Aneuploidogenic and clastogenic potential of the mycotoxins citrinin and patulin

Erika Pfeiffer, Kerstin Groß and Manfred Metzler

Institute of Food Chemistry, University of Karlsruhe, D-76128 Karlsruhe, Germany

To whom correspondence should be addressed
Email: manfred.metzler@chemie.uni-karlsruhe.de

The aneuploidogenic and clastogenic potentials of the mycotoxins citrinin (CIT) and patulin (PAT) were studied by determining inhibition of microtubule assembly under cell-free conditions and by measuring induction of mitotic arrest and micronuclei in cultured Chinese hamster V79 cells. Both CIT and PAT inhibited cell-free microtubule polymerization in a concentration-dependent manner. PAT, but not CIT, bound covalently to reactive thiol groups of microtubule proteins. At concentrations without gross cytotoxicity, mitotic arrest and CREST-positive microcnes, i.e. micronuclei containing whole chromosomes/chromatids, were induced by CIT and PAT in V79 cells. The time course of micronucleus induction and positive CREST staining indicate the aneuploidogenic potential of CIT and PAT. CREST-negative micronuclei, i.e. micronuclei containingacentric chromosomal fragments, were induced by PAT but not by CIT, implying a clastogenic potential of PAT. The aneuploidogenic and clastogenic potential of PAT and the aneuploidogenic potential of CIT may well contribute to the putative carcinogenicity of these mycotoxins in long-term animal studies.

Introduction

Citrinin (CIT*; chemical formula illustrated in Figure 1) and patulin (PAT; Figure 1) are mycotoxins produced by several strains of Aspergillus, Penicillium and Byssoschlamys (1). CIT is often associated with PAT or ochratoxin A in food of plant origin, in particlar crops (2). PAT is preferentially found in moldy fruits, especially apples, but also occurs in tomatoes and other vegetables as well as in cereals (3).

The toxicology of CIT and PAT is poorly understood and therefore the health risk posed to consumers of contaminated food is unclear. CIT has been shown to induce benign kidney tumors in Fischer 344 rats (4,5). It caused single- and double-strand breaks in the chromosomal DNA of intact E.coli and induced DNA repair synthesis in permeabilized E.coli cells (13). Furthermore, PAT enhanced the reversion frequency of the amber mutant of phage M13 to the wild-type phenotype in E.coli and induced DNA repair synthesis in Tetrahymena pyriformis (14), implying a direct attack on DNA in these bacterial systems. DNA repair assays in rat and mouse hepatocytes in vitro were negative with PAT (15,16), whereas structural chromosomal damage was caused in cultured mammalian cells (17–19). In cultured hepatoma cells, PAT caused disorganization of the cytoplasmic microfilaments comparable with the effects exerted by colchicine (20).

In order to further clarify the potential of CIT and PAT to induce structural and numerical chromosomal aberrations, we have tested both compounds for their ability to inhibit cell-free microtubule assembly, to arrest cultured V79 cells in metaphase and to induce micronuclei in these cells. Our studies show that CIT is a potential aneugen, whereas PAT has both aneugenic and clastogenic potential.

Materials and methods

Chemicals, microtubule proteins and cultured cells

CIT and PAT were purchased from Sigma (St Louis, MO). The crystalline materials were of >98% purity according to HPLC and GC/MS after trimethylsilylation with N,O-bis(trimethylsilyl)acetamide. Microtubule proteins (MTP) were prepared from bovine brain by two cycles of assembly and disassembly according to the method of Shelanski (21). The final protein pellet was suspended in assembly buffer [100 mM morpholinooethane sulfonic acid, 1 mM ethyleneglycol bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, 0.5 mM MgCl₂, pH 6.4] and stored in liquid nitrogen. MTP concentration was determined by the Bio-Rad (Munich) protein assay based on the method of Bradford (22), with bovine serum albumin (BSA) as the standard. The absorbance was measured at 276 nm and an extinction coefficient of 120 mM/cm was used.

Male Chinese hamster V79 lung fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM), pH 7.5, containing 4500 mg/l glucose, 100 U/ml penicillin, 100 mg/ml streptomycin and supplemented with 10% fetal calf serum. The cells were cultured at 37°C in a 10% CO₂ atmosphere.

Solutions of the test compounds in dimethylsulfoxide (DMSO) were added to DMEM in amounts to yield a final DMSO concentration of 0.1% (v/v). Control experiments were carried out with DMEM containing 0.1% DMSO without test compounds.

Assembly of microtubules

Polymerization of microtubules (MT) was carried out with freshly thawed MTP. The test compound (final concentration 20–200 µM) dissolved in ethanol (final concentration 2%) was added to 0.5 ml (final volume) assembly buffer containing 10 µM MTP. After 20 min at 35°C, MT assembly was started by adding 0.5 mM GTP and the increase in turbidity was measured at 350 nm for 30 min. The control incubation containing all components except the test...
compound was used as a reference (100% assembly). Disassembly at 4°C and a second assembly cycle were carried out to distinguish between normal MT formation and aggregation.

**Determination of free sulfhydryl groups**

Free thiol groups were determined by the method of Ellman (23) using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Formation of 2-nitro-5-thiolbenzoate (NTB) was measured by absorbance at 412 nm.

Incubation mixtures with PAT or CIT and the thiol compounds were prepared under the same conditions as the assembly assay. For analysis of free thiol groups, 200 µl aliquots of the incubation mixture were diluted with 800 µl 0.1 M phosphate buffer, pH 7.4, containing 8 M urea. Aliquots of 50 µl 10 mM DTNB in phosphate buffer were then added and the absorbance at 412 nm was measured after 10 min. The concentration of NTB was calculated using an extinction coefficient of 13.6/nM/cm.

**Cytotoxicity testing**

Cytotoxicity of the mycotoxins was tested using the trypan blue assay. V79 cells (7×10⁴ cells/flask) were incubated for 6 h in DMEM containing various concentrations of the test compounds. The medium was then collected, the cells were washed with phosphate-buffered saline (PBS) free of calcium and magnesium (PBS-CMF) and trypsinized by treatment with 5 ml PBS-CMF, containing 0.25% trypsin and 0.02% EDTA, for 1.5 min. The cells were collected from the combined supernatants in a small centrifuge at 1200 r.p.m. for 5 min. The cell pellet was resuspended in 5 ml PBS, pH 7.5, at 37°C and aliquots of 370 µl were added to 270 µl of a 0.4% solution of trypan blue in 0.9% saline. Following incubation at 37°C for 3 min, the numbers of cells without the dye (colorless viable cells) and with the dye (blue dead cells) were scored in a Neubauer chamber.

**Mitotic arrest**

V79 cells were plated on microscope glass slides in a quadriperm vial (30 000 cells/ml DMEM, 5 ml corresponding to 150 000 cells/slide) and grown in DMEM for 24 h. The medium was subsequently replaced by DMEM containing various concentrations of the test compounds and the cells were incubated at 37°C for 3, 6, 9 or 12 h. The slides were then immersed in methanol/acetic acid 3:1 (v/v) at –20°C for 1 h for fixation, air-dried, stained with 30 µl 0.9% saline. Following incubation at 37°C for 3 min, the numbers of cells without the dye (colorless viable cells) and with the dye (blue dead cells) were scored in a Neubauer chamber.

**Microtubule assembly and reactivity with thiols in vitro**

Polymerization of MTP to MT under cell-free conditions is inhibited by numerous established aneugens, e.g. colchicine, vinblastine and diethylstilbestrol (25). When this assay was performed under standard conditions (26), i.e. incubation with MTP for 30 min at 35°C in assembly buffer, pH 6.4, prior to starting MT polymerization, an inhibitory effect of both PAT and CIT was observed in a concentration-dependent manner (Figure 2). Half-maximal inhibition required 100 µM PAT and 400 µM CIT. Depolymerization and a second polymerization were possible, indicating that the MTP were not denatured by the mycotoxins. Extension of the incubation time increased the inhibitory effect of PAT (e.g. complete inhibition with 100 µM PAT after 2 h incubation) but not of CIT. Increasing the MTP concentration from 10 to 20 µM decreased inhibition of MT assembly by 30%.

In order to clarify whether the inhibitory effect of PAT on MT formation was due to its well-known reactivity with thiol groups, cysteine, N-acetylcysteine and glutathione (GSH), as well as native and denatured MTP with a thiol content of 0.9% saline. Following incubation at 37°C for 3 min, the numbers of cells without the dye (colorless viable cells) and with the dye (blue dead cells) were scored in a Neubauer chamber.

**Results**

**Inhibition of cell-free microtubule assembly and reactivity with thiols in vitro**

Polymerization of MTP to MT under cell-free conditions is inhibited by numerous established aneugens, e.g. colchicine, vinblastine and diethylstilbestrol (25). When this assay was performed under standard conditions (26), i.e. incubation with MTP for 30 min at 35°C in assembly buffer, pH 6.4, prior to starting MT polymerization, an inhibitory effect of both PAT and CIT was observed in a concentration-dependent manner (Figure 2). Half-maximal inhibition required 100 µM PAT and 400 µM CIT. Depolymerization and a second polymerization were possible, indicating that the MTP were not denatured by the mycotoxins. Extension of the incubation time increased the inhibitory effect of PAT (e.g. complete inhibition with 100 µM PAT after 2 h incubation) but not of CIT. Increasing the MTP concentration from 10 to 20 µM decreased inhibition of MT assembly by 30%.

In order to clarify whether the inhibitory effect of PAT on MT formation was due to its well-known reactivity with thiol groups, cysteine, N-acetylcysteine and glutathione (GSH), as well as native and denatured MTP with a thiol content of...
120 µM, were incubated with 100 µM PAT under the standard conditions of the MT polymerization assay, i.e. in assembly buffer, pH 6.4 (see above). In control incubations PAT was omitted. Subsequently, the number of thiol groups was determined with DTNB. Native MTP was denatured with 8 M urea prior to reaction with DTNB. The results are summarized in Table I.

Reaction of PAT was about three times more extensive with cysteine than with N-acetylcysteine and ~25% faster than with GSH. Denatured MTP and GSH exhibited the same loss of thiol groups. Native MTP reacted with PAT to a lesser extent than denatured MTP (Table I). The number of thiol groups available for reaction with DTNB was 12 per tubulin dimer (27). In our experiments, 4.3 thiol groups/tubulin dimer reacted with PAT in denatured MTP, but only 1.6 in native MTP. The reduced accessibility of thiol groups in native MTP is well established in the literature. For example, alkylation of >3 thiols with iodoacetamide has been reported to cause denaturation of MTP (28). It is also known that the few accessible sulfhydryl groups of native MTP are essential for polymerization and their alkylation causes inhibition of MT assembly (29).

In order to compare the reactivity of these important thiol groups of MTP with that of cysteine, polymerization assays with PAT were conducted in which 100, 200 and 400 µM cysteine was added simultaneously with the MTP. Addition of cysteine under these conditions did not affect the inhibitory effect of PAT on MT assembly (data not shown). When 100 µM PAT was incubated with 100 µM cysteine for 30 min at 35°C prior to adding MTP, inhibition was 24%, as compared with 48% without cysteine (Figure 2). Preincubation of PAT with a 10-fold excess of cysteine for 2 h at 35°C abolished the inhibitory effect of PAT on cell-free MT polymerization. These data suggest that the essential thiol groups of tubulin are more reactive than the sulfhydryl group of cysteine.

The loss of thiol groups as determined with DTNB was always accompanied by an equivalent decrease in the extinction at 275 nm, the maximum absorbance of PAT. This is further evidence of a chemical reaction of PAT with the thiol groups.

In order to study the possibility that PAT might also react with amino groups, incubations were carried out under the same conditions but with alanine instead of cysteine. No indication of a reaction of this amino acid with PAT could be obtained, even after 2 h incubation.

In contrast with PAT, CIT did not bind covalently to MTP or the other thiol compounds. No loss of thiol groups could be found even after preincubation with a 10-fold excess of cysteine for 48 h at 35°C, and MT assembly was inhibited to the same extent as without cysteine. Therefore, the inhibitory effect of CIT on cell-free MT must be due to other mechanisms, which remain to be elucidated, e.g. colchicine-like non-covalent binding to tubulin or complexation of magnesium ions or interaction with GTP.

Cytotoxicity and metaphase arrest in V79 cells

To measure cytotoxicity, exponentially growing cells were incubated with various concentrations of PAT and CIT for 6 h, then stained with trypan blue and the number of vital (unstained) and dead (stained) cells determined. PAT proved to be much more cytotoxic than CIT, as shown in Table II. To ensure a survival rate of >90% of the cells after 6 h, the maximum concentration was 1 µM for PAT and 60 µM for CIT.

To study metaphase arrest, cells were incubated with various concentrations of CIT and PAT for 6 h. Following fixation and staining with DAPI, the number of metaphase cells per 1000 cells was scored.

Figure 3 depicts the results with CIT. No effect was observed at concentrations <20 µM, whereas metaphase cells increased in number in a concentration-dependent manner at higher concentrations. Incubation of the cells with CIT for 3 or 12 h gave rise to similar numbers of metaphase cells as incubation for 6 h, but incubation for only 1 h reduced metaphases to one-third (data not shown).

Metaphase arrest caused by CIT proved to be completely reversible up to 40 µM; upon incubation of arrested cells in CIT-free medium, complete transition into anaphase was observed within 30 min. After 60 min, the ratio of metaphase to anaphase cells was the same as in untreated control cells.
Table III. Ratio of metaphase to anaphase V79 cells at various time points after treatment with PAT

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Time (h)</th>
<th>1.5</th>
<th>3</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>12:6</td>
<td>12:7</td>
<td>12:6</td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>10:9</td>
<td>12:7</td>
<td>13:7</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>19:2</td>
<td>21:1</td>
<td>21:2</td>
</tr>
</tbody>
</table>

One thousand cells were scored in each experiment.

Table IV. Induction of MN by PAT in V79 cells as determined without and with cytochalasin B (CYB)

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Without CYB (MN/2000 cells)</th>
<th>With CYB (MN/1000 BNC*)</th>
<th>CREST-negative</th>
<th>CREST-positive</th>
<th>Total</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14 ± 1.4</td>
<td>3 ± 0.6</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>13 ± 1.5</td>
<td>13 ± 1.5</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>15 ± 1.5</td>
<td>20 ± 1.2</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represent means ± standard deviation of three independent experiments.

Experiments to induce metaphase arrest with PAT were carried out using 0.1, 0.5 and 1 µM concentrations and incubation times of 1.5, 3 and 6 h (Table III). No clear increase in the number of metaphase cells, but a morphological alteration in mitotic cells, could be observed. PAT at 0.1 µM had no effect, whereas 1 µM PAT caused cytotoxic effects with conglomerates of mitotic cells. Metaphases seemed to be elevated at 0.5 µM PAT; however, this increase may not be real, due to the fact that no clear distinction between metaphase and anaphase cells was possible since ~50% of all anaphase cells exhibited incomplete separation of sister chromatids at this concentration of PAT (Figure 4). These cells may possibly give rise to interphase cells with chromatin bridges (Figure 4), which account for ~2% of all interphase cells 6 h after treatment with 0.5 µM PAT.

Induction of micronuclei in V79 cells

Based on the results of the cytotoxicity and metaphase arrest studies, the concentrations used in the micronucleus (MN) assay were 30 and 40 µM for CIT, and 0.1 and 0.5 µM for PAT. After 6 h incubation with the mycotoxins, the cells were kept for another 3 h in fresh medium and then the numbers of micronucleated cells per 3000 cells determined and further characterized by CREST staining.

Both CIT and PAT induced MN in a concentration-dependent manner (Figure 5). Only the number of CREST-positive MN, i.e. MN containing whole chromosomes/chromatids, was induced under these conditions, whereas the number of CREST-negative MN was the same as in untreated cells.

Based on the results of the metaphase arrest studies, the CIT-induced MN arise from dividing cells. In order to examine whether the small number of micronucleated cells observed after PAT have also undergone cell division, the MN assay was repeated using the cytochalasin B (CYB) method. CYB inhibits cytokinesis without affecting karyokinesis and thus allows the detection of cells after division as binucleated cells, whereas mononucleated cells have not undergone mitosis (30). V79 cells were incubated with 0.1 and 0.5 µM PAT for 6 h in the presence and absence of 3 µg/ml CYB and then in PAT-free medium with and without CYB for another 3 h. MN were then determined in the binucleated cells. In order to clearly identify these cells, the cytoplasm has to be stained. As this staining interferes with CREST staining, only the total number of MN was determined in the CYB studies (Table IV).

The data obtained for CREST-negative and CREST-positive MN in the experiments without CYB in Table IV are in good agreement with those in Figure 5, when extrapolated to 3000 cells. The total number of MN per 2000 cells corresponds to
of two independent experiments.

This suggests strongly that the MN induced by PA T under these conditions arise from dividing cells. The formation of CREST-positive MN implies an aneugenic potential of PA T and CIT.

In order to analyze PA T and CIT for clastogenic potential, the time course of MN formation was studied. V79 cells were treated for 6 h with 0.5 µM CIT, followed by incubation with fresh medium for various lengths of time up to 24 h. The same experiment was carried out with the established clastogen NQO at 0.5 µM concentration. The results are depicted in Figure 6. With PA T, the number of CREST-positive MN per 3000 cells showed a peak at 6 h post-treatment with a fairly constant level of ~35 MN before and after this time point. In contrast, no CREST-negative MN were observed at 3 or 6 h, but their number increased steeply between 12 and 24 h, indicating that PA T has clastogenic potential. As expected, NQO gave rise only to CREST-negative MN and exhibited a typical time course with a steady increase over 24 h.

When the time course of MN induction was studied with 30 µM CIT, the number of CREST-positive MN was about the same at 3, 6 and 12 h, with a slight decrease at 24 h (data not shown). No CREST-negative MN were induced by CIT even at 24 h, indicating that CIT has no clastogenic potential.

**Discussion**

The mycotoxins CIT and PA T are contaminants of various foodstuffs and can reach concentrations above the parts per million levels. However, the genotoxic and carcinogenic potential of CIT and PA T have not been fully elucidated. According to studies published in the literature, both compounds exhibit mutagenicity in some bacterial systems (6,7,13,14) but not in others (3,12). Structural aberrations of metaphase chromosomes have been reported for PAT (17–19) and CIT (8) in mammalian cells, but for CIT only after metabolic activation. PA T has been reported to cause disruption of the cytoskeleton of hepatoma cells, which may be due to an aneugenic effect (20).

Our study provides evidence that both mycotoxins have aneuploidogenic potential, i.e. the ability to induce numerical chromosomal aberrations. This proposition is based on the inhibition of cell-free MT assembly and on the induction of mitotic arrest and of MN containing whole chromosomes/chromatids. Different mechanisms account for the aneugenic potential of CIT and PAT. CIT interacts with MTP or other components of the MT system in a non-covalent manner. In contrast, PAT binds covalently to thiol groups, which are essential for polymerization of tubulin heterodimers to MT (28). At pH 6.4, which is the optimum for MT assembly, the order of increasing reactivity was N-acetylcysteine < GSH = denatured MTP < cysteine < native MTP. When added to a mixture of cysteine and native MTP with equal thiol content PAT preferentially bound to MTP. Other studies in our laboratory have shown that the initial step in the reaction of PAT with thiols is a Michael-analogous 1,6-addition of the thiol group to the α,β,γ,δ-unsaturated carbonyl system of PAT (R.Fliege and M.Metzler, in preparation). The thiol reactivity may also explain the much higher cytotoxicity for V79 cells of PA T as compared with CIT: after 6 h at pH 7.5, the concentrations causing 40% cell death were 5 µM for PAT but 100 µM for CIT. It is likely that the low level of GSH, namely 11.3 nmol/mg cellular protein (31), and the high activity of GSH S-transferase (32) contribute to the susceptibility of V79 cells to PAT cytotoxicity, as cellular GSH may become rapidly depleted by this mycotoxin.

Induction of mitotic arrest and MN were the other endpoints measured in our study. CIT behaved like a typical aneugen. At concentrations >20 µM, a concentration-dependent increase in mitotic arrest was noted that was independent of the time of incubation (ranging from 3 to 12 h) and reversible after removal of CIT (at non-toxic concentrations). MN appeared in the cells with a maximum at 3 h after removal of CIT. The number of cells containing MN at this time corresponded to approximately one-third of the cells arrested in metaphase.

With PAT, a clear induction of mitotic arrest could not be found. This may be due to the fact that the range between the concentration without effect and that with pronounced cytotoxicity is quite small. In spite of the lack of observable mitotic arrest, CREST-positive MN are clearly induced by PAT, indicating loss of whole chromosomes/chromatids during mitosis.

CREST-negative MN, i.e. MN containingacentric chromosomal fragments and indicating clastogenic potential, were induced by PAT but not by CIT. The lack of clastogenicity of CIT in our study is in agreement with a previous report showing that CIT needs metabolic activation by microsomal enzymes to become a clastogen (8). The CREST-negative MN induced by PAT were first detectable 12 h after removal of PAT from the cell culture medium. Their number at 24 h post-treatment was comparable with the level induced by the established clastogen NQO, used as a reference compound in our study. The time course implies that the clastogenic event occurs after the second cell cycle, thus rendering a direct attack on DNA by PAT unlikely. It is more likely that PAT interacts with chromosomal proteins, as has already been proposed by Thust et al. (17). The chromatin bridges between interphase cells and in anaphase cells after treatment with PAT are also indicative of clastogenic potential and may arise from interaction with chromosomal proteins leading to cross-links.

In conclusion, both CIT and PAT have the potential to cause genetic damage at the chromosomal level. The induction of numerical chromosomal aberrations leading to aneuploidy is thought to contribute significantly to the carcinogenicity of
established human and animal carcinogens, e.g., benzene, asbestos and diethylstilbestrol (33,34). However, for aneugenic carcinogens indications for threshold concentrations have recently been obtained (35,36). Similarly, a threshold response may be assumed for the clastogenic effect of PAT if the underlying mechanism involves interaction of PAT with chromosomal proteins rather than with DNA. Further studies are needed to define the threshold concentrations and to address the question of possible combined actions of CIT and PAT, as these mycotoxins are frequently found together in food.

**Acknowledgements**

We thank Brigitte Rosenberg for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (grant Me 574/14-1).

**References**


Received on January 6, 1998; revised and accepted on February 20, 1998

E.Pfeiffer, K.Groß and M.Metzler