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Patulin: Mechanism of genotoxicity

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ABSTRACT

Patulin is a frequently found food contaminant mainly produced by the fungi *Aspergillus* and *Penicillium*. Patulin is suspected to be clastogenic, mutagenic, teratogenic and in higher concentrations cytotoxic. Here, we investigate the mechanism of the patulin-induced genotoxicity. Chromosomal damage was detected as micronucleus and nucleoplasmic bridge formation. Due to the activity of patulin on SH-groups, glutathione is a major compound in the cellular defense against patulin and the depletion of glutathione level with buthionine sulfoximine led to a strong increase in the genoxicity of patulin. A modified version of the alkaline comet assay was carried out to show the cross-linking properties of patulin. As a mechanistic hypothesis, we suspect patulin to cause structural DNA damage by cross-linking, yielding nucleoplasmic bridges and as a later consequence, micronucleus formation. The structural DNA damage may also lead to cell cycle delays, the consequence of which may be the observed centrosome amplification and formation of multipolar mitotic spindles.

1. Introduction

Patulin is a mycotoxin mainly produced by *Aspergillus* and *Penicillium*. It is a frequently found contaminant in spoiled fruits, especially apples and related products. The World Health Organisation established a safety level of 50 μg/L (0.32 μM) for apple juice (World Health Organization, 1999) which was taken over by the European Union and many other countries (Commission of the European Communities, 2006). However, several publications documented the exceedance of this safety level (Chao-Ling Lai, 2000; SCOOP, 2002; Yurdun et al., 2001).

In vivo patulin caused severe damage in several organ systems like kidney, intestinal tissue (McKinley et al., 1982; Speijers et al., 1988) and immune system (Escoula et al., 1988), if applied at a range between 2.5 and 41 mg/kg bw. Regarding carcinogenicity, the International Agency for Research on Cancer classified patulin in group C, since the evidence of carcinogenicity was considered limited in experimental animal (International Agency for Research on Cancer, 1998). However, *in vitro* mutagenicity was shown in different mammalian cell types like Chinese hamster lung fibroblast V79 cells or mouse lymphoma L5178Y cells (Schumacher et al., 2005). The frequency of chromosomal aberrations in HepG2 (Ayed-Boussema et al., 2011) and V79 cells (Alves et al., 2000) was increased after patulin treatment, whereas no elevated level of sister-chromatid exchanges was detected in V79-E cells (Thust et al., 1982). Patulin induced both kinetochore-positive and -negative micronuclei in V79 cells (Pfeiffer et al., 1998).

The genotoxic (Alves et al., 2000; de Melo et al., 2011; Liu et al., 2003; Pfeiffer et al., 1998) and cytotoxic (Schumacher et al., 2005) properties are believed to be due to the high reactivity of patulin to cellular nucleophiles. It reacts fast with sulfhydryl groups and more slowly with amino functions (Lee and Roschenthaler, 1986) of proteins and glutathione (Pfeiffer et al., 1998; Schumacher et al., 2006). Up to three molecules of glutathione can bind to one molecule patulin and the structures of the main adducts were reported in the literature (Fliege and Metzler, 2000; Schebb et al., 2009). A reduction of the cellular glutathione content by the glutathione synthesis inhibitor buthionine sulfoximine (BSO) is known to increase the cytotoxicity and genotoxicity of patulin (Schumacher et al., 2005; Zhou et al., 2009). Here, we identify some additional steps of the mechanistic pathway of patulin-induced genotoxicity.

2. Materials and methods

2.1. Materials

If not otherwise mentioned, chemicals were purchased from Sigma–Aldrich, Taufkirchen, Germany. Cell culture medium and supplements were purchased from PAA.

Patulin, cytochalasin B and cisplatin (cispt) were dissolved in dimethyl sulfoxide (DMSO), whereas H_2O_2 and BSO were dissolved in phosphate buffered saline (PBS). Compounds were added to the medium to a final DMSO concentration of ⩽1%. Control experiments were carried out with medium containing an equal amount of solvent without test compound.

Abbreviations: BSO, buthionine sulfoximine; CBPI, cytokinesis block proliferation index; Cispt, cis-platin; Co, control; DMSO, dimethyl sulfoxide; FBS, fetal bovin serum; HUMN, human micronucleus project; MN, micronuclei; MEM, minimum essential medium; NPB, nucleoplasmic bridge, Pat, patulin; PBS, phosphate buffered saline.

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2.2. Cell culture

V79 cells (Chinese hamster fibroblasts) were routinely grown in MEM (Minimum Essential Medium) with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotics (penicillin, streptomycin) at 37 °C in a water-saturated atmosphere containing 5% CO_2.

2.3. Micronucleus and nucleoplasmic bridge analysis

2×10^5 cells, seeded the day before in 3 mL well plates, were incubated for 20 h with 20 μM BSO. Subsequently, cells were washed and treated with 0.5 μM patulin. After 4 h patulin was removed and the cytokinesis inhibitor cytochalasin B (5 μg/mL) was added, yielding a high number of binucleated cells after a 20 h post-incubation time. By limiting the analysis to such binucleated cells, it can be ensured that these cells have actively divided since the treatment. For the time course of micronucleus and nucleoplasmic bridge formation, cells seeded the day before, were incubated for the indicated time with 0.5 μM patulin and 5 μg/mL cytochalasin B simultaneously. Cells were brought onto glass slides by cytospin centrifugation and fixed in methanol (−20 °C, ⩾1 h). Slides were stained with Gel Green (Biochrom, 1:1000 in PBS for 3 min). From each of two slides, 1000 binucleated cells were evaluated with regard to frequencies of micronucleus-containing and nucleoplasmic bridge-containing cells.

Cytokinesis block proliferation index (CBPI) was calculated in 1000 cells per slide using the formula CBPI = [MI + 2MII + 3 (MIII + MIV)] with MI–MIV representing the number of cells with one to four nuclei (Surralles et al., 1995).

Micronuclei and nucleoplasmic bridges were scored according to the criteria defined by the members of HUman MicroNucleus (HUMN) project (Fenech et al., 2003). Structures were defined as micronuclei if they were round or oval, had the same staining intensity as the main nuclei and were not connected to them. Continuous links between the nuclei in binucleated cells were scored as nucleoplasmic bridges. The damaged cell was considered as endpoint, therefore cells containing more than one micronucleus or nucleoplasmic bridge were scored as one cell with one or more micronuclei or bridge, respectively.

For kinetochore analysis cells were treated for 4 h with 0.5 μM patulin followed by 20 h post-incubation with cytochalasin B (5 μg/mL). Cells were brought onto glass slides by cytospin centrifugation and fixed in methanol (−20 °C, ⩾1 h). Kinetochores were stained with a first antibody against centrosomes (Positive Control Serum (Centromere), Antibodies Incorporated; undiluted, 37 °C, over night) and a rhodamine conjugated secondary antibody (Santa Cruz, sc-2457; 1:20, 37 °C, 2 h). Counter staining of nuclei was done with chromomycin A (50 μM, 3 min). About 5627 cells were evaluated for the presence of kinetochore-positive or -negative micronuclei.

2.4. Comet assay

The comet assay is a widely accepted method for quantification of DNA lesions of different nature. For the detection of cross-links a modified protocol has be proposed by Olive et al. (1990). By creating DNA cross-links DNA fragments resulting from treatment with radiation or strand breaking agents are artificially increased in size and their migration in an electrical field is impeded. V79 cells (5×10^5), seeded the day before, were treated for 4 h with 0.5 μM patulin or 10 μM cis-platin. After a washing step 100 μM H_2O_2 was added for 30 min. Subsequently, the cells were harvested and used in the comet assay as described earlier (Schupp et al., 2011) using a fluorescence microscope at 200-fold magnification and computer-aided image analysis (Komet 5, Kinetic Imaging Ltd.). Twenty five cells from each of two slides stained with Gel Red (Biochrom, 20 μg/mL in PBS) were measured, with percent tail DNA as the evaluation parameter.

2.5. α/γ-Tubulin staining

2×10^5 cells, seeded the day before were incubated with 0.5 μM patulin for 4 h. After 20 h substance-free post incubation cells were harvested and brought onto glass slides as described above. Fixed slides were washed with PBS containing 0.5% Tween 20 and incubated at 4 °C overnight with FITC labeled Sigma mouse anti-α-tubulin (F2168) 1:50 in 5% FBS–PBS or Sigma mouse anti-γ-tubulin (T6557) 1:50 in 5% FBS–PBS. Slides for γ-tubulin were subsequently washed and incubated with Alexa 488 labeled goat anti-mouse antibody (Invitrogen A11001) 1:200 in 5% FBS–PBS for 3 h at room temperature. For evaluation α- and γ-tubulin dyed slides were counter stained with Hoechst 33258 for 3 min. Two hundred mitotic figures from each of two slides were counted and classified as normal or multipolar mitosis.

3. Results

The micronucleus assay and an analysis of nucleoplasmic bridges were carried out to evaluate the genotoxicity of patulin in V79 cells (Fig. 1). The formation of micronuclei increased dose-dependently in BSO-pretreated (glutathione-depleted) cells, but increased only slightly without BSO pre-treatment. Nucleoplasmic bridges increased dose-dependently in BSO-pretreated as well as in not pretreated cells. However, the increase occurred at lower concentrations in the BSO-pretreated cells.

Analyzed from more than 5600 cells 37.2% of micronuclei showed kinetochores after patulin treatment and 62.8% were kinetochore-negative.

Cell proliferation was slightly reduced with increasing concentrations of patulin and much stronger in BSO-pretreated cells (Fig. 1a). At the highest concentration of 1 μM patulin, proliferation of the BSO-pretreated cells was almost completely blocked, making an evaluation of binucleated cells for micronuclei/nucleoplasmic bridges impossible. Comparing the time course of formation (Fig. 2) revealed that nucleoplasmic bridges were already present 3 h after patulin treatment, while micronuclei did not appear within the first 6 h after treatment.

A modified version of alkaline comet assay was performed to investigate the potential cross-linking properties of patulin. Fig. 3 shows the DNA damage induced by H_2O_2 with or without pretreatment with the known cross-linking agent cis-platin or patulin. If there are cross-links, the DNA can migrate less after H_2O_2 treatment. DNA migration was decreased in patulin pretreated cells and significantly reduced in cis-platin-pretreated cells compared to H_2O_2 alone.

To investigate if the high reactivity of patulin on cellular macromolecules also affects the formation of tubulin fibers from its subunits, a well known mechanism of genotoxic micronucleus forming spindle poisons such as colcemide, α- and γ-tubulin staining for detection of mitotic spindles (α) and centrioles (γ) was employed. Microscopic inspection did not reveal a compromised formation of spindle fibers after patulin treatment, but the number of mitoses containing multipolar spindles was about 25 times increased compared to control cells (Fig. 4, left side). This was associated with the presence of supernumerary centrosomes in patulin-treated cells (Fig. 4, right side).

4. Discussion

The objective of this study was to investigate the mechanisms by which patulin unfolds its genotoxic effects in V79 cells. The micronucleus assay revealed a dose dependent formation of micronuclei. After staining with an antibody against centromeres, kinetochore-positive and -negative cells were present, which is in agreement with the results of Pfeiffer et al. (1998). Further evaluation revealed a striking number of the so called nucleoplasmic bridges, which were formed shortly after patulin treatment. Nucleoplasmic bridges are generally explained by the disturbed distribution of dicentric chromosomes during mitosis. Dicentric chromosomes which are pulled to opposite poles during mitosis lead to anaphase bridges, which in the absence of breakage form nucleoplasmic bridges. However, for the formation of dicentric chromosomes, a breakage and reunion event is needed (Thomas et al., 2003). This cannot be achieved within 3 h, the shortest time after which nucleoplasmic bridges were observed. Furthermore, the mechanism of breakage-fusion-bridge-cycles is thought to include the generation of micronuclei accompanying the formation of nucleoplasmic bridges (Thomas et al., 2003). Such simultaneous appearance of micronuclei and nucleoplasmic bridges was rarely seen in patulin-treated cells during our study. Therefore, another mechanism must be responsible for the formation of nucleoplasmic bridges after patulin treatment. We hypothesize that the cross-linking ability of patulin, by connecting sister-chromatids before mitosis, provides an explanation. If sister chromatids cannot separate, but are pulled apart from their kinetochores being attached to the two opposite spindle poles, the chromatin must