Fate and effects of cytostatic pharmaceuticals in the environment and the identification of biomarkers for and improved risk assessment on environmental exposure

Reporting

Project Information

CYTOTHREAT

Grant agreement ID: 265264

🌐 **Project closed**

Start date
1 January 2011

End date
31 December 2014

Funded under
Specific Programme "Cooperation": Environment (including Climate Change)

Total cost
€ 3 293 999,80

EU contribution
€ 2 582 959,30

Coordinated by
NACIONALNI INSTITUT ZA BIOLOGIJO NATIONAL INSTITUTE OF BIOLOGY
🇸🇮 Slovenia

Final Report Summary - CYTOTHREAT (Fate and effects of cytostatic pharmaceuticals in the environment and the identification of biomarkers for and improved risk assessment on environmental exposure)

Executive Summary:
CytoThreat (www.cytothreat.eu) project addresses the needs of the European society for assessing the

risks associated with the release of pharmaceuticals into environment focusing on cytostatic pharmaceuticals. The mechanisms of action of most of the anticancer drugs are by interference with genetic material and cell signalling, which are very similar in all organisms and theoretically exposure to anticancer drug residues may affect also nontarget organisms. The aims are to provide new analytical methods needed for to determine the actual environmental exposure of these drugs, their metabolites and transformation products detection, to provide missing ecotoxicity data needed for accurate environmental risk assessment and identify biomarkers of delayed effects that may be used for development of early warning systems.

The partners developed a multi-residue methods based on on-line SPE-LC-MS/MS and direct sample injection LC-MS/MS method for analysis of up to 43 compounds and metabolites with detection and quantification limits in the range of nano to pico gram per liter. In degradation/transformation studies they evaluated different methods and conditions, and identified a number of transformation products including 13 not known before. The analysis of real samples confirmed the presence of certain anticancer drugs and metabolites in wastewaters, but except cis-platin, not in recipient waters.

The ecotoxicity studies were performed with four selected drugs (5-fluorouracil, etoposide, cisplatin and imatinib mesilate) with aquatic organisms at three trophic levels (phytoplankton, invertebrate and vertebrate). We specifically focused on genotoxic and reproductive effects of these contaminants in aquatic organisms at concentrations relevant for environmental exposure. The results showed different sensitivity of different aquatic species. The most sensitive were curustacean. Significant reduction of reproduction was observed at concentrations of several hundreds of ng/L (5-fluorouracil and cis-platin) to µg/L concentration range (etoposide and imatinib mesilate), whereas significant increase in DNA damage (comet assay) was already after 24h exposure for all four compounds observed at lower concentrations than inhibition of reproduction. Algae and cyanobacteria were less sensitive; significant growth inhibition was detected at concentration ranges of µg/L to mg/L. Zebrafish were relatively insensitive to the acute exposure as well as sub-chronic exposure to the selected anticancer drugs. The chronic two generation toxicity study with 5-fluorouracil revealed histopathological changes in liver and kidney, induction of micronuclei in blood cells and changes in gene expression in liver in fish exposed to 10 ng 5-fluorouracil/L. This result indicates that the Early life stage toxicity test in fish, that is currently proposed by EMA may not be appropriate for prediction of the effects of chronic exposure to pharmaceuticals with genotoxic potential. In higher plants all four tested cytostatics induced chromosomal damage and pollen abortion however at concentrations several order higher from those in the environment.

In vitro fish and human cell models were applied with the aim of developing predictive assays for chronic effects, and exploring predictive value of results obtained in zebrafish for humans. The studies showed that ZFL cells are sensitive to the cytotoxic and genotoxic effects of cytoststatics and are an in vitro model of choice for studying environmental pollutants, however transcriptional biomarkers for predicting long-term or delayed effects in vitro with ZFL were not identified.

Special attention has been paid to the combined effects of the mixtures of cytostatics using the same experimental models as for single compound studies. It was found in the toxicity and in genotoxicity tests that certain anticancer drugs act synergistically. Nevertheless, the increase of the effects was maximally 2-3 fold higher as those expected on basis of the activities of individual compounds and the overall effect is not likely to lead to significantly different consequences.

The environmental risk assessment based on the data obtained within the Cytothreat project indicate that residues of cytostatics may represent risk to the aquatic organisms when the genotoxicity endpoints (DNA damage in crustacean and zebrafish erythrocytes) are considered as the most sensitive endpoints.

Therefore for the environmental and human health protection it should be recommended to establish a monitoring programme for the most consumed anti-cancer drugs. It should also be recommended that in the ecotoxicity studies of pharmaceuticals genotoxicity endpoints are included.

Project Context and Objectives:

Pharmaceuticals have brought enormous benefits to humanity in terms of healthier and longer lives. Thus it is not surprising that pharmaceutically active substances are emerging as a new group of environmental contaminants the potential adverse environmental and human health effects of which are yet to be fully investigated. The project CytoThreat (www.cytothreat.eu) focused on the evaluation of the environment and human health risks posed by residues of cytostatic pharmaceuticals that due to their genotoxic mechanism of action belong to highly hazardous compounds. The occurrence and distribution of cytostatics in the environment has not been systematically studied as they are, compared to other pharmaceuticals, used in much smaller quantities. Consequently, so far not much research efforts have been directed into development of ultra-sensitive analytical methods to allow their determination in the environment. The side effects of cytostatic drugs in cancer treatment are in humans thoroughly investigated during preclinical and clinical studies required for a marketing authorisation permit, whereas data on their potential effects on non-target organisms and studies that would allow the prediction of their long term and delayed affects due to chronic exposure through environmental contamination is missing. Presence of cytostatic residues in the environment may lead to systemic environmental effects that in the worst case could lead to extinction of susceptible organisms, while in exposed humans increased cancer incidence and reproductive defects may occur. Thus, data is needed to reliably assess the potential risks and introduce appropriate risk management.

This is why the main objectives of CytoThreat are:

To assess the occurrence and fate of cytostatic pharmaceuticals, their metabolites and transformation products in wastewater treatment systems and in the environment.

• To explore potential delayed and irreversible effects of cytostatic pharmaceuticals at environmentally relevant concentrations in aquatic experimental models, and compare the data to those obtained in human experimental models.

• To explore combined effects of mixtures of cytostatic pharmaceuticals, their excreted metabolites and transformation products formed in the environment or in wastewater treatment systems, and real environmental samples.

• To develop, based on the obtained results, guidance on how to improve the environmental and human risk assessment of cytostatics released into the environment.

To fulfil the objectives the EU-funded project CytoThreat has brought together chemists, biologists and toxicologists from research institutes and universities across Europe. In the project we used diverse state of the art chemical and biological techniques and advanced technologies to analyze the presence and distribution of selected cytostatics and their degradation products in the ecosystem and their adverse effects as single compounds and in mixtures to aquatic organisms. Through the aim of developing new chemical analytical methods and studying formation of degradation products with concurrent application of whole range of toxicological tests, including identification of early biomarkers for long term effects based on state of the art toxicogenomic approaches and bioinformatics, the project has produced new information that will contribute to increased understanding of potential adverse effects of the residues of cytostatic drugs to aquatic organisms. In the long term this will enable improvement of the risk assessment for

human health and ecosystems for cytostatic pharmaceuticals by providing accurate data on their environmental concentrations and toxicological data on key parameters (LC50, NO(A)EC) required in risk assessment procedures.

Project Results:
The research is organized into two main segments: chemical analytical and ecotoxicological.

This division is based on different methodological approaches and types of information. While chemical analysis provides occurrence data, the toxicological tests give data on adverse effect to aquatic organisms. Both types of data are complementary and inevitably required for risk assessment.
The chemical part is focused on the development of new sensitive GC/MS and LC/MS based analytical methods that are necessary for the assessment of the occurrence of cytostatic drugs in different aquatic compartments and on cycling of cytostatic compounds and their transformation products (TPs) in wastewater treatment plants (WWTPs) and receiving waters.

Chemical analytical studies:
A fully automated on-line solid-phase extraction-liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS) method has been developed for the determination of 13 cytostatics and 4 metabolites in aqueous matrices, including groundwater, surface water, and raw and treated wastewater. The main advantages of the method are high sensitivity, with limits of determination in groundwater, surface water, and raw and treated wastewater below 5ngL-1 for all compounds except for gemcitabine, temozolomide, imatinib and etoposide, repeatability, with relative standard deviations in most cases below 15%, and selectivity and reliability of results. To the best of our knowledge, this method constitutes the first multiresidue method based on on-line SPE developed for the determination of cytostatics in the aquatic environment. In addition temozolomide, imatinib, erlotinib, capecitabine, hydroxytamoxifen, desmethylhydroxytamoxifen and hydroxypaclitaxel have not been included in previously optimized methods for environmental samples (Negreira et al., 2013a). Moreover, a multianalyte liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) method for determination of 19 cytostatics and 5 metabolites, from 6 different therapeutic families, has been developed, and the structures of the main characteristic fragment ions have been proposed. The method was applied for studying the stability of the compounds in aqueous solutions under different conditions. Five of the analytes investigated have never been searched for in the aquatic environment (imatinib, hydroxypaclitaxel, endoxifen, (Z)4-hydroxytamoxifen, and temozolomide). For of these analytes the stability data and the analytical LC-MS/MS conditions were provided and published for the first time (Negreira et al., 2013b).

In order to evaluate existing methods and as well as the influence of the analytical method and sample matrices on the results an interlaboratory study on the determination of the most common cytostatic compounds in aqueous matrices was performed. Different matrices of spiked samples (with 5-FU, CP, IF, MTX, CDDP, GEM and ET in total 40 samples) were sent to 9 laboratories that responded to the invitation. Only 8 laboratories returned the results and none of them analyzed all the 7 compounds, which prevented reliable statistical evaluation of the results (see D2.2). On the other hand such low response clearly indicates that methods for the determination of cytostatic in environmental samples are still poorly developed.

The developed analytical methods were applied to assessed the occurrence of cytostatics (including transformation products) in waste and surface water samples from Spain and Slovenia, two very dissimilar European countries in terms of water resourses and reuse, and in different periods of time (winter and summer) in order to being able to assess potential geographical and temporal variations.

The concentrations of cytostatics were higher in the Slovenian than in the Spanish wastewaters analysed in both campaigns, and while in Slovenia the total concentration of the compounds appears to decrease in the order hospital wastewater > WWTP influent > WWTP effluent (at least in June), in Spain it was rather similar in all three matrices. This is rather expected since the Slovenian hospital effluent samples were collected from an oncological ward, in contrast to the general hospital sampled in Barcelona.

The relative contribution of the identified cytostatics revealed cyclophosphamide (CP), and especially its human metabolites, as major contributors to the total load of cytostatics in the Slovenian hospital effluent. CAP, MET and OH-MET dominate in the WWTP influent and CP and Pt-based cytostatics in the effluent. In Spanish samples, the most predominant compounds were MET, ERL, TAM, OH-D-TAM and Pt-based cytostatics, without a clear pattern being observed for each kind of matrix.

The above described wastewater samples were characterized also toxicologicaly by in vitro determination of cytotoxicity and genotoxicity with zebrafish liver cells (ZFL), by determination of acute and chronic toxicity in crustacean and by determination of genotoxicity in higher plants. In ZFL cells none of the samples reduced cells viability by more than 30%, indicating low cytotoxic activity. On the other hand all the samples were genotoxic; they induced DNA strand breaks in exposed cells. The highest genotoxic potential had wastewater samples form oncological ward followed by influent and effluent samples. The results reflect dilution of oncological ward wastewater in the wastewater treatment plant influent and reduction of genotoxic activity in the effluent after the wastewater treatment. In crustacean Ceriodaphnia dubia the samples showed relatively low acute toxicity and high chronic toxicity. The WWTP effluents showed a decrease of chronic toxicity when compared to influents, indicating a partial removal of pollutants through the WWTP. In higher plants (Tradescantia and Allium), only wastewater form oncological ward and influent to WWTP were genotoxic.

These studies indicate that residues of cytostatics contribute to the genotoxicity of wastewaters. However, it is possible that also other, chemically not characterised substances such as detergents and disinfectants are responsible for observed positive effects.

Ecotoxicological studies:
This part of the project is focused on thorough ecotoxicological evaluation of cytostatics in aquatic organisms from different trophic levels.

Four cytostatics were selected for the ecotoxicological testing: 5-fluorouracil (5-FU), cisplatin (or cis-diamminedichloroplatinum; CDDP), etoposide (ET) and imatinib mesylate (IM). In the absence of the data on the occurrence of different cytostatics in aquatic environment at the beginning of the project the selection was based on the predicted environmental concentrations (PEC) that were derived from the data of their consumption in Slovenia and literature data. According to their mechanisms of action, the selected compounds represent different classes of anticancer drugs.

5-Fluorouracil is a pyrimidine analog that belongs to the group of antimetabolites. It blocksf DNA synthesis and replication, by inhibition of thymidylate synthase and incorporation of its metabolites into DNA and RNA. Such antimetabolites that are mainly represented by 5-FU and its prodrug capecitabin are among the most consumed anticancer drugs in developed countries. 5-FU is a bacterial mutagen, induces chromosomal abarrations and DNA damage and is according to IARC classified to as carcinogen group 3.

Cisplatin belongs to the group of platinum complexes. It forms DNA and protein adducts and crosslinks that block DNA transcription and replication, which leads to cell death. CDDP is a bacterial mutagen, induces chromosomal aberrations and DNA damage and is according to IARC classified to as carcinogen group 2A.

Etoposide is a topoisomerase inhibitor that inhibits topoisomerase II and causes an increase in DNA and chromosomal breakage and cell death. As a cancer treatment, ET is most often used in combination with other antineoplastic drugs, including 5-FU and CDDP. ET is not a bacterial mutagen, but it induces chromosomal aberrations and DNA damage and is according to IARC classified to as carcinogen group 1.

Imatinib mesylate was the first of the protein kinase inhibitors, which are anti-neoplastic drugs that were developed for targeted chemotherapy. It selectively inhibits the BCR-ABL tyrosine kinase, and has become the therapy of choice for Philadelphia-chromosome-positive leukemia. Imatinib mesylate also inhibits some other tyrosine kinase activities (e.g. c-KIT, the PDGF receptor), which indicates its potential use for treatment of other cancers. The consumption of protein kinase inhibitors, including IM, is increasing very rapidly at present. No data are available for bacterial mutagenicity, limited data on clastogenicity and no data onm carcinogenicity.

Induction of the adverse effects after acute and chronic exposure to the model cytostatic drugs selected has been studied in algae, plants, crustacea (Daphnia magna and Ceriodaphnia dubia), mussel (Unio sp) and zebrafish. Where available the standardized assays were applied. In addition to standard lethal and sublethal toxic effects, the specific endpoints of genomic stability such as DNA integrity, strand breaks and micronuclei, induction were evaluated as indicators of adverse long term effects associated with damage of the genetic material. Toxicogenomic approach was applied to investigate the relationship between transcriptome alterations and observed toxiological effects (i.e. reproductive effects and genotoxicity biomarker responses) with the aim to identify early biomarkers of delayed effects and to determine the so called NOTEL values.

In primary producers alga Pseudokirchneriella subcapitata, and the cyanobacterium Synechococcus leopoliensis the four drugs showed different toxic potential, and the two species examined also showed differences in their susceptibilities towards the tested drugs and their mixtures. With P. subcapitata, the most toxic of these drugs was 5-FU (EC50, 0.13 mg/L), followed by CDDP (EC50, 1.52 mg/L), IM (EC50, 2.29 mg/L), and the least toxic, ET (EC50, 30.43 mg/L). With S. leopoliensis, the most toxic was CDDP (EC50, 0.67 mg/L), followed by 5-FU (EC50, 1.20 mg/L) and IM (EC50, 5.36 mg/L), while ET was not toxic up to 351 mg/L. The toxicities of the binary mixtures (5-FU + CDDP, 5-FU + IM, CDDP + ET) were predicted by the concepts of 'concentration addition' and 'independent action', and were compared to the experimentally determined toxicities. The measured toxicity of 5-FU + CDDP with P. subcapitata and S.

leopoliensis was higher than that predicted, while the measured toxicity of CDDP + ET with both species was lower than that predicted. The measured toxicity of 5-FU + IM with P. subcapitata was higher, and with S.leopoliensis was lower, than that predicted. These data show that these mixtures can have compound-specific and species-specific synergistic or antagonistic effects, and they suggest that single compound toxicity data are not sufficient for the prediction of the aquatic toxicities of such anticancer drug mixtures.

The predicted no effect concentrations (PNEC) obtained in this study are higher than the predicted environmental concentrations of these anti-neoplastic drugs, which suggest that they do not represent a risk for the aquatic phytoplankton. However, the concentrations of 5-FU in wastewaters have been reported to be higher than the predicted no effect concentrations, and the mixtures of 5-FU with CDDP or IM show synergistic effects, which implies that at local sites a potential environmental risk to phytoplankton cannot be ruled out.

Acute and chornic toxicity of the 5-FU, CDDP, ET, IM as well as capecitabine (CAP) and doxorubicine (DOX) has been tested in primary consumers of the aquatic chain Daphnia magna, Ceriodaphnia dubia, Brachionus calyciflorus, and Thamnocephalus platyurus. Acute ecotoxicological effects occurred at concentrations in the range of mg L-1, which are much higher than those predicted in the environment. For chronic toxicity, CDDP and 5-FU showed the highest toxic potential in all test organisms, inducing 50% reproduction inhibition in crustaceans at concentrations on the order of µg L-1. The low effective concentrations in the chronic exposure suggest a potential environmental risk of 5-FU and CDDP (Parrella et al., 2014b).

The genotoxicity of the six anti-neoplastic drugs, was studied applying the in vivo comet assay on cells from whole organisms of Daphnia magna and Ceriodaphnia dubia. For the first time, this test was performed in C. dubia. The comet assay results showed that all drugs induced DNA damage, in both organisms. DOX, 5-FU and CDDP induced DNA damage at concentrations close to those expected in the environment near hospital and WWTP effluents (Parrella et al., 2015).

The results show that crustacean are highly sensitive to the exposure to cytostatic drugs and that in crustacean the induction of DNA damage is an early biomarker of adverse reproductive effects. Thus, this study could be an important starting point for establishing the real environmental impact of these substances.

Mussels are often employed in the ecogenotoxicological studies. They have several characteristics, such as wide distribution, filter feeding, a sessile life form and an ability to accumulate pollutants, which makes them favorable organisms for estimating the environmental pollution level and the bioavailability of various types of pollutants. In freshwater mussels U. pictorum and U. tumidus we determined induction of DNA strand breaks in haemacytes after in vivo exposure (72h) or in vitro exposure of mussel haemocytes (24h) (Gacic et al., 2014). 5-FU, CDDP and ET induced DNA damage after in vivo, but not after in vitro exposure, while IM was ineffective. However, the overall sensitivity of mussel was lower from that of crustacean.

Plants are primary producers that play key role in terrestrial and aquatic eco systems. Therefore acute

toxicity and genotoxicity of 5-FU, ET, CDDP, IM, carboplatin, vincristine and cyclophospahmide micronuclei formation in Tradescantia and Allium cepa and for mincronuceli induction in Tradescantia (Misik et al., 2014; Pichler et al., 2014). The acute toxic properties of the drugs were bonitored as inhibition of cell division (mitotic indices and retardation of root growth) in Allium cepa. All compounds caused in both indicator plants genotoxic effects. The order of genotoxic potencies expressed as NOELs in mM was CDDP (0.1)≥ Et (0.5)>CP (1.0)>CaPt (10)> 5FU (30)> VinS (100) in Tradescantia. A similar order was seen in Allium MN but ET was less active (5.0 mM). Four compounds caused alterations of the mitotic indices under the present conditions namely CDDP (0.5) ET (10.0) 5FU (10.0) and VinS(100). Inhibition of root growth decreased in the order CDDP (0.5)>ET (1.0)≥VinS (1.0)> 5FU (5.0) > CaPt (33.0)> CP (41000).

Comparisons of the NOELs with the predicted environmental concentrations (PEC) show that the latter values are at least 5 orders of magnitude lower and indicate that it is unlikely that their release in the environment may cause adverse effects in higher plants. However, it is notable that the levels of both platinum compounds and of 5FU in hospital effluents may reach levels which may induce damage of the genetic material of plants. We are also the first who described genotoxic and acute toxic properties of IM in higher plants (Pichler et al., 2014) . Although comparisons with the genotoxic potencies of other commonly used cytostatic drugs and with highly active heavy metal compounds show that IM is an extremely potent genotoxin in higher plants, it is evident that the environmental concentrations are > 5 orders of magnitude lower as the levels which are required to cause adverse effects.

In addition we assessed the effect of the exposure to 5-FU, ET and CDDP on the reproductive system of higher plants. Pollen abortion experiments were conducted with species which belong to major plant families, namely with Tradescantia paludosa (Commelinaceae), Arabidopsis thaliana (Brassicaceae), Chelidonium majus (Papaveraceae) and Allisma plantago-aquatica (Alismataceae). All compounds increased the frequencies of abortive grains. The lowest effective doses were in general in a narrow range (i.e. 1 and 10 mg/kg of dry soil). The effects of the individual drugs were similar in T. paludosa, A. plantago-aquatica and Ch. majus, while A. thaliana was consistently less sensitive. The highest abortion rate was obtained in most experiments with CDDP, followed by 5FU and Et. Comparisons of the doses which caused effects in the present experiments in the different species with the predicted environment concentrations and with the levels of the cytostatics which were detected in hospital waste waters show that the realistic environmental concentrations of the drugs are 4 – 6 orders of magnitude lower. Therefore, it is unlikely that these drugs affect the fertility of higher plants in aquatic and terrestrial ecosystems (Misik et al., in press).

In zebrafish (Danio rerio) the acute and embryo toxicty as well as early life stage toxicity of the four cytostatics was low. In the acute toxicity test with adult zebrafish the NOEC for 5-FU and ET was > 100mg/L, whereas thel love values for CDDP and IM were 50 and 10 mg/L, respectively. In the zebrafish embryotest tehe NOEC values for 5-FU, CDDP, ET and IM were 1000 mg/L, 50 mg/L, 200 mg/L and 50 mg/L, respectively. In the fish early life stage toxicity assay 5-FU and IM were tested. Regarding mortalities, the NOEC for 5-FU is 1 mg/L and for IM 10 mg/L. No other adverse effects were observed. However, none of these assays is appropriate to detect long term and delayed effects that may be associated with the genotoxic activity of cytostatic drugs. Thus 5-FU and IM were tested in a two generation study, which has not been performed before with cytostatic compounds. The concentration

range used was 0.01 µg/L, which was selected because according to EMA (2006) for pharmaceuticals with PEC > 0.01 µg/L Phase II environmental effect analysis and fate has to be performed. The higher two concentrations were 1 and 100 µg/L.

In addition to standard toxicological endpoints, such as the 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. Complementary to the comet assay, the micronucleus assay was applied to determine chromosomal damage in erythrocytes. Both of these assays have broad applicability in aquatic animals and they have been applied in numerous laboratory exposure and environmental monitoring studies. Furthermore, 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; they can indicate harmful impacts of chemicals in cases where classical toxicological endpoints show no obvious adverse effects.

The study with 5-FU showed that 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-responsive genes and oncogenes (i.e. jun, myca) (Kovacs et al., in press).

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.

The study with IM showed that multigeneration exposure of zebrafish to this compound reduced their early stage survival at exposure to 1 and 100 µgL-1 IM. Only marginal increase in DNA damage was observed and the induction of micronuclei formation was significant only in fish exposed to 1 µgL-1, Compared to 5-FU, IM exerts lower genotoxic potential which is probably associated with different mode of action. The transcriptomic analyses reviled that IM treatment interfered with endocrine responses. We have observed decreased expression of vitellogenin genes only in males regardless of concentration, while deregulation of sterol (cyp51, hsd) and hormone metabolism (ar, esr2a, greb, adm, hsd, apoeb) as well as genes involved in regulation of circadian rhythm (per1, per 2, cryp1,2,5, arnt, NPAS) were gender and concentration independent. On the contrary to the 5-FU treatment, IM did not affect expression of genes involved in DNA damage response and oncogenesis, confirming different mode of action of IM.

In vitro fish and human cell models were applied with the aim of developing predictive assays for chronic

effects, and exploring predictive value of results obtained in zebrafish for humans. The studies showed that ZFL cells are sensitive to the cytotoxic and genotoxic effects of cytoststatics and are an in vitro model of choice for studying environmental pollutants (Geric et al., in press). To identify biomarker transcripts of long-term effects we compared transcriptional responses in vitro in ZFL cell line to those obtained during the in vivo exposure. The results with 5-FU however showed that ZFL cells do not express any transcriptional biomarker that could be used as an early specific marker of the exposure to this drug.

In the case of IM the comparison of transcriptional responses in vitro in ZFL cell line to those obtained during the in vivo exposure revealed elevated expression of vitellogenin as common biomarker. This was a significant new finding that indicate potential endocrine disrupting activity of IM. High endocrine disrupting activity of IM was then confirmed with E-screen assay that is based on determination of the 17β-Estradiol (E2) dependent proliferation of human breast cancer ER+MCF-7 cells (Parrella et al., 2014c). This finding actually opens the question whether the endocrine disrupting potential is specific for IM or is it a general property of kinase inhibitors.

Environmental risk assessment
The EMA guideline (CMPH, 2006) describes a stepwise tiered procedure for environmental risk assessment of pharmaceuticals for human uses (ERA). The Phase I is a pre-screening assessment aiming to estimate exposure and has an action limit for PEC at 0.01 µgL-1. If the PEC in surface water is below this limit, it is assumed that the compound is unlikely to represent a risk for the environment. If the PEC is equal to or above 0.01 µgL-1, then a Phase II environmental effect analysis and fate has to be performed.

ERA for the four cytostatics was performed based on the toxicological data obtained in this study and the data on the predicted and measured environmental concentrations (PEC and MEC) for 5-FU, ET, CDDP and IM that were obtained from the literature data survey and form the consumption data obtained for Slovenia and the predicted no effect concentrations (PNEC) that were obtained within this project.

For two compounds (5FU and IM) the PEC levels assessed with conservative approach (which does not take in account excretion rates that take into account human metabolism of the drug) were ≥ 10 ng/L. However, when the excretion rates are taken into consideration the levels are bellow this limit.

The results of the chemical analysis of environmental samples including ours showed that 5FU, CDDP and ET were detected in different environmental compartments, while no data are available for IM. The reason is low sensitivity of current analytical methods for IM detection with limit of detection at 22 ngL-1 (Negreira et al., 2013a).

The data on LOEC and NOEC values for the most sensitive organism were collected from the ecotoxicity studies with different organisms and cell lines performed within this project to calculate the predicted no effect concentration (PNEC). Our study showed that regarding the reproductive effect the most sensitive organism were crustacean, thus LOEC values for this organism were used for further risk assessment. The PNEC values are: 5-FU 206 ngL-1, CDDP 100 ngL-1, IM 27 ngL-1 and ET 98 ngL-1. The environmental risk was characterized based on PEC/PNEC ratios where the ratio values above 1 indicate potential risk from the compound towards the environment. The corresponding PEC/PNEC ratios are 0.04 0.005 0.19 and 0.009 for 5-FU, CDDP, IM and ET, respectively. It can be seen that the ratio of PEC/PNEC

for all four drugs is <1 when the refined PEC (which take excretion into account) is divided by the PNEC and thus do not represent environmental risk.

However, the PNEC values above were calculated on the basis of the effect of long term treatment of the different indicator organisms on reproduction. This approach is in agreement with the suggestion of the EMA (2006) to use "the lowest NOEC result from the base set long-term toxicity tests. However, our experiments showed that the DNA damage (COMET assay) in crustacean (which is based on short term treatment for ≤ 24hrs) and micronucleus assay in peripheral blood cells of zebrafish chronically exposed to 5-FU through F0 and F1 generation were more sensitive endpoints that gave lower NOEC results. Therefore, we calculated additionally the PEC/PNEC rates for these endpoints. The PNEC values based on genotoxic effects are: for 5-FU 1 ngL-1 based on micronuclei detection in adult F1 generation zebrafish, for CDDP 0.1 ngL-1 based on comet assay in D. magna, for IM 3 ngL-1 based on comet assay in C. dubia and for ET 10 ngL-1 based on comet assay in ZFL cells. The corresponding PEC/PNEC ratios are for 5-FU, CDDP and IM ≥1 (7.9 5.2 and 1.7 respectively). Only PEC/PNEC for ET is < 1 (0.09). These results indicate that certain cytostatic may be threat to the environment and according to the regulation of EMA further (more detailed) risk assessment has to be conducted when this is the case.

The overall environmental risk assessments are currently based on experiments with individual compounds. However, in the environment pharmaceuticals always occur as multi-component mixtures. Therefore ERA was extended to the effect of combinations of the studied cytostatic drugs. It was found in toxicity experiments and in genotoxicity tests that certain anticancer drugs act synergistically (Brezovšek et al., 2014; Parrella et al., 2014a). Nevertheless, the increase of the effects was maximally 2-3 fold higher as those expected on basis of the activities of individual compounds. Therefore we conclude the overall effect is not likely to lead to major consequences.

On the other hand, the bioindication experiments with untreated hospital wastewaters from two hospitals which contained elevated levels of anticancer drugs and their metabolites, yielded positive results in experiments with Crustacea, cell lines and higher plants. These findings underline the importance of control of the release of the drugs and the need for adequate removal by waste water treatment.

Based on the results of the Cytothreat project we conclude, that certain highly consumed cytostatic do represent risk for the environment. Thus further studies and activities of the academic, industial and regulatory body stakeholders are needed to introduce appropriate protective measures.

Potential Impact:
a) Contribution of project results to improved risk assessment for human health and ecosystems for pharmaceuticals
Distinctiveness of the CytoThreat project is reflected in the emphasis on the evaluation of the risks posed by cytostatic pharmaceuticals that were so far more or less ignored. In the CytoThreat project we used diverse chemical and biological techniques and advanced technologies to analyze the presence and distribution of selected cytostatics and their degradation products in the ecosystem and their adverse effects as single compounds and in mixtures to aquatic organisms in order to evaluate their potential threat to ecosystems and human health when released into the environment. We specifically focused on genotoxic and reproductive effects of these contaminants in aquatic organisms at concentrations relevant

for environmental exposure that may in the long-term lead to the decline of susceptible populations of aquatic organisms, whereas indirect human exposure to these compounds may lead to severe health disorders particularly in more susceptible populations (for instance children and pregnant women). For four cytostatics we provided ecotoxicological parameters (ECx, LO(A)EC, NO(A)EC) for the aquatic organisms at all trophic levels (phytoplankton, crustacean, vertebrate) as well as higher plants that were not available so far. In addition we provided the LOAEC values based on the detection of genotoxic endpoints (DNA damage by Comet assay and choromosomal damage by micronucleus assay) that were also not available so far. These are crucial data for the reliable environmental risk assessment.

In human health risk assessment where the subject of protection is individual the genotoxicity endpoints are an important parameters in the risk assessment. 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. However, in crustacean DNA damage was detected after 24h exposure at lower concentrations than inhibition of reproduction. Thus by our opinion genotoxic endpoint should not be neglected in ERA, however further studies are needed to clarify the significance.

The developed sensitive analytical methods for detection of numerous cytostatics and transformation products in the environmental matrices that were not available so far are important contribution to the future environmental monitoring and research.

B) Contribution of project results to the relevant EU policies/strategies

Results of the CytoThreat project contributed to the implementation of actions within the 2nd main theme of the Environment and Health Action Plan 2004-2010: Filling the knowledge gaps by strengthening research on environment and health and identifying emerging issues. The project results provided scientifically grounded information that is needed to help all 25 EU member States to recognize and reduce the potential adverse health impact of cytostatic pharmaceuticals. In particular the results of the studies of the adverse effects of mixtures of cytostatics, combined exposures and cumulative effects rendered the overall assessment of environmental impact on human health more efficient, and contributed to the goal to develop an environment and health "cause-effect framework".

The results of the studies of the effects of cytostatics on gene transcription and identification of transcriptional markers predictive for long term effects in zebrafish experimental models can be applied for prediction of long term effects also in humans. The up-regulation of several onco-genes after chronic exposure of zebrafish to 5-FU indicate oncogenic potential of this compound that can be expected also in humans after chronic exposure (i.e. occupational exposure).

In the perspective the results of Cytothreat project are likely to lead to a considerable improvement of the quality of environment and human health. The associations between particular cytostatic and adverse environmental effects give scientific grounds for introduction of surveillance and responses to their threats.

The substantive activities in this project are of direct relevance to the scientific priorities of each partner, each of which has a key national role in their respective country. In most of the countries the work complemented the ongoing research in the area of environmental contaminants and adverse environmental and health outcomes and strengthens the evidence base by pooling resources and results. Thus the results of the project will be in the future exploited directly by the partner countries, and could be

integrated in their respective national governmental policies. The results will also be directly usable for the EC for ongoing risk assessment of the impact of pharmaceuticals released into environment, and will contribute to policymaking including EMA guidelines on environmental risk assessment of medicinal products for human use.

Cytothreat also contributed to the development of skills in the area of assessment of the occurrence and effects cytostatic pharmaceuticals in the environment that is generally present in relatively small areas of research interest and expertise. The collaboration of researches with expertises and available infrastructure and equipment in the fields of analytical chemistry, toxicology, ecotoxicology, 'omic' technologies and bioinformatics form different parts of Europe enabled exchange of experience knowledge and specific technologies between the partners. This can be clearly seen from the scientific publications produced by partners.

To disseminate the achieved results as well as experience and skills to wider public three workshops were organized during the project. The Workshop on the effects of residues of cytostatics and other pharmaceuticals on non-target organisms was organized in Naples in October 2012. Number of distinguished invited speakers responded to the invitation and more than 60 participants attended the workshop. The Workshop: "Pharmaceutical residues: parent compounds, metabolites and transformation products as environmental contaminants" was organized in Nimes in June 2013. in December 2014 final workshop with the aim to present the compilation of the achieved results to wider public was organized. To attract wider interested stakeholders three EU projects (Globaqua, Cytothreat, Endetech) funded under the European Union's Seventh Framework Programme together with SCARCE from the Consolider Programme (Spanish Ministry of Economy and Competitiveness), have joined the efforts to prepare a unique Workshop in which pharmaceuticals were considered from a multidisciplinary perspective. The conference was attended by 50 attendees predominantly from research and environmental management sector.

List of Websites:
www.cytothreat.eu

Contact
Prof. Metka Filipic, PhD
Head Department for Genetic Toxicology and Cancer Biology
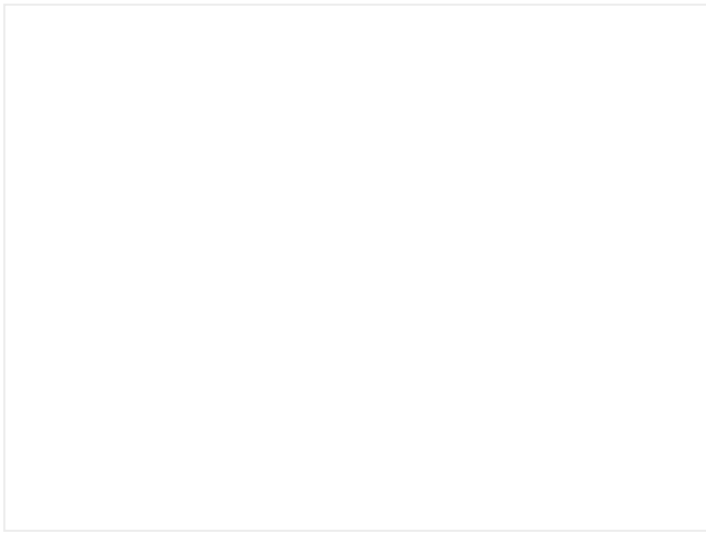National Institute of Biology
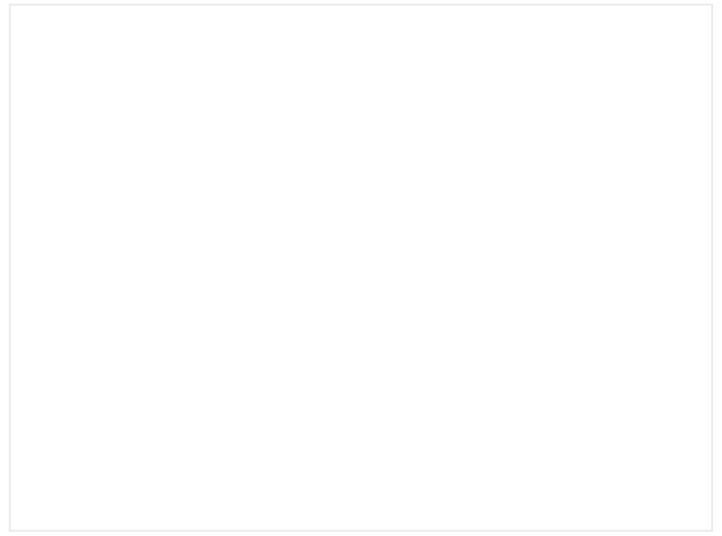Vecna pot 111, Ljubljana
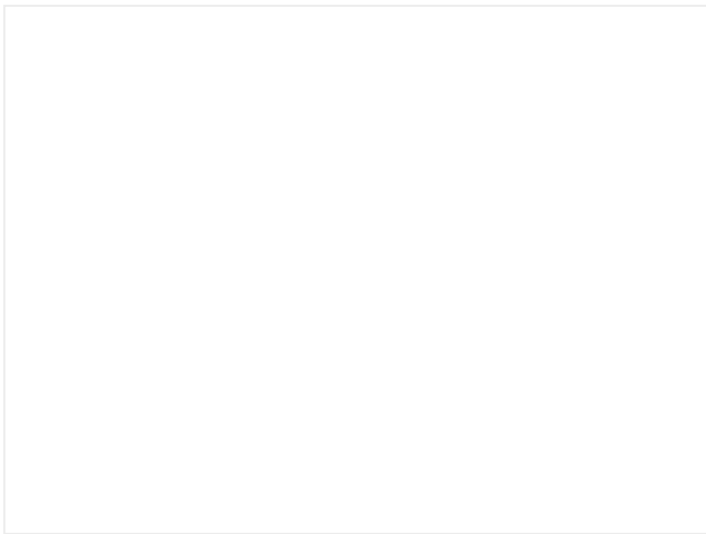SLOVENIA
Tel: +386 5 9232 861
e-mail: metka.filipic@nib.si
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Last update: 27 July 2015

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European Union, 2024