Environmental Pollution 219 (2016) 275–287

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

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

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ARTICLE INFO

Article history:
Received 15 July 2016
Received in revised form
11 October 2016
Accepted 12 October 2016

Keywords:
Anticancer drugs
Wastewater influents and effluents
Hospital effluents
Toxicity
Genotoxicity
Cytotoxicity

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.

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

☆ This paper has been recommended for acceptance by Charles Wong.

☆☆ Electronic Supplementary information (ESI) available.

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http://dx.doi.org/10.1016/j.envpol.2016.10.039
0269-7491/

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 (Kovacs 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 (Toolaram 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) (Ferk 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-Climent 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-Climent 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 (*cis*-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-d_6,; 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-d_3, 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-d_6 (CAS 90344-84-6; 98%) was purchased from LGC Standards GmbH (Wesel, Germany) while 5-Chlorouracil (5-CU, 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