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Estrogenic activity and cytotoxicity of six anticancer drugs detected in water systems

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HIGHLIGHTS

- Estrogenic activity and cytotoxicity of six cytostatics were assessed.
- Imatinib, cisplatin and 5-fluorouracil had the highest estrogenic effect.
- This study contributes to cytostatic environmental risk evaluation.

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ABSTRACT

The aim of the present study was to investigate the *in vitro* estrogenic and the cytotoxic activity of six cytostatics (5-fluorouracil, capecitabine, cisplatin, doxorubicin, etoposide, and imatinib) belonging to the five classes of Anatomical Therapeutic Classification (ATC) detected in wastewater systems. The estrogenic activity was assessed by YES-assay on *Saccharomyces cerevisiae*-RMY326 and E-screen on MCF-7 cells. The cytotoxic activity was assessed by MTT Cell Proliferation Assay on the MCF-7 and the MDA-MB-231 cells.
The results of estrogenic activity, detected by E-screen and expressed as EC_{50}, showed a high potential of imatinib (10^{-7} μM) followed by cisplatin and 5-fluorouracil. Capecitabine was poorly estrogenic while etoposide and doxorubicin EC_{50} values were not possible to determine. Cytotoxicity was found at concentrations far from those detected in effluents. The potential endocrine activity of the most active drugs could be associated with human and wildlife risk when considering their occurrence in the environment.

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1. Introduction

Cancer incidence is increasing in developed and, particularly, in developing countries not only because of the progressive increase of population aging but also because of risk factors such as tobacco and alcohol consumption, nutritional habits and environmental pollution.

Due to the worrying increase of cancer rates, the use of chemotherapy treatments is also rising with a related growing concern over the presence of cytostatics in water systems putting humans and aquatic organisms at risk (Johnson et al., 2008; Rowney et al., 2009). Although most anticancer drugs are administered in clinics or in hospitals with also healthcare worker occupational exposure concern (Castiglia et al., 2008; Pieri et al., 2010), from few years, home and day-hospital therapies are increasing, causing a continuous release of these chemicals directly into the municipal wastewater-treatment plants, usually not designed to treat such pollutants (Kosjek and Heath, 2011).

In light of the above-mentioned, the detection of cytostatics in wastewaters is rapidly growing and the concentrations found are worldwide from sub-ng to μg/L as reported in Table 1. Although these drugs are generally present in the environment at concentrations lower than those of other pharmaceutical classes (Kosjek and Heath, 2011), each living organism, humans included, may potentially be affected by their peculiar molecular mode of action. Recent studies showed sub-lethal and sub-organismal level effects of cytostatics on non-target organisms because of their mutagenic, genotoxic and teratogenic properties (Zounková et al., 2007, 2010) and, as all drugs in the environment, these chemicals might have chronic toxic effects on whole aquatic organisms acting as pseudo-persistent pollutants due to their continuous introduction into the environment (Fent et al., 2006a; Constantine and Huggett, 2010). Another effect of drugs, in any case detectable at very low concentrations, is the endocrine disruptor activity that has been drawing the attention of researchers in the last years. In fact, some xenoestrogens have the capability to mimic the female steroid hormone, 17β-Estradiol (E_2). Different compounds act as Endocrine Disrupting Chemicals (EDCs) and their effects on the aquatic environment are known (Sumpter, 2005). In

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Table 1
Occurrence of cytostatic pharmaceuticals in wastewater systems detected in different countries. 5-Fluorouracil (5-FU), capecitabine (CAP), cisplatin (CisPt), doxorubicin (DOX), etoposide (ET) and imatinib (IM).

Cytostatic	Matrix	Concentration detected	Countries	Ref.
5-FU	Hospital effluent	<5.0–27 ng/L	Switzerland	Kovalova et al. (2009)
	Hospital effluent	20–122 μg/L	Austria	Mahnik et al. (2004)
	Hospital effluent	<8.6–124 μg/L	Austria	Mahnik et al. (2007)
	Hospital wastewater	35–92 ng/L	Slovenia	Kosjek et al. (2013)
	Municipal wastewater	4.7–14 ng/L	Slovenia	Kosjek et al. (2013)
CAP	Wastewater effluent	8.2–27.0 ng/L	Spain	Negreira et al. (2013)
CisPt as Pt compound	Hospital influent	3–250 μg/L	Austria	Lenz et al. (2007)
	Hospital effluent	2–150 μg/L	Austria	Lenz et al. (2007)
DOX	Hospital effluent	0.1–0.5 μg/L	Austria	Mahnik et al. (2006)
	Hospital effluent	<10 ng/L	China	Yin et al. (2010)
	Hospital effluent	<0.26–1.35 μg/L	Austria	Mahnik et al. (2007)
	Wastewater influent	4.5 ng/L	Spain	Martin et al. (2011)
ET	Hospital effluent	6–380 ng/L	China	Yin et al., 2010
	Hospital effluent	110–600 ng/L	France	Catastini et al. (2008)
	Wastewater effluent	3.4 ng/L	Spain	Martin et al. (2011)
	Wastewater influent	15 ng/L	Spain	Martin et al. (2011)
IM	–	–	–	–

different studies (Fent et al., 2006b; Isidori et al., 2009), some anticancer drugs such as tamoxifen, a selective estrogen receptor modulator (SERM) used in many estrogen-dependent cancers with a high estrogenic activity, were tested. Although hormone therapies with SERMs or selective estrogen receptor down-regulators (SERDs) are used for breast cancer treatment, drugs such as anthracyclines, taxanes and antimetabolites are also often used for breast chemotherapy and dispensed in combination regimens to increase the single drug efficacy (De Angelis et al., 2013; Lukyanova et al., 2009). But, do these agents have any estrogenic activity? Nowadays very little information is available about the endocrine disruption activity of anticancer drugs and their potential consequences for wildlife and humans when these compounds enter the aquatic sewer network.

In light of the increasing environmental concentrations of cytostatics and in view of the possible exposure to aquatic organisms and humans, the aims of the present study were to assess the *in vitro* estrogenic activity and the cytotoxic activity of six cytostatics belonging to the five classes of the World Health Organization (WHO) Anatomical Therapeutic Classification (ATC) scheme. The anticancers studied were: the antimetabolites 5-fluorouracil (5-FU) and its pro-drug orally administered capecitabine (CAP), the anthracycline doxorubicin (DOX), etoposide (ET), a topoisomerase II inhibitor belonging to the class of mitotic inhibitors, cisplatin (CisPt), a platinum derivative DNA cross-link agent and imatinib mesylate (IM), a potent and selective tyrosine kinase inhibitor. The estrogenic activity was investigated by two *in vitro* assays: a recombinant yeast system (YES test) expressing the human estrogen receptor α and the E-screen which measures estrogen-dependent growth stimulation in the human breast cancer cell line, MCF-7, ERα and ERβ positive. The estrogen receptor antagonist ICI 182,780, also known as Fulvestrant, was used to confirm the ER-related activity. Although *in vitro* estrogenic tests cannot fully predict a hazard to humans and particularly to wildlife, they are able to give an overall view concerning the estrogen mimetic potential of test compounds (Vanparys et al., 2010). The cytotoxic activity was assessed by the MTT Cell Proliferation Assay on two human breast cancer cell lines: the estrogen-dependent MCF-7 and the estrogen-independent MDA-MB-231 (ER^-) cells to measure the cell viability.

2. Materials and methods

2.1. Chemicals

5-FU (CAS: 51-21-8), CisPt (CAS: 15663-27-1), DOX (CAS: 25316-40-9), 17β-Estradiol (CAS: 50-28-2), ET (CAS: 33419-42-0), 7α,17β-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780, CAS: 129453-61-8), 2-nitrophenyl-β-D-galactopyranoside (ONPG, CAS: 369-07-3) and 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide (MTT, CAS: 298-93-1) were purchased from Sigma-Aldrich (Milano, Italy). CAP (CAS: 154361-50-9) and IM (CAS: 220127-57-1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Yeast Nitrogen Base was purchased by BD Difco™ (Milan, Italy). Dulbecco's modified Eagle's medium phenol red-free (DMEM), HEPES and Roswell Park Memorial Institute medium (RPMI 1640) were supplied by Lonza BioWhittaker (Verviers, Belgium).

2.2. Yeast estrogen screen (YES)

The YES-assay was carried out on *Saccharomyces cerevisiae*-RMY326 which was kindly supplied by Prof. Picard, Geneva University, Switzerland. This strain expresses a human estrogen receptor (hERα) and includes expression plasmids carrying the reporter gene *lac-Z*, encoding the β-galactosidase, used to measure the receptor activity (Routledge and Sumpter, 1996). The yeast cells were grown for 24 h at 26 °C with shaking in the Yeast Nitrogen Base minimal medium enriched with amino acids and glucose. An aliquot of the culture was diluted in the fresh minimal medium and grown in the presence of five serial dilutions of pharmaceuticals for 16–18 h until growth reached the exponential phase (2×10^7 cells/mL). E_2 was assayed as the positive control from 10^{-5} to 10^{-1} μM. Then, yeast cells were harvested by centrifugation at 4000 rpm for 5 min and the pellet re-suspended in 1 mL of Z-buffer (30 mM Na_2HPO_4, 20 mM NaH_2PO_4, 5 mM KCl, 0.5 mM $MgSO_4$) plus a 0.025% β-mercaptoethanol and centrifuged again. The pellet was re-suspended in 150 μL Z-buffer. CH_2Cl_2 (50 μL), 0.1% sodium dodecyl sulfate (20 μL) and Z-buffer (30 μL) were added to the cells; the mixture was incubated for 5 min at 26 °C. The β-galactosidase reaction was started by adding 700 μL of ONPG (4 mg/mL in Z-buffer) and stopped by adding 500 μL of Na_2CO_3 1 M. The β-galactosidase activity was determined by adding the colorimetric substrate, 2-nitrophenyl-β-galactoside. The absorbance of the sample was measured at 420 nm (Garabedian et al., 1999). The β-gal units (Miller units) were determined using the following formula: $OD420 \times 1000 / t \times V \times OD600$; where t = elapsed incubation time (min); V = culture volume (mL); and OD600 = absorbance of culture at 600 nm. All experiments, in two replicates, were repeated three times and the median effective concentration (EC_{50}) was calculated by a non-linear regression (curve fit) model by GraphPad Prism 5 analysis. The Relative Inductive Efficiency (RIE) was determined by dividing the maximal β-galactosidase activity induced by the sample and the maximal activity induced by E_2 and expressing this ratio in a percentage.

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