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Acute toxic and genotoxic activities of widely used cytostatic drugs in higher plants: Possible impact on the environment

Miroslav Mišík [a], Clemens Pichler [a], Bernhard Rainer [a], Metka Filipic [b], Armen Nersesyan [a], Siegfried Knasmueller [a,*]

[a] Institute for Cancer Research, Department of Internal Medicine I, Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria
[b] National Institute of Biology, Ljubljana, Slovenia

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ABSTRACT

Cytostatic drugs are highly toxic pharmaceuticals and it was repeatedly postulated that they may cause adverse effects in ecosystems. The acute toxic and genotoxic properties of these drugs have not been adequately investigated in higher plants so far; therefore, we studied the most widely used drugs (5-flurouracil, 5FU; etoposide, Et; cisplatin, CisPt; carboplatin, CaPt; vincristine sulfate, VinS and cyclophosphamide monohydrate, CP) in micronucleus (MN) assays with meiotic pollen tetrad cells of Tradescantia and with root cells from *Allium cepa*. MNi are formed as a consequence of chromosome breaks and aneuploidy. We monitored also the acute toxic properties of the drugs, i.e. inhibition of cell division (mitotic indices and retardation of root growth) in the latter species. All compounds caused in both indicator plants genotoxic effects. The order of genotoxic potencies expressed as NOELs in μM was CisPt (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 μM). Four compounds caused alterations of the mitotic indices under the present conditions namely CisPt (0.5), Et (10.0), 5FU (10.0) and VinS (100). Inhibition of root growth decreased in the order CisPt (0.5) > Et (1.0) ≥ VinS (1.0) > 5FU (5.0) > CaPt (33.0) > CP (> 1000). 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.

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1. Introduction

Cytostatic drugs have been developed to kill cancer cells. They are amongst the most toxic chemicals which are produced and used worldwide. The basis of the therapeutic efficiency of most of these drugs is the (direct or indirect) interaction with the genetic material (McKnight, 2003). It was postulated, that the release of genotoxic compounds in the environment may reduce the viability of species and their offspring and affect the stability of ecosystems (Hamlin and Guillette, 2010; Hebert and Luiker, 1996; Medina et al., 2007). Several recent papers concerning the contamination of surface waters with cytostatics postulated that waste waters from hospitals and households may cause adverse effects (see for example Besse et al. (2012); Booker et al. (2014)).

In order to assess the magnitude of this problem, information concerning the cytotoxic and genotoxic properties of these chemicals is required. Most compounds have been tested intensively in bacterial indicator cells, in mammalian and human cell lines and also in laboratory rodents (IARC, 1987, 2000; Ishidate et al., 1988; Mavournin et al., 1990). However, information on their effects in plants, which play a key role in terrestric and aquatic ecosystems is scarce and confined to a few studies which were conducted with outdated and non-standardized experimental systems three to four decades ago (for details see discussion section).

Aim of the present study was the investigation of the cytotoxic and genotoxic properties of six cytostatics with two widely used plant bioassays, namely in micronucleus (MN) assays with meiotic tetrads of Tradescantia and with mitotic root tip cells of *Allium cepa*. These tests are the most frequently used plant based assays for the detection of environmental genotoxins (Leme and Marin-Morales, 2009; Ma et al., 2005; Misik et al., 2011). MN are formed as a consequence of chromosome breakage (clastogenicity) and/or aneuploidy (Fenech et al., 2011; Schmid, 1975) and are one of the

* Corresponding author. Fax: +43 1 40160 957500.
E-mail address: siegfried.knasmueller@meduniwien.ac.at (S. Knasmueller).

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most commonly used endpoints in genetic toxicology (Heddle et al., 2011). Experiments with tetrads reflect DNA damage in meiotic cells which may have an impact on fertility while experiments with root tip meristems reflect damage in mitotic cells. We included in the present study both types of cells, since it has been postulated that meiotic and mitotic cells differ in regard to their sensitivity towards chemically induced DNA damage (Majer et al., 2005; Rodrigues et al., 1997).

The tested compounds are among the most widely used cytostatics in Europe (Besse et al., 2012; Booker et al., 2014; Johnson et al., 2008; Kümmer et al., 2009). All of them cause damage of the genetic material but the molecular mechanisms differ substantially: 5-Fluorouracil (5-FU) is a base analog which is misincorporated into DNA (Straub, 2010), etoposide (Et) interferes with topoisomerase II activity (Jackson et al., 1996), vincristine sulfate (VinS) bind to tubulin dimers causing dissolution or disassembly of the microtubules which leads to destruction of the mitotic spindle (Jackson et al., 1996) while platinum compounds (cis-platinum CisPt and carbo-platinum CaPt) cause DNA intrastrand cross-links (Jackson et al., 1996). Cyclophosphamide (CP) requires metabolic activation by cytochrome P450 and causes alkylation of DNA bases (Jackson et al., 1996).

In order to compare the cytotoxic properties (inhibition of cell division and root growth) and the genotoxic activities of the different drugs, all of them were tested under identical conditions with standardized protocols (Ma et al., 1994, 1995).

To assess their potential adverse effects at the environmental level, the NOEL (no-observed-effect level) and LOEL (lowest-observed-effect level) values which were determined in the present experiments were compared with the predicted environmental concentrations (PEC) and rPEC (refined PEC value where also excretion rate of drug was considered).

2. Materials and methods

2.1. Chemicals

5-Fluorouracil (5FU, CAS 51-21-8), cyclophosphamide monohydrate (CP, CAS 6055-19-2), maleic hydrazide (MH, CAS 123-33-1), hydrochloric acid (HCl, CAS 7647-01-0), DMSO, ethanol (99%), acetic acid and carmine were purchased from Sigma-Aldrich (Munich, Germany). Etoposide (Et, CAS 33419-42-0) came from Santa Cruz Biotechnology (Heidelberg, Germany), cis-diammine-platinum(II) dichloride (CisPt, CAS 15663-27-1) and carboplatin (CaPt, CAS 41575-94-4) were obtained from Sandoz Co. EBEWE Pharma (Unterach, Austria). Vincristine sulfate (VinS, CAS 2068-78-2) was purchased from Tecoland Corporation (Irvine, CA).

2.2. Tradescantia micronucleus (Trad MN) assays

Tradescantia MN assays were performed according to the protocol of Ma et al. (1994). Clone #4430 was exclusively used in this study. Only young inflorescences with at least nine individual flower buds were exposed in aqueous solutions of the drugs. Per dose, 15 cuttings were treated for 24 h followed by a 24 h recovery period. The cuttings were placed into glass beakers with 100 ml tap water or in aqueous solutions of the test substance. The beakers were covered with perforated aluminum foils to separate the stems. After exposure, the inflorescences were fixed for 24 h in a mix of ethanol/acetic acid (3:1) and stored in 70% ethanol. Per experimental point, tetrad preparations of at least five buds were made and stained with 2% acetocarmine. 1500 early phase tetrads (i.e. five inflorescences from individual plants and 300 tetrads per bud) were scored in each experimental group. All experiments were performed at least twice. Tap water was used in all experiments as a control with all drugs except for Et. The final DMSO concentration in the test solutions of this drug and in the negative controls was 0.5%. Maleic hydrazide (MH, 20 mg/L) was used in all experimental series as a positive control.

The results were analyzed with one-way ANOVA followed by Dunnett's multiple comparison test. P-values ≤ 0.05 were considered as significant.

2.3. Allium micronucleus assay

The experiments were carried out according to the standard protocol published by Ma et al. (1995). Briefly, young onion bulbs (diameter 12–21 mm, Schneeball Weiss, Austrosaat, Vienna, Austria) were placed in 13 ml glass tubes filled with tap water for 24 h in the dark. Subsequently, the roots (length ca. 1 cm) were exposed to different doses of the cytostatics in dark for 24 h and then transferred to fresh tap water for further 24 h. At the end of the recovery period, the roots were fixed in a mix of ethanol and glacial acetic acid (3:1) for 24 h and stored in 70% ethanol. All experiments were performed at least twice. Tap water was used as a control for experiments with all drugs except in the experiments with Et. For this drug, DMSO was used as solvent (0.5% in each experiment and in the control group as well). Maleic hydrazide (MH) 10 mg/L was used in all experimental series as a positive control.

The root tips were hydrolyzed in a mix (1:1) of HCl (5.0 N) and ethanol (99%) for 3 min and washed in tap water before staining with 2% acetocarmine. MNi were scored according to the criteria described by Ma et al. (1995). 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). Furthermore, also the mitotic indices (MIs) were determined in 1000 cells (100 cells/root) per experimental point. The microscopic evaluation was carried out under a light microscope (Nikon YS200, Japan) with 400-fold magnification.

The results were analyzed with one-way ANOVA followed by Dunnett's multiple comparison test. The results of experiments concerning the mitotic indices (MI) were analyzed with Kruska–Wallis followed by Dunn's comparison test. P-values ≤ 0.05 were considered as significant.

2.4. Cytotoxicity assay with A. cepa

Experiments regarding the acute toxicity of the drugs in *A. cepa* were conducted according to the protocol of Fiskesjo (1995) with small modifications. Young onion bulbs (diameter 12–21 mm, Austrosaat, Vienna, Austria) were placed in 13 ml glass tubes (VWR International, Wien, Austria) which were filled with tap water in the dark for 24 h. The roots (length ca 1.0 cm) of 10 onions were exposed to at least five doses of the different cytostatics in the dark (26 °C) for 72 h. Solutions were changed daily. The root lengths were measured after the exposure. All experiments were performed at least twice. Tap water was used as a control in experiments with all drugs except in experiments with Et. For this drug, DMSO was used as a solvent (0.5% in each experimental series and in the control). Maleic hydrazide (100 mg/L) was used of in all experimental series as a positive control.

The results were analyzed statistically with Prism 5 (GraphPad Inc., CA, USA). EC_{50} values (concentrations which cause 50% inhibition of root growth) were calculated by non-linear regression (log agonist vs. normalized response-variable slope). The No Observed Effect Concentrations (NOECs) were estimated by ANOVA and followed by Dunnett's multiple comparisons test. P-values ≤ 0.05 were considered as significant.

3. Results

The results which were obtained with meiotic tetrad cells of Tradescantia are summarized in Fig. 1A–F. All compounds caused significant effects but the activity of the drugs varied over a broad range. The most pronounced genotoxic activities were detected with CisPT and Et which induced MN formation at concentrations ≥ 1.0 μM and with CP (≥ 5.0 μM). With 5FU and CaPt, clear induction of MN was seen at 100-fold higher doses. VinS caused only at the highest dose (500 μM) a moderate effect.

The findings which were obtained in Allium MN assays are in general similar to those obtained in Trad MN tests (see Figs. 2A–H and 3A–D). A notable exception is that the root cells were distinctively less sensitive towards Et.

We determined in the Allium experiments also the impact of the drugs on the division rates of the primary roots. In contrast to results which were found in the genotoxicity experiments, acute toxic effects were detected under identical experimental conditions only with four drugs, namely with CisPt (1.0), Et (10.0), 5FU (10.0) and VinS (100). Numbers in parenthesis indicate the NOELs in μM.

The impact of the cytostatics on the growth of the roots was tested over a broad dose range and higher concentrations were included as in the genotoxicity tests. The findings are summarized in Fig. 4A–F. It can be seen that significant effects were obtained with five compounds; only CP was devoid of activity under all experimental conditions.

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