecological risk

It is well recognized that DNA damaging agents have a significant ecological relevance since they cause genetic alterations in somatic and germ cells that are associated with serious adverse effects, which may occur even at low exposure levels (Bolognesi and Cirillo, 2014).

Our study showed that chronic two-generation exposure of zebrafish to 5-FU at concentrations that are relevant for environmental exposure induced DNA strand breaks, micronuclei, whole transcriptome changes and histopathological changes, while the reproductive parameters were not affected. The question is the significance of these effects in the context of ecological risk assessment.

On the contrary to human risk assessment that is aimed to protect individuals, ecological risk assessment is aimed to protect natural populations. In ecotoxicology the detrimental outcomes of the exposure to DNA damaging compounds are considered to be gamete loss, embryo mortality, abnormal development, neoplasia that may affect reproductive success of exposed populations and heritable mutations that may cause increase or decrease in genetic diversity (Anderson and Wild, 1994). Although there is a clear evidence linking exposure of aquatic organisms to genotoxic contaminants and adverse effects such as DNA damage, chromosome aberrations, and cancer, it is still not clear how these effects are expressed as adverse effect at the level of populations. From the ecological point of view, reproduction failure is seen as a crucial link between the genotoxic effects in individuals and long-term population changes.

At the applied exposure conditions in our study we did not observe any effect on the reproduction of exposed fish. A similar two-generation study with the model genotoxicant 4-nitroquinoline-1-oxide have shown some evidence of causal relationships between genotoxic effects and impairment of zebrafish reproduction although it was not clear whether the effect was related to genotoxic pathway of 4-nitroquinoline-1-oxide (Diekmann et al., 2004a, b). In a study with model genotoxin methyl methanesulphonate paternal exposure to this compound resulted in DNA damage in spermatozoa and a large array of morphological abnormalities during embryonic and larval development (Devaux et al., 2013). In our study 5-FU did not induce significant increase in DNA damage in gonads which may explain the lack of reproductive and developmental effects.

Genotoxic effects in somatic cells are associated with initiation and development of cancer and other degenerative diseases (Bonassi et al., 2011). In association with exposure to specific classes of DNA-damaging pollutants, it is not uncommon to find epizootic neoplasms in aquatic species, such as shellfish, echinoderms and fish (Pinkney et al., 2011; Myers et al., 2003). Therefore the observed up-regulation of the expression of several oncogenes indicate that chronic exposure of fish to 5-FU may induce neoplasia. The presence of tumours in populations of wild fish is for more than 20 years used as a bioindicator of fish and ecosystem health (Pinkney et al., 2011), however so far no evidence has been found for correlation between high incidence of tumours and steady decline in population size in the environment.

Although 5-FU remains to be one of the most extensively used chemotherapeutics the data on its environmental occurrence is limited. In the sewers of oncological wards 5-FU has been detected at concentration ranges from <5 ng/L to up to 150 ng/L (Kosjek and Heath, 2011; Lin et al., 2014). There are only two studies in which municipal wastewaters and surface waters were analyzed for the occurrence of 5-FU. In the influent of municipal wastewater plant 4.7–35 ng/L 5-FU was reported while it was not detected in the effluent and the river water downstream the treatment plant (Kosjek et al., 2013). Recently Lin et al. (2014) analyzed the occurrence of 5-FU in hospital wastewater and river samples in two regions in Taiwan. They reported presence of 5-FU in 10 of 13 hospital wastewater samples and in 18 out of 20 river water samples. The median concentration values were in the high populated region 120 ng/L (48–1500 ng/L) in hospital water and 6.2 (5.0–70 ng/L) in river water samples whereas in the low populated region the median concentrations in hospital and river water samples were 24 ng/L (46–510 ng/L) and 56 ng/L (35–160 ng/L), respectively. This study, at least for Taiwan, indicate ubiquitous presence of 5-FU in surface waters at concentrations that are in the range of the concentrations applied in our study. This implies that potential ecological risk of aquatic exposure of vertebrate to 5-FU cannot be ruled out.

4. Conclusions

Our study demonstrates for the first time that chronic two-generation exposure of zebrafish to 5-FU at concentrations that are relevant for environmental exposure impairs their

DNA integrity and induced massive whole-transcriptome changes, which over long-term exposure of several generations might affect fish populations. We suggest that for the prediction of the adverse effects of genotoxic cytostatic drugs to aquatic vertebrate, long-term, multiple generation studies that include detection of genotoxic effect and transcriptomic changes are warranted. To enable environmental risk assessment more data on the occurrence of cytostatic drugs in surface waters are needed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2015.03.025>.

REFERENCES

- Adhikary, S., Silers, M., 2005. Transcriptional regulation and transformation by MYC proteins. *Nature Reviews. Mol. Cell. Biol.* 6, 635–645.
- Amatruda, J.F., Zon, L.I., 1999. Dissecting hematopoiesis and disease using the zebrafish. *Dev. Biol.* 216, 1–15.
- Anderson, S.L., Wild, G.C., 1994. Linking genotoxic responses and reproductive success in ecotoxicology. *Environ. Health Perspect.* 102, 9–12.
- Besse, J.P., Latour, J.F., Garric, J., 2012. Anticancer drugs in surface waters. What can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? *Environ. Int.* 39, 73–86.
- Bolognesi, C., Cirillo, S., 2014. Genotoxicity biomarkers in aquatic bioindicators. *Curr. Zool.* 60, 273–284.
- Bolognesi, C., Hayashi, M., 2011. Micronucleus assay in aquatic animals. *Mutagenesis* 26, 205–213.
- Bonassi, S., El-Zein, R., Bolognesi, C., Fenech, M., 2011. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis* 26, 93–100.
- Brezovsek, P., Elser, T., Filipic, M., 2014. Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Synchococcus leopoldensis*. *Water Res.* 52, 168–177.
- Choudhury, R.C., Misra, S., Jagdale, M.B., Palo, A.K., 2001. S-phase dependent cytogenetic toxicity of 5-fluorouracil in bone marrow cells of mice. *J. Exp. Clin. Cancer Res.* 20, 57–62.
- Devaux, A., Fiat, L., Gillet, C., Bony, S., 2013. Reproduction impairment following paternal genotoxin exposure in brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*). *Aquat. Toxicol.* 101, 405–411.
- DeYoung, D.J., Bantle, J.A., Hull, M.A., Burks, S.L., 1996. Differences in sensitivity to developmental toxicants as seen in *Xenopus* and *Pimephales* embryos. *Bull. Environ. Contam. Toxicol.* 56, 143–150.
- Diekmann, M., Hultsch, V., Nagel, R., 2004a. On the relevance of genotoxicity for fish populations I: effects of a model genotoxicant on zebrafish (*Danio rerio*) in a complete life-cycle test. *Aquat. Toxicol.* 68, 13–26.
- Diekmann, M., Waldmann, P., Schnurstein, A., Grummt, T., Braunbeck, T., Nagel, R., 2004b. On the relevance of genotoxicity for fish populations II: genotoxic effects in zebrafish (*Danio rerio*) exposed to 4-nitroquinoline-1-oxide in a complete life-cycle test. *Aquat. Toxicol.* 68, 27–37.
- Edgar, R., Domrachev, M., Lash, A.E., 2002. Gene expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 30, 207–210.
- El-Sayyad, H.I., Ismail, M.F., Shalaby, F.M., Abou-El-Magd, R.F., Gaur, R.L., Fernando, A., Raj, M.H.G., Ouhit, A., 2009. Histopathological effects of cisplatin, doxorubicin and 5-fluorouracil (5-FU) on the liver of male albino rats. *Int. J. Biol. Sci.* 5, 466–473.
- European Medicines Agency, 2006. Guidelines on the Environmental Risk Assessment of Medicinal Products for Human Use. European Chemical Agency. Doc ref. EMEA/CHMP/SWP/4447/00.
- Fassbender, C., Braunbeck, T., 2013. Reproductive and genotoxic effects in zebrafish after chronic exposure to methyl methanesulfonate in a multigeneration study. *Ecotoxicology* 22, 825–837.
- Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The comet assay for the evaluation of genotoxic impact in aquatic environments. *Mutat. Res. Rev. Mutat. Res.* 681, 80–92.
- Girardot, F., Monnier, V., Tricoire, H., 2004. Genome wide analysis of common and specific stress responses in adult *Drosophila melanogaster*. *BMC Genomics* 5, 16.
- Grisolia, C.K., Cordeiro, C.M.T., 2000. Variability in micronucleus induction with different mutagens applied to several species of fish. *Genet. Mol. Biol.* 23, 235–239.
- Hernando, M.D., Mezcu, M., Fernandez-Alba, A.R., Barólo, D., 2006. Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. *Talanta* 69, 334–342.
- Huber, W., von Heydebreck, A., Sultmann, H., Poustka, A., Vingron, M., 2002. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 18 (1), S96–S104.
- Johnson, A.C., Jurgens, M.D., Williams, R.J., Kummer, K., Kortenkamp, A., Sumpter, J.P., 2008. Do cytotoxic chemotherapy drugs discharged into rivers pose a risk to the environment and human health? an overview and UK case study. *J. Hydrol.* 348, 167–175.
- Knežević, A.H., Đikić, D., Lisičić, D., Kopjar, N., Orsolić, N., Karabeg, S., Benković, V., 2011. Synergistic effects of irinotecan and flavonoids on Ehrlich ascites tumour-bearing mice. *Basic Clin. Pharmacol. Toxicol.* 109, 343–349.
- Kosjek, T., Heath, E., 2011. Occurrence, fate and determination of cytostatic pharmaceuticals in the environment. *Trends Anal. Chem.* 30 (7), 1065–1087.
- Kosjek, T., Perko, S., Žigon, D.A., Heath, E., 2013. Fluorouracil in the environment: analysis, occurrence, degradation and transformation. *J. Chromatogr. A* 1290, 62–72.
- Kumar, S., Aninat, C., Michaux, G.G., Morel, F., 2010. Anticancer drug 5-fluorouracil induces reproductive and developmental defects in *Caenorhabditis elegans*. *Reprod. Toxicol.* 29, 415–420.
- Lau, C., Andersen, M.E., Crawford-Brown, D.J., Kavlock, R.J., Kimmel, C.A., Knudsen, T.B., Muneoka, K., Rogers, J.M.,

- Setzer, R.W., Smith, G., Tyl, R., 2000. Evaluation of biologically based dose-response modeling for developmental toxicity: a workshop report. *Regul. Toxicol. Pharmacol.* 31, 190–199.
- Lenz, K., Mahnik, S.N., Weissenbacher, N., Mader, R.M., Krenn, P., Hann, S., Koellensperger, G., Uhl, M., Knasmüller, S., Ferk, F., Bursch, W., Fierhacker, M., 2007. Monitoring, removal and risk assessment of cytostatic drugs in hospital wastewater. *Water Sci. Technol.* 56, 141–149.
- Lin, A.Y., Lin, Y.C., Lee, W.N., 2014. Prevalence and sunlight photolysis of controlled and chemotherapeutic drugs in aqueous environments. *Environ. Pollut.* 187, 170–181.
- Loeb, L.A., Bielas, J.H., Beckman, R.A., 2008. Cancers exhibit a mutator phenotype: clinical implications. *Cancer Res.* 68, 3551–3557.
- Longley, D.B., Harkin, D.P., Johnston, P.G., 2003. 5-Fluorouracil: mechanisms of action and clinical strategies. *Nat. Rev. Cancer* 3, 330–338.
- Lorge, E., Thybaud, V., Aardema, M.J., Oliver, J., Wakata, A., Lorenzon, G., Marzin, D., 2006. SFTG international collaborative study on in-vitro micronucleus test - I. General conditions and overall conclusions of the study. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 607, 13–36.
- Macdonald, J.S., 1999. Toxicity of 5-fluorouracil, vol. 13. *Oncology*, New York, pp. 33–34.
- Meyer, N., Penn, L.Z., 2008. MYC – Timeline reflecting on 25 years with MYC. *Nat. Rev. Cancer* 8, 976–990.
- Myers, M.S., Johnson, L.L., Collier, T.K., 2003. Establishing the causal relationship between polycyclic aromatic hydrocarbon (PAH) exposure and hepatic neoplasms and neoplasia-related liver lesions in English sole (*Pleuronectes vetulus*). *Hum. Ecol. Risk Assess.* 9, 67–94.
- Negreira, N., Mastroianni, N., López de Alda, M., Barceló, D., 2013. Multianalyte determination of 24 cytostatics and metabolites by liquid chromatography-electrospray-tandem mass spectrometry and study of their stability and optimum storage conditions in aqueous solution. *Talanta* 116, 290–299.
- Ohuchida, A., Furukawa, A., Yoshida, J., Watanabe, M., Aruga, F., Miwa, Y., Shinkawa, K., Kinase, N., 1992. Micronucleus assays on 5-fluorouracil and 6-mercaptopurine with mouse peripheral-blood reticulocytes. *Mutat. Res.* 278 (2–3), 139–143.
- Oka, H., Ikeda, K., Yoshimura, H., Ohuchida, A., Honma, M., 2006. Relationship between p53 status and 5-fluorouracil sensitivity in 3 cell lines. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 606, 52–60.
- Parrella, A., Lavorgna, M., Criscuolo, E., Russo, C., Fiumano, V., Isidori, M., 2014. Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans. *Chemosphere* 115, 59–66.
- Pinkney, A.E., Harshbarger, J.C., Karouna-Renier, N.K., Jenko, K., Balk, L., Skarphéðinsdóttir, H., Liewenborg, B., Rutter, M.A., 2011. Tumor prevalence and biomarkers of genotoxicity in brown bullhead (*Ameiurus nebulosus*) in Chesapeake bay tributaries. *Sci. Total Environ.* 410–411, 248–257.
- Reinardy, H.C., Dharamshi, J., Jha, A.N., Henry, T.B., 2013. Changes in expression profiles of genes associated with DNA repair following induction of DNA damage in larval zebrafish *Danio rerio*. *Mutagenesis* 28, 601–608.
- Schuetz, J.D., Wallace, H.J., Diasio, R.B., 1984. 5-Fluorouracil incorporation into DNA of CF-1 mouse bone-marrow cells as a possible mechanism of toxicity. *Cancer Res.* 44, 1358–1363.
- Singh, N.P., McCoy, M., Tice, R., Schneider, E., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175, 184–191.
- Smyth, G.K., 2005. Limma: linear models for microarray data. In: Gentleman, R., Carey, V.J., Huber, W., Irizarry, R.A., Dudoit, S. (Eds.), *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*, Statistics for Biology and Health. Springer-Verlag, pp. 397–420.
- Straub, J.O., 2010. Combined environmental risk assessment for 5-fluorouracil and capecitabine in Europe. *Integr. Environ. Assess. Manag.* 6, 540–566.
- Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U. S. A.* 102, 15545–15550.
- Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in-vitro and in-vivo genetic toxicology testing. *Environ. Mol. Mutagen.* 35, 206–221.
- Ud-Daula, A., Pfister, G., Schramm, K.W., 2012. 5-Fluorouracil accumulation in green microalgae and its biogenic transfer into ciliate protozoan. *Bull. Environ. Contam. Toxicol.* 88 (4), 548–554.
- van Kuilenburg, A.B.P., Maring, J.G., 2013. Evaluation of 5-fluorouracil pharmacokinetic models and therapeutic drug monitoring in cancer patients. *Pharmacogenomics* 14, 799–811.
- Vasquez, M.Z., 2012. Recommendations for safety testing with the in vivo comet assay. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 747, 142–156.
- Williams, T.D., Mirbahi, L., Chipman, J.K., 2014. The toxicological application of transcriptomics and epigenomics in zebrafish and other teleosts. *Brief. Funct. Genomics* 13, 157–171.
- Zhou, C., Wang, Q., Wang, Z., Chang, Y., 2013. Comparison of three-colour flow cytometry and slide-based microscopy for the scoring of micronucleated reticulocytes in rat bone-marrow and peripheral blood. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 758, 12–17.
- Zuniga-Gonzalez, G.M., Torres-Bugarin, O., Zamora-Perez, A.I., Gomez-Meda, B.C., Ramos-Ibarra, M.L., Gallegos-Arreola, P., Flores-Garcia, A., Lopez-Urbe, A., 2003. Induction of micronucleated erythrocytes in mouse peripheral blood after cutaneous application of 5-fluorouracil. *Arch. Med. Res.* 34, 141–144.

Chapter 4

Conclusions

Cytostatics, due to their mechanism of action, are highly hazardous chemicals. However, very little is known about potential risks for environmental and human health of their residues when released into the environment. In this study we performed *in vitro* and *in vivo* toxicity and genotoxicity studies of selected highly consumed cytostatics (5-FU, CDDP, ET and IM) and their mixtures, which provided valuable new data relevant for environmental and human hazard and risk and risk assessment.

The *in vitro* genotoxicity studies of 5-FU, CDP and ET with human (human hepatoma HepG2 cells, human peripheral blood lymphocytes; HPBLs) and fish (zebrafish (*Danio rerio*) liver (ZFL) cells) derived *in vitro* cell models demonstrated a different toxic and genotoxic potential of different cytostatics as well as a different susceptibility of fish-derived ZFL cells compared to human-derived HepG2 cells and HPBLs to cytotoxic and genotoxic effects of selected cytostatics. ZFL cells were the most susceptible, which raises the question of a negative impact of these pharmaceuticals on fish populations if discharged into aquatic environments excessively. The high sensitivity of non-target ZFL cells also indicates that these cells could be a good model for testing the genotoxic potential of pharmaceuticals as well as other aquatic pollutants and could serve as an alternative to animal testing in preliminary ecotoxicological studies and for environmental screening.

Surprisingly we discovered that IM induced DNA strand breaks and micronuclei formation in fish and human cells despite its different mechanism of therapeutic action, which involves inhibition of mutated tyrosine kinases in certain types of cancer and not direct interaction with DNA. Comparing the effects of IM, 5-FU, CDDP and ET on the expression of genes that are typically deregulated by exposure to genotoxic compounds (genes involved in apoptosis, DNA damage response and oncogenes) revealed that 5-FU, CDDP and ET, but not IM deregulated the expression of these genes. This confirms the indirect mechanism of genotoxic action of IM, which most probably does not involve a direct interaction with DNA. Nevertheless, considering genotoxicity of IM, an increased risk for humans and aquatic organisms at chronic exposure to low concentrations cannot be completely excluded. On the other hand, because IM is not likely to directly interact with the genetic material, exposure to IM during the cancer treatment represents for the patients a lower risk for delayed effects such as secondary cancer, reproductive effects and heritable disease than cancer treatment with 5-FU, CDDP or ET.

In wastewaters and in the aquatic environment the residues of different cytostatics as well as their metabolites and transformation products are present simultaneously in complex mixtures in which different interactions may occur leading to additive, synergistic, antagonistic or potentiating effects. This is particularly relevant in cases of combined exposure to the residues of cytostatics, which are, in cancer treatment regimens, very often used in combinations to obtain improved efficiency of the treatments. The results of the cytotoxicity and genotoxicity testing of a complex mixture that consisted of four cytostatics (CP, IF, CDDP and 5-FU) at their maximal detected concentrations in the effluents of the Oncological Ward induced the increased formation of DNA strand breaks in exposed ZFL cells. The concentrations of the individual compounds were in the mixture much lower than the effective concentrations when tested as individual compounds. The results very clearly demonstrated that cytostatic residues, when they occur in a mixture, may pose a threat to aquatic organisms even at low environmentally relevant concentrations and contribute to the accumulating evidence that it is not always possible

to predict adverse effects of complex mixtures based on the toxicological data for individual compounds.

On the other hand, information regarding toxicity of metabolites and transformation products (TPs), especially their mixtures with parent compounds, is almost completely missing. We showed that certain metabolites/TP of CP and IF exhibit higher ecotoxicity and genotoxicity than the parent compounds. These results indicate the importance of toxicological evaluation and monitoring of metabolites and TPs as some of them may be for certain aquatic organisms more hazardous than the parent compounds.

In the study of the chemical and toxicological characterization of cytostatics in the hospital and municipal wastewaters we confirmed that their residues typically occur as complex mixtures of parent compounds, their metabolites and TPs. Even though the measured concentrations of an individual compound were low, eco/genotoxic effects of the samples were observed in daphnids, higher plants and *in vitro* in ZFL cells. A statistical correlation between the results of toxicity tests and the concentration of identified single drug in the sample was found, however the total concentration of the residues within the sample did not correlate with their toxicities. The observed toxic effects could be due to specific interactions between the cytostatic residues and their TPs as well as due to the presence of other pollutants that not been chemically characterized. These findings underline the importance of control of the release of the cytostatics and the need for adequate removal by waste water treatment.

In order to explore whether cytostatics at chronic exposure to low concentrations that are relevant for environmental contamination induce adverse effects in vertebrate we performed a chronic two-generation exposure study of 5-FU in zebrafish. To the best of our knowledge this is the first such study with cytostatics. The results demonstrated 5-FU induced histopathological changes in the liver and kidney, genotoxic effects in liver and blood cells and massive deregulation of whole transcriptome including up-regulation of several DNA-damage-responsive genes and oncogenes at exposure concentrations that are in the range of predicted or measured environmental concentrations. The up-regulation of several onco-genes after chronic exposure of zebrafish to 5-FU indicates oncogenic potential of this compound that can be expected also in humans after chronic exposure (i.e. occupational exposure). Thus, these findings are not relevant only for the risks assessment for aquatic environment but also for humans.

The results of this thesis filled important knowledge gaps and contributed to a better understanding of the mechanisms of action and potential adverse effects of cytostatic residues to aquatic organisms as well as humans. It can be concluded that certain highly consumed cytostatics do represent a risk for the environment and humans. Of particular importance are genotoxic effects induced by these compounds, which are important parameters in the human risk assessment where the subject of protection are individuals. On the contrary, in the environmental risk assessment the subject of protection are populations of aquatic organisms, therefore the significance of this parameter is not clear. Nevertheless, because genotoxic effects lead to severe consequences, our opinion is that genotoxic endpoint should not be neglected in environmental risk assessment, however further studies are needed to clarify the significance. Moreover, for genotoxic compounds it is not possible to establish safe limits, therefore within the current legislation it should be considered to exclude genotoxic cytostatics from the action limit (i.e. EMA, 2006: 10 ng/L) and make ERA for these chemicals obligatory. In addition to standard toxicological endpoints also genotoxicity as well as relevant transcriptomic endpoints should be included in ERA.

The work presented in this thesis was reported in 7 scientific papers, published in SCI international journals, and presented at 9 national and international conferences.

References

- Aherne GW, Hardcastle A, Nield AH. Cytotoxic drugs and the aquatic environment: estimation of bleomycin in river and water samples. *J Pharm Pharmacol* 1990; 42: 741-2.
- Araldi RP, de Melo TC, Mendes TB, de Sa Junior PL, Heidi B, Nozima N, Ito ET, de Carvalho RF, de Souza EB, Stocco RC. Using the comet and micronucleus assays for genotoxicity studies: A review. *Biomedicine and Pharmacotherapy* 2015; 72: 74-82
- Arora A, Scholar EM. Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther* 2005; 315: 971-9.
- Besse JP, Latour JF, Garric J. Anticancer drugs in surface waters What can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? *Environment International* 2012; 39: 73-86.
- Bonassi S, El-Zein R, Bolognesi C, Fenech M. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis* 2011; 26: 93-100.
- Booker V, Halsall C, Llewellyn N, Johnson A, Williams R. Prioritising anticancer drugs for environmental monitoring and risk assessment purposes. *Sci Total Environ* 2014; 473-474: 159-70.
- Bound JP, Voulvoulis N. Pharmaceuticals in the aquatic environment--a comparison of risk assessment strategies. *Chemosphere* 2004; 56: 1143-55.
- Boxall AB, Rudd MA, Brooks BW, Caldwell DJ, Choi K, Hickmann S, et al. Pharmaceuticals and personal care products in the environment: what are the big questions? *Environ Health Perspect* 2012; 120: 1221-9.
- Brezovsek P, Elersek T, Filipic M. Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Synechococcus leopoliensis*. *Water Res* 2014; 52: 168-77.
- Brock N. Oxazaphosphorine cytostatics: past-present-future. Seventh Cain Memorial Award lecture. *Cancer Res* 1989; 49: 1-7.
- Buerge IJ, Buser HR, Poiger T, Muller MD. Occurrence and fate of the cytostatic drugs cyclophosphamide and ifosfamide in wastewater and surface waters. *Environ Sci Technol* 2006; 40: 7242-50.
- Burden DA, Osheroff N. Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. *Biochimica Et Biophysica Acta-Gene Structure and Expression* 1998; 1400: 139-154.
- Commission of the European Communities, 1996. Technical Guidance Document in Support of Commission Directive 93/ 67/EEC on Risk Assessment for New Notified Substances and Commission Regulation (EC) No 1488/94 on Risk Assessment for Existing Substances. Part II; Environmental Risk Assessment. Office for Official Publications of the European Communities, Luxembourg.
- Daughton CG. Environmental stewardship and drugs as pollutants. *Lancet* 2002; 360: 1035-6.
- Dong Z, Senn DB, Moran RE, Shine JP. Prioritizing environmental risk of prescription pharmaceuticals. *Regul Toxicol Pharmacol* 2013; 65: 60-7.
- Druker B. Imatinib (Gleevec) as a paradigm of targeted cancer therapies. *Keio J Med* 2010; 59: 1-3.
- Emadi A, Jones RJ, Brodsky RA. Cyclophosphamide and cancer: golden anniversary. *Nat Rev Clin Oncol* 2009; 6: 638-47.
- Ellinger-Ziegelbauer H, Aubrecht J, Kleinjans JC, Ahr HJ. Application of toxicogenomics to study mechanisms of genotoxicity and carcinogenicity. *Toxicology Letters* 2009; 186: 36-44.

- Fent K. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. *Toxicol In Vitro* 2001; 15: 477-88.
- Fent K, Weston AA, Caminada D. Ecotoxicology of human pharmaceuticals. *Aquat Toxicol* 2006; 76: 122-59.
- Ferrando-Climent L, Rodriguez-Mozaz S, Barcelo D. Incidence of anticancer drugs in an aquatic urban system: from hospital effluents through urban wastewater to natural environment. *Environ Pollut* 2014; 193: 216-23.
- Fleming RA. An overview of cyclophosphamide and ifosfamide pharmacology. *Pharmacotherapy* 1997; 17: 146S-154S.
- Franquet-Griell H, Gomez-Canela C, Ventura F, Lacorte S. Predicting concentrations of cytostatic drugs in sewage effluents and surface waters of Catalonia (NE Spain). *Environ Res* 2015; 138: 161-72.
- Gacic Z, Kolarevic S, Sunjog K, Kracun-Kolarevic M, Paunovic M, Knezevic-Vukcevic J, et al. The impact of *in vivo* and *in vitro* exposure to base analogue 5-FU on the level of DNA damage in haemocytes of freshwater mussels *Unio pictorum* and *Unio tumidus*. *Environ Pollut* 2014; 191: 145-50.
- Gomez-Canela C, Ventura F, Caixach J, Lacorte S. Occurrence of cytostatic compounds in hospital effluents and wastewaters, determined by liquid chromatography coupled to high-resolution mass spectrometry. *Anal Bioanal Chem* 2014; 406: 3801-14.
- Guainazzi A, Scharer OD. Using synthetic DNA interstrand crosslinks to elucidate repair pathways and identify new therapeutic targets for cancer chemotherapy. *Cell Mol Life Sci* 2010; 67: 3683-97.
- Hagner N, Joerger M. Cancer chemotherapy: targeting folic acid synthesis. *Cancer Manag Res* 2010; 2: 293-301.
- Heberer T. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicol Lett* 2002; 131: 5-17.
- Jamieson ER, Lippard SJ. Structure, Recognition, and Processing of Cisplatin-DNA Adducts. *Chem Rev* 1999; 99: 2467-98.
- Jha AN. Genotoxicological studies in aquatic organisms: an overview. *Mutat Res* 2004; 552: 1-17.
- Johnson AC, Jürgens MD, Williams RJ, Kümmere K, Kortenkamp A, Sumpter JP. Do cytotoxic chemotherapy drugs discharged into rivers pose a risk to the environment and human health? An overview and UK case study. *Journal of Hydrology* 2008; 348: 167-175.
- Johnson AC, Oldenkamp R, Dumont E, Sumpter JP. Predicting concentrations of the cytostatic drugs cyclophosphamide, carboplatin, 5-fluorouracil, and capecitabine throughout the sewage effluents and surface waters of Europe. *Environ Toxicol Chem* 2013; 32: 1954-61.
- Kelland L. The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* 2007; 7: 573-84.
- Kosjek T, Heath E. Occurrence, fate and determination of cytostatic pharmaceuticals in the environment. *Trac-Trends in Analytical Chemistry* 2011; 30: 1065-1087.
- Kovacs R, Bakos K, Urbanyi B, Kovesi J, Gazsi G, Csepeli A, et al. Acute and sub-chronic toxicity of four cytostatic drugs in zebrafish. *Environ Sci Pollut Res Int* 2015.
- Kovalova L, Mc Ardell CS, Hollender J. Challenge of high polarity and low concentrations in analysis of cytostatics and metabolites in wastewater by hydrophilic interaction chromatography/tandem mass spectrometry. *Journal of Chromatography A* 2009; 1216: 1100-1108.
- Kovalova L, Siegrist H, Singer H, Wittmer A, Mc Ardell CS. Hospital wastewater treatment by membrane bioreactor: performance and efficiency for organic micropollutant elimination. *Environ Sci Technol* 2012; 46: 1536-45.
- Kračun-Kolarević M, Kolarević S, Atanacković A, Marković V, Gačić Z, Paunović M, et al. Effects of 5-Fluorouracil, Etoposide and CdCl₂ in Aquatic Oligochaeta *Limnodrilus udekemianus* Claparede (Tubificidae) Measured by Comet Assay. *Water, Air and Soil Pollution* 2015; 226.
- Kruhlak NL, Benz RD, Zhou H, Colatsky TJ. (Q)SAR Modeling and Safety Assessment in Regulatory Review. *Clinical Pharmacology and Therapeutics* 2012: 529-534.
- Kummerer K. Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources--a review. *Chemosphere* 2001; 45: 957-69.

- Kummerer K. The presence of pharmaceuticals in the environment due to human use--present knowledge and future challenges. *J Environ Manage* 2009; 90: 2354-66.
- Kummerer K, Al-Ahmad A. Estimation of the cancer risk to humans resulting from the presence of cyclophosphamide and ifosfamide in surface water. *Environ Sci Pollut Res Int* 2010; 17: 486-96.
- Kümmerer K, Al-Ahmad A. Biodegradability of the Anti-tumour Agents 5-Fluorouracil, Cytarabine, and Gemcitabine: Impact of the Chemical Structure and Synergistic Toxicity with Hospital Effluent. *Acta Hydrochim Hydrobio* 1997; 25: 166-172.
- Kummerer K, Haiss A, Schuster A, Hein A, Ebert I. Antineoplastic compounds in the environment-substances of special concern. *Environ Sci Pollut Res Int* 2014; 23: 14791-14804.
- Kuster A, Adler N. Pharmaceuticals in the environment: scientific evidence of risks and its regulation. *Philos Trans R Soc Lond B Biol Sci* 2014; 369.
- Levitzki A. Tyrosine kinase inhibitors: views of selectivity, sensitivity, and clinical performance. *Annu Rev Pharmacol Toxicol* 2012; 53: 161-85.
- Levitzki A, Mishani E. Tyrophostins and other tyrosine kinase inhibitors. *Annu Rev Biochem* 2006; 75: 93-109.
- Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 2003; 3: 330-8.
- Lutterbeck CA, Kern DI, Machado EL, Kummerer K. Evaluation of the toxic effects of four anti-cancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation. *Chemosphere* 2015; 135: 403-10.
- Mahnik SN, Lenz K, Weissenbacher N, Mader RM, Fuerhacker M. Fate of 5-fluorouracil, doxorubicin, epirubicin, and daunorubicin in hospital wastewater and their elimination by activated sludge and treatment in a membrane-bio-reactor system. *Chemosphere* 2007; 66: 30-7.
- Martin J, Camacho-Munoz D, Santos JL, Aparicio I, Alonso E. Simultaneous determination of a selected group of cytostatic drugs in water using high-performance liquid chromatography-triple-quadrupole mass spectrometry. *J Sep Sci* 2011; 34: 3166-77.
- Martin J, Camacho-Munoz D, Santos JL, Aparicio I, Alonso E. Occurrence and Ecotoxicological Risk Assessment of 14 Cytostatic Drugs in Wastewater. *Water Air and Soil Pollution* 2014; 225: 10.
- Misik M, Pichler C, Rainer B, Filipic M, Nersesyan A, Knasmueller S. Acute toxic and genotoxic activities of widely used cytostatic drugs in higher plants: Possible impact on the environment. *Environ Res* 2014; 135: 196-203.
- Mughal TI, Schrieber A. Principal long-term adverse effects of imatinib in patients with chronic myeloid leukemia in chronic phase. *Biologics* 2010; 4: 315-323.
- Nussbaumer S, Bonnabry P, Veuthey JL, Fleury-Souverain S. Analysis of anticancer drugs: a review. *Talanta* 2011; 85: 2265-89.
- Ortiz de Garcia S, Pinto Pinto G, Garcia Encina P, Irusta Mata R. Consumption and occurrence of pharmaceutical and personal care products in the aquatic environment in Spain. *Sci Total Environ* 2013; 444: 451-65.
- Parrella A, Lavorgna M, Criscuolo E, Russo C, Fiumano V, Isidori M. Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans. *Chemosphere* 2014; 115: 59-66.
- Parrella A, Lavorgna M, Criscuolo E, Russo C, Isidori M. Eco-genotoxicity of six anticancer drugs using comet assay in daphnids. *J Hazard Mater* 2015; 286: 573-80.
- Perez-Herrero E, Fernandez-Medarde A. Advanced targeted therapies in cancer: Drug nanocarriers, the future of chemotherapy. *Eur J Pharm Biopharm* 2015; 93: 52-79.
- Pichler C, Filipic M, Kundi M, Rainer B, Knasmueller S, Misik M. Assessment of genotoxicity and acute toxic effect of the imatinib mesylate in plant bioassays. *Chemosphere* 2014; 115: 54-8.
- Pinkney AE, Harshbarger JC, Karouna-Renier NK, Jenko K, Balk L, Skarpheethinsdottir H, et al. Tumor prevalence and biomarkers of genotoxicity in brown bullhead (*Ameiurus nebulosus*) in Chesapeake Bay tributaries. *Sci Total Environ* 2011; 410-411: 248-57.
- Pomati F, Cotsapas CJ, Castiglioni S, Zuccato E, Calamari D. Gene expression profiles in zebrafish (*Danio rerio*) liver cells exposed to a mixture of pharmaceuticals at environmentally relevant concentrations. *Chemosphere* 2007; 70: 65-73.
- Pommier Y. Topoisomerase I inhibitors: camptothecins and beyond. *Nat Rev Cancer* 2006; 6: 789-802.

- Pommier Y. Drugging Topoisomerases: Lessons and Challenges. *Acs Chemical Biology* 2013; 8: 82-95.
- Rowney NC, Johnson AC, Williams RJ. Cytotoxic drugs in drinking water: a prediction and risk assessment exercise for the thames catchment in the United kingdom. *Environ Toxicol Chem* 2009; 28: 2733-43.
- Scholz S, Fischer S, Gundel U, Kuster E, Luckenbach T, Voelker D. The zebrafish embryo model in environmental risk assessment--applications beyond acute toxicity testing. *Environ Sci Pollut Res Int* 2008; 15: 394-404.
- Seiler JP. Pharmacodynamic activity of drugs and ecotoxicology--can the two be connected? *Toxicol Lett* 2002; 131: 105-15.
- Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 2003; 22: 7265-7279.
- Steger-Hartmann T, Kummerer K, Hartmann A. Biological degradation of cyclophosphamide and its occurrence in sewage water. *Ecotoxicol Environ Saf* 1997; 36: 174-9.
- Toolaram AP, Kummerer K, Schneider M. Environmental risk assessment of anti-cancer drugs and their transformation products: A focus on their genotoxicity characterization-state of knowledge and short comings. *Mutat Res Rev Mutat Res* 2014; 760: 18-35.
- Usawanuwat J, Boontanon N, Boontanon SK. Analysis of Three Anticancer Drugs (5-Fluorouracil, Cyclophosphamide and Hydroxyurea) in Water samples by HPLC-MS/MS. *International Journal of Advances in Agricultural and Environmental Engineering* 2014; 1: 2349-1523.
- Verlicchi P, Al Aukidy M, Galletti A, Petrovic M, Barcelo D. Hospital effluent: Investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. *Science of The Total Environment* 2012; 430: 109-118.
- Walker CH, Hopkin SP, Sibly RH, Peakall DB. *Principle of toxicology*. London: New York: Taylor and Francis 2001.
- Walko CM, Lindley C. Capecitabine: a review. *Clin Ther* 2005; 27: 23-44.
- Webb S, Ternes T, Gibert M, Olejniczak K. Indirect human exposure to pharmaceuticals via drinking water. *Toxicol Lett* 2003; 142: 157-67.
- Xie H. Occurrence, Ecotoxicology, and Treatment of Anticancer Agents as Water Contaminants. *Journal of Environmental & Analytical Toxicology* 2012.
- Yin J, Shao B, Zhang J, Li K. A preliminary study on the occurrence of cytostatic drugs in hospital effluents in Beijing, China. *Bull Environ Contam Toxicol* 2010; 84: 39-45.
- Zegura B, Heath E, Cernosa A, Filipic M. Combination of *in vitro* bioassays for the determination of cytotoxic and genotoxic potential of wastewater, surface water and drinking water samples. *Chemosphere* 2009; 75: 1453-60.
- Zhang J, Chang VW, Giannis A, Wang JY. Removal of cytostatic drugs from aquatic environment: a review. *Sci Total Environ* 2013; 445-446: 281-98.
- Zoukova R, Odraska P, Dolezalova L, Hilscherova K, Marsalek B, Blaha L. Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals. *Environmental Toxicology and Chemistry* 2007; 26: 2208-2214.
- Zuccato E, Calamari D, Natangelo M, Fanelli R. Presence of therapeutic drugs in the environment. *Lancet* 2000; 355: 1789-90.

Bibliography

Publications Related to the Thesis

- Cesen M, Elersek T, Novak M, Zegura B, Kosjek T, Filipic M, Heath E. Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites/trans-formation products and their mixtures. *Environmental Pollution* 2016; 210: 192–201.
- Gajski G, Geric M, Zegura B, Novak M, Nunic J, Bajrektarevic D, Garaj-Vrhovac V, Filipič M. Genotoxic potential of selected cytostatic drugs in human and zebrafish cells. *Environmental Science and Pollution Research* 2016; 23: 14739–14750.
- Isidori M, Lavorgna M, Russo C, Kundi M, Zegura B, Novak M, Filipic M, Misik M, Knasmuller S, Lopez de Alda M, Barcelo D, Zonja B, Česen M, Scančar J, Kosjek T, Heath E. Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain. *Environmental Pollution* 2016; 219:275–287.
- Kovacs R, Csenki Z, Bakos K, Urbanyi B, Horvath A, Garaj-Vrhovac V, Gajski G, Geric M, Negreira N, Lopez de Alda M, Barcelo D, Heath E, Kosjek T, Zegura B, Novak M, Zajc I, Baebler S, Rotter A, Ramsak Z, Filipic M. Assessment of toxicity and genotoxicity of low doses of 5-fluorouracil in zebrafish (*Danio rerio*) two-generation study. *Water Research* 2015; 7: 201-212.
- Novak M, Zegura B, Baebler S, Stern A, Rotter A, Stare K, Filipic M. Influence of selected anti-cancer drugs on the induction of DNA double-strand breaks and changes in gene expression in human hepatoma HepG2 cells. *Environmental Science and Pollution Research* 2016; 23: 14751-14761.
- Novak M, Zegura B, Modic B, Heath E, Filipic M. Cytotoxicity and genotoxicity of anticancer drug residues and their mixtures in experimental model with zebrafish liver cells. *Science of The Total Environment* 2017; 601-602: 293-300.
- Novak M, Zegura B, Nunic J, Gajski G, Geric G, Garaj-Vrhovac V, Filipic M. Assessment of the genotoxicity of the tyrosine kinase inhibitor imatinib mesylate in cultured fish and human cells 2017; 814: 14-21.

Biography

The author of this thesis was born on January 27, 1987 in Murska Sobota, Slovenia. He finished his primary and secondary education in Murska Sobota, Slovenia. In 2005 he started his study at the Biotechnical Faculty, Study Programme in Biology, University of Ljubljana, Slovenia and finished the study in 2011. He performed his graduation thesis under the supervision of Prof. Dr. Kristina Sepčič and co-supervision of Assist. Prof. Bojana Žegura. The laboratory part of the research for his graduation thesis was performed at the National Institute of Biology, department of Genetic Toxicology and Cancer Biology. The title of the graduation thesis was "Protective activity of xanthohumol against genotoxic activity of heterocyclic amine MeIQx".

In 2011 he started to work at the National Institute of Biology, Ljubljana, Slovenia as technician. He was mainly working on the European FP7 Project "Cytothreat" where he studies side effects of cytostatics on zebrafish.

In 2012 he was selected as a young researcher candidate at the Ecological Engineering Institute, Maribor Slovenia, under supervision of Prof. Dr. Metka Filipič, department of Genetic Toxicology and Biology of Cancer. At the same time he started his postgraduate studies in Ecotoxicology at the Jožef Stefan Institutional Postgraduate School, Ljubljana, Slovenia, also under supervision of Prof. Dr. Metka Filipič and Assist. Prof. Bojana Žegura.

His research focuses on the cytotoxic and genotoxic effects of cytostatics on different non-target human and fish cells. He attended several international workshops and congresses on the topic of environmental toxicology and genetic toxicology where he also presented his work.

He is currently involved in the Slovenian Research Agency funded projects, and he is a management committee substitute for Slovenia in the EU-funded COST project CA15132: "The comet assay as a human biomonitoring tool (hCOMET)".