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Cytotoxic and antimitotic effects of *N*-containing *Monascus* metabolites studied using immortalized human kidney epithelial cells

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Recently the first *Monascus* metabolites with a pyridine ring were detected, the monascopyridines A and B. They are formally dehydrogenated derivatives of the red rice pigments rubropunctamine and monascorubramine. Because of their structural similarity, the toxicological effects of these secondary metabolites were studied using immortalized human kidney epithelial cells. The cytotoxicity was determined with the following different endpoint detection methods: metabolic activity, trypan blue exclusion, and electronic cell counting. The compounds led to EC_{50} values between 11 and 31 µmol/L but the pigments caused a stronger reduction of the cell viability. Also, the apoptotic potential was examined by measuring caspase 3 activity and detecting apoptotic bodies, but none of the tested compounds induced apoptosis. All four substances caused a rise of the mitotic index to about 9% (100 µmol/L monascopyridine A and B) and 20% (25 µmol/L rubropunctamine and monascorubramine). The significant decrease of the ratio of cells in the ana- and telophase to cells in the prometa- and metaphase proved a stop of the mitosis at the meta- to anaphase control point. The compounds caused mitotic arrest and the formation of structural damages like c-mitosis through interaction with the mitotic spindle. These effects point to an aneuploidy inducing potential, which is linked to cancer formation.

Keywords: Cytotoxicity / Mitotic arrest / *Monascus* / Pigments / Red fermented rice

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1 Introduction

Red fermented rice, which is also known as red yeast rice, koji, anka, angkak, and ben-koji, has been used as a traditional food additive for improving the color and aroma of meat, fish, and soybean products. It is also described to have conserving properties [1]. Its medicinal application to lower the blood cholesterol levels has turned red rice into an interesting natural but unapproved dietary supplement [2, 3]. Red yeast rice is produced by the fermentation of rice with *Monascus* sp., such as *M. purpureus* and *M. anka.* Fungi of the genus *Monascus* are known to produce several secondary metabolites among other pigments and monascopyridines [4–6]. For *M. purpureus*, six main pigments have been identified: rubropunctamine, monascorubramine, rubropunctatin, monascorubrin, monascin, and ankaflavin (for structures see Fig. 1). The red pigments rubropunctamine and monascorubramine are probably formed on the polyketide pathway *via* the orange pigments rubropunctatin and monascorubrin by reaction with amines [7,

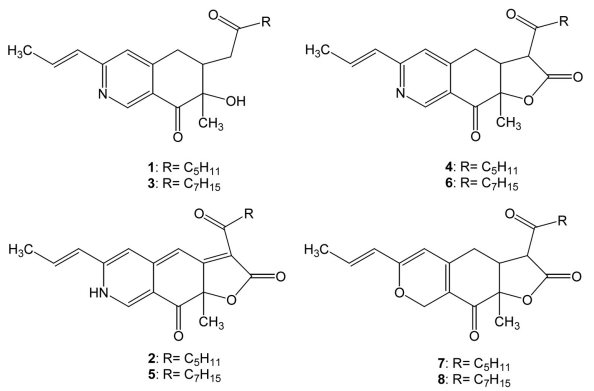

Figure 1. Structure of (A) monascopyridine C (**1**) and D (**3**), (B) rubropunctamine (**2**) and monascorubramine (**5**), (C) monascopyridine A (**4**) and B (**6**), (D) monascin (**7**) and ankaflavin (**8**).

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Abbreviations: **AcDEVD-AFC**, *N*-Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin; **AT/PM ratio**, ratio of cells in anaphase + telophase/prometaphase + metaphase; **CY3 conjugated**, carbocyanin 3 conjugated; **EC**, effective concentration; **IHKE cells**, immortalized human kidney epithelial cells

8]. The amount of red pigments in *Monascus* products is unknown but varies strongly depending on the fungus and fermentation method used [9]. The biological properties of rubropunctamine and monascorubramine were examined as a mixture of both pigments, in which they displayed weak antibacterial effects against *Bacillus subtilis.* Rubropunctatin and monascorubrin showed stronger effects, which could be lowered by the reaction with ammonia and thus turning them into the red pigments [10, 11]. For the monascopyridines A and B, a biosynthetic way similar to the red pigments' is proposed. The monascopyridines A and B are produced by *M. purpureus* up to amounts of 6 mg/g red rice. Their pharmacological and toxicological properties have not been examined yet [5].

Because of the structural analogy of the monascopyridines A and B on the one hand and the red pigments rubropunctamine and monascorubramine on the other hand, their toxicological properties were compared. Therefore the red pigments were isolated and the four compounds were studied in cell culture studies with immortalized human kidney epithelial (IHKE) cells, which have shown a sensitive induction of apoptosis in studies of another *Monascus* metabolite, the mycotoxin citrinin.

2 Materials and methods

2.1 Chemicals

ACN (HPLC grade) and phosphoric acid (analytical grade) were obtained from Merck (Darmstadt, Germany). HPLC-grade water was prepared with a MilliQ Gradient A10 system (Millipore, Schwalbach, Germany). DMEM/Ham F-12 media, PBS, fetal calf serum, and trypan blue were obtained from Biochrom (Berlin, Germany). Accutase™ was purchased from PAA Laboratories (Cölbe, Germany), mouse epidermal growth factor from BD Biosciences (Heidelberg, Germany), goat serum from Gibco BRL (Paisley, UK), and CASY®Ton from Schärfe System (Reutlingen, Germany). Monoclonal anti-α-tubulin-antibody from mouse ascites fluid was obtained from Sigma, carbocyanin 3 conjugated (CY3 conjugated) antimouse antibody of the goat from Jackson Immuno Research (Soham, UK). All other chemicals were obtained from Merck and Sigma-Aldrich (Taufkirchen, Germany).

2.2 NMR spectroscopy

^{1}H NMR data were acquired in $CDCl_3$ on a Bruker DPX-400 (Bruker BioSpin, Rheinstetten, Germany).

2.3 Preparation of red fermented rice and HPLC analysis

Preparation of red yeast rice, extraction, and HPLC analysis (Fig. 2) were performed as described in [6].

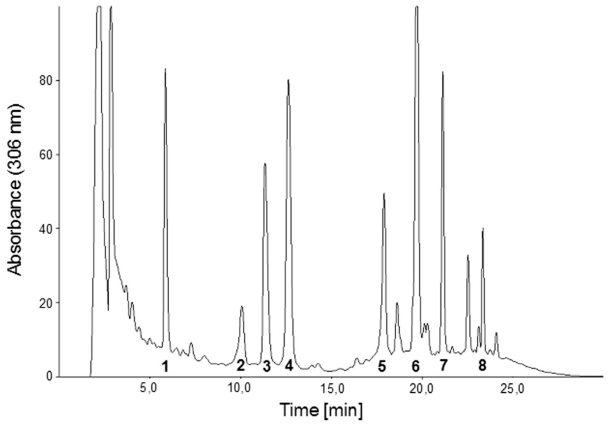

Figure 2. HPLC chromatogram of red fermented rice obtained from *M. purpureus* (DSM1603) grown on rich rice medium: (**1**) monascopyridine C, (**2**) rubropunctamine, (**3**) monascopyridine D, (**4**) monascopyridine A, (**5**) monascorubramine, (**6**) monascopyridine B, (**7**) monascin, (**8**) ankaflavin. λ = 306 nm.

2.4 Isolation of rubropunctamine and monascorubramine

Aliquotes of 0.5 g of the pulverized red rice [6] were extracted with 1.5 mL of a mixture of 0.25 M phosphoric acid and ACN (1:1) in a ultrasound water bath and subsequently centrifuged (12 000 × *g*, 5 min, 15°C). The supernatant was directly used for HPLC separation.

Extracts were fractionated by HPLC on a 250 × 16 mm id Eurospher-100 C18 column (Knauer GmbH, Berlin, Germany) using the following gradient of (A) 0.25 M phosphoric acid and ACN (1:1) and (B) ACN: 0–10 min 100% A, 60 min 20% A, 70 min 20% A, 80 min 100% A, 90 min 100% A; flow: 3 mL/min. Rubropunctamine (**2**) eluted at 32.6 and monascorubramine (**5**) after 45.4 min. (The retention times are different compared to the chromatogram in Fig. 2, since another HPLC method was used.)

Fractions eluting at 31–34 min (**2**) and 44–47 min (**5**) were further fractionated using an (A) ACN and (B) water gradient on the same column: start 100% A, 0–40 min; 0% A, 50 min; 0% A, 70 min; 100% A, 75 min; 100% A; flow: 3 mL/min; **2** eluted at 25.0 min, **5** at 41.1 min. Solvent was removed and ^{1}H-NMR spectra were measured for purity check. The NMR data were in agreement with published data [12].

2.5 Cell culture

IHKE cells (passage 150–180) were kindly provided by M. Gekle (Würzburg, Germany). They were cultured as described by Tveito *et al.* [13] in DMEM/Ham's-F12 medium (100 μL/cm^2) enriched with 13 mmol/L $NaHCO_3$,

15 mmol/L HEPES, 36 µg/L hydrocortisone, 5 mg/L human apotransferrin, 5 mg/L bovine insulin, 10 µg/L mouse epidermal growth factor, 5 µg/L sodium selenite, 10% fetal calf serum, and in addition 1% penicillin/streptomycin under standard cell culture conditions (37°C, 5% CO_2).

To exclude any binding of the tested compounds to serum proteins, cytotoxicity assays were carried out in serum-free medium. Under these conditions, cells were still proliferating, as determined with electronic cell counting. The control cells and the cells incubated with the test compounds were treated with the same amount (<1%) of organic solvent (ACN/0.25 M H_3PO_4).

2.6 Cytotoxicity assay

2.6.1 CCK8 assay

Photometrical determination of the cytotoxicity was determined using the CCK8 assay (Dojindo Laboratories, Tokyo, Japan) as described in [14]. Results are presented as mean values from three replicate experiments with different passages enclosing ten wells each.

2.6.2 Electronic cell counting

Electronic cell counting analyses were performed utilizing a CASY TT cell counter (Schärfe System, Reutlingen, Germany) with the same sample preparation as described in [6]. In brief, cells were incubated in Petri dishes for 24 h with the test compounds. For viability determination, cell medium was collected and adherent cells were harvested by incubation with Accutase for 15 min. Cell suspension was combined with the previously collected cell medium and centrifuged (120 × *g*, 5 min, 20°C). The pellet was resuspended in 1 mL of PBS and 200 µL of this suspension were mixed with 10 mL of CASY-Ton for measurement. Results are presented as mean values from two replicate experiments with different passages enclosing three Petri dishes each.

2.6.3 Trypan blue exclusion

For studying the cell viability with the trypan blue exclusion method, a suspension of cells in PBS obtained as described in Section 2.6.2 was used. Hundred microliters of the suspension was mixed with 100 µL 0.5% trypan blue solution and incubated for 2 min at 37°C. Cells were counted in a Neubauer hemocytometer. The viability of the treated cells was compared to the viability of an untreated control. Results are presented as mean values from three replicate experiments.

2.7 Caspase 3 assay

Determination of the caspase 3 activation was performed as stated before according to the manufacturer (Clontech Laboratories, Heidelberg, Germany) with slight modifications. The monascopyridines A and B were incubated for 12, 24, and 48 h, the pigments for 12 and 24 h. The sample protein content was quantified with a bicinchoninic acid assay kit from Sigma. Results are presented as mean values from three replicate experiments with different passages enclosing three Petri dishes each.

2.8 Determination of the antimitotic potential and detection of apoptotic bodies

The antimitotic potential was determined as described before [6] with colchicine as a positive control [15]. DNA was stained with a methanolic Hoechst 33258 solution and was viewed with an excitation wavelength of 365 nm and emission at 420 nm. The mitotic index and the proportion of cells in ana- and telophase to cells in prometa- and metaphase (AT/PM ratio – ratio of cells in anaphase + telophase/prometaphase + metaphase) were calculated as parameters of mitotic arresting activity. Additionally, cells in the prometa- and metaphase were further divided into normal prometa- and metaphases and cells with structural damages, such as c-mitosis, dislocated chromosomes, and multipolar spindles. The different metaphases were classified as percent of all prometa- and metaphases. Results are presented as mean values of three replicate experiments with different passages enclosing two slides each. Using the same method, apoptotic bodies can be detected.

2.9 Immunological staining of the mitotic spindles

Cells were cultured and incubated as described in Section 2.8. They were fixed and stained according to [6] with Hoechst 33258 and the monoclonal anti-α-tubulin-antibody from mouse ascites fluid (diluted 1:500 with 1% BSA solution), the CY3 conjugated antimouse antibody of the goat, (diluted 1:250 with 1% BSA solution). The cells were viewed with different wavelengths: Hoechst staining was viewed as stated in Section 2.8, mitotic spindles were observed with an excitation wavelength of 546 nm and emission at 590 nm.

2.10 Data analysis

Data are represented as means ± SD. The *n*-value indicates the number of wells, Petri dishes, or slides used for the measurements and is given in the figures. The median effective concentration (EC_{50}) values were calculated using

SigmaPlot 8.0. Statistical significance of changes in the mitotic index and AT/PM ratio were determined by unpaired Student's *t*-test. Differences were considered statistically significant when $p \leq 0.01$.

3 Results and discussion

In Fig. 2, the HPLC chromatogram of an extract of red rice fermented with *M. purpureus* DSM 1603 is shown. All compounds were identified by comparing their retention times and UV-Vis absorption spectra to references. In addition to the monascopyridines A (**4**), B (**6**), C (**1**), and D (**3**), the pigments rubropunctamine (**2**), monascorubramine (**5**), monascin (**7**), and ankaflavin (**8**) were produced (for structures see Fig. 1). The red pigments (**2**) and (**5**) were isolated by preparative HPLC.

In order to study the toxicity of the secondary metabolites **2**, **4**, **5**, and **6** cell culture experiments using IHKE cells were performed. Cytotoxicity was evaluated with three different endpoint detection methods. The CCK8 assay allows the determination of the cell viability by measuring the metabolic activity of the cells: mitochondrial and cytoplasmatic dehydrogenases of living cells transform the tetrazolium salt WST8 into a water-soluble formazan, which is quantified. The trypan blue exclusion method is based on the fact that the dye is negatively charged and does not interact with cells unless their membrane is damaged, so all cells excluding the dye are viable. The electronic cell counting method determines viability through different electronic properties of dead and viable cells. Cells with an intact membrane are electrical insulators and therefore measured by the volume of the whole cell. In contrast, cells with damaged membranes are measured by their nuclei size only, so dead and viable cells can be distinguished by their size.

The EC_{50} values of all four compounds varied between 11 and 31 μmol/L (Table 1). There was only a small difference between the values determined for the monascopyridines **4** and **6** on the one side and the pigments **2** and **5** on the other side. Also, the different length of the hexanoyl or octanoyl side chain did not seem to have an influence on the toxicity. This is different from findings for the yellow and orange pigments monascin (**7**), ankaflavin (**8**), rubropunctatin, and monascorubrin. When observing embryotoxicity using chicken embryos, these pigments showed differences in their effects depending on the length of the side chain, but there was no uniform impact such as higher or lower toxicity with the longer C_7H_{15} side chain. However, only the C_5H_{11} side chain homologs rubropunctatin and monascin (**7**) were teratogenic, which was attributed to the higher hydrophility [11]. Also, in cell culture studies with the human cancer cell lines Hep G2 and A549, only ankaflavin (**8**) showed strong cytotoxicity, whereas monascin (**7**) had no effect, which was caused by apoptotic cell death [16].

However, when looking at the viability reduction, there is a large difference between the effects exhibited by the monascopyridines and the pigments (Fig. 3A–C). The highest concentration of the monascopyridines (100 μmol/L **6** and 150 mmol/L **4**) reduced cell viability to 87% (**4**) compared to the untreated control and 77% (**6**) with the trypan blue endpoint detection, and to 55% (**4**) and 47% (**6**) using the CCK8 assay. When measuring the viability with electronic cell counting, it was reduced to about 80% by both **4** and **6**. Pigment concentrations of 150 μmol/L also reduced the viability to 80% when using the electronic cell counting method. However, applying the other two assays, **2** and **5** reduced the viability with trypan blue exclusion to about 4% and using the CCK8 assay to about 13%. This is an indication that the pigments interfere with the cellular metabolism before affecting the cell membrane, since the CCK8 assay showed a higher cytotoxicity compared to electronic cell counting. The differences in the viability reduction between the trypan blue exclusion and the electronic cell counting method can be explained by the toxicity of trypan blue itself. This could lead to further dying of already damaged cells whose membranes were not destroyed enough to be detected as dead by the electronic cell counter.

The monascopyridines interfered with the viability of IHKE cells without showing strong cytotoxic effects, whereas the pigments were much more toxic and led to the complete dying of the cells. This is probably due to the 2,4-dihydropyridine ring of the pigments, which is more reactive compared to the pyridine ring. The observed reductions

Table 1. EC_{50} values of the monascopyridines A (**4**) and B (**6**), rubropunctamine (**2**) and monascorubramine (**5**) in IHKE cells (24 h incubation, for details see Section 2.6)

Compound	EC_{50} CCK8 assay (μmol/L)	EC_{50} cell counting (μmol/L)	EC_{50} Trypan blue (μmol/L)
Monascopyridine A	21.8 ± 1.1	25.8 ± 1.4	21.6 ± 0.9
Monascopyridine B	10.8 ± 1.2	16.5 ± 0.4	17.5 ± 0.2
Rubropunctamine	18.2 ± 1.3	31.4 ± 0.5	31.1 ± 0.4
Monascorubramine	20.2 ± 0.9	20.1 ± 0.3	31.1 ± 1.0

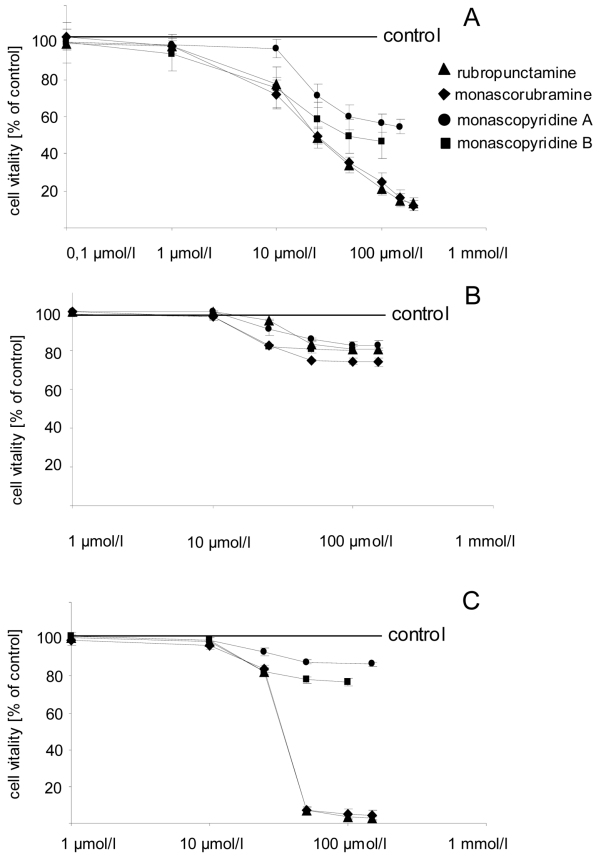

Figure 3. Cytotoxicity with (A) CCK8 assay, (B) electronic cell counting (C) trypan blue exclusion. IHKE cells were incubated for 24 h with the monascopyridines (**4**, **6**) and pigments (**2**, **5**) in concentrations of (A) 0.1–200 µmol/L, (B) and (C) 1–150 µmol/L ($n = 30$ (A), $n = 6$ (B), $n = 3$ (C), mean ± SD).

of the cell viability can be caused by substances with an apoptosis inducing or mitotic arresting potential, so those two possibilities were examined more closely.

A marker enzyme for the induction of apoptosis is caspase 3. Its activity can be measured specifically by using the substrate AcDEVD-AFC (*N*-Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin), from which the fluorescent AFC (7-amino-4-trifluoromethylcoumarin) is liberated. The occurrence of apoptotic bodies is another sign for apoptosis. This can be observed after fluorescence staining of the DNA with bisbenzimide (Hoechst 33258) or DAPI [17]. Monascopyridine A and B (**4**, **6**) were incubated for 24 h in concentrations of 1, 10, 25, and 50 µmol/L and for 12 and 48 h with 50 µmol/L only. The pigments **2** and **5** were incubated for 24 h in concentrations of 1, 10, 25 µmol/L and for 12 h with 25 µmol/L, due to their higher cytotoxicity. None of the tested compounds induced a change of the caspase 3 activity (data not shown). When detecting mitotic cells after an incubation time of 24 h, only cells treated with 25 nmol/L colchicines as positive control formed apoptotic

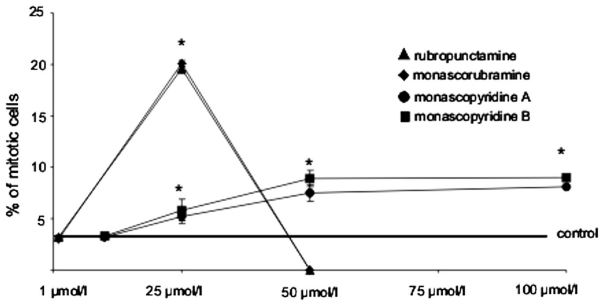

Figure 4. Mitotic arresting activity expressed as the mitotic index in percentage of IHKE cells after 24 h of incubation with the monascopyridines (**4**, **6**) and pigments (**2**, **5**). Mitotic index of the control: 3.21 ± 0.27%; mitotic index of the positive control colchicine at 25 nmol/L: 24.15 ± 1.79% ($n = 6$, mean ± SD). *, indicates significant difference from control ($p < 0.05$).

bodies. So, it can be concluded that neither the monascopyridines **4** and **6** nor the red pigments **2** and **5** are capable of inducing apoptosis in IHKE cells.

Another explanation for the observed cytotoxic effects is an arrest of cells during mitosis with an accumulation of cells in the metaphase. This effect can occur when the function of the spindle, the kinetochores, chromosome alignment, or their attachment to the kinetochores are affected [18]. Figure 4 shows the mitotic index of IHKE cells treated with different concentrations of **2**, **4**, **5,** and **6**. Both the monascopyridines and the pigments led to a significant increase of the mitotic index compared to the control in concentrations over 25 µmol/L. However, the pigments enhanced the mitotic index to 20% whereas the monascopyridines only caused an increase to 5%. Concentrations of 50 µmol/L of the pigments **2** and **5** were so cytotoxic that there were no cells in mitosis. As shown in Fig. 5A and B, the pigments caused a stronger condensation of chromosomes, which can be considered as an indicator of cytotoxicity [19]. An additional index of the antimitotic potential is the AT/PM ratio, as shown in Fig. 6. All compounds led to a significant decrease of the AT/PM ratio, which proves that mitosis is arrested in the metaphase. The pigments caused a complete mitotic arrest with an AT/PM ratio of 0.00, an effect that also occurs with colchicine. After incubation with the monascopyridines a small number of cells were in the ana- and telophase, so a few cells can complete mitosis.

Both monascopyridines caused the same abnormal mitotic figures. As example, Fig. 7A shows abnormal metaphases caused by **4** as a representative example. Predominant defects were structural damages like c-mitosis (see also Fig. 5), dislocated chromosomes to a lower extent, and a small amount of tripolar spindles. The metaphase damages caused by the incubation with 25 µmol/L of all four compounds are shown in Fig. 7B. The pigments led to a higher number of cells in the metaphase with structural damages

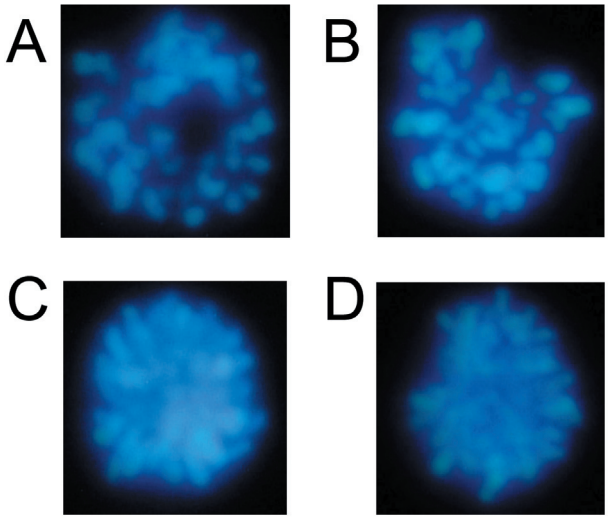

Figure 5. Fluorescent microscopic photographs of IHKE cells after 24 h incubation stained with Hoechst 33258. (A) Metaphase after incubation with 25 μmol/L **2**, (B) metaphase after incubation with 25 μmol/L **5**, (C) c-mitosis induced by 25 μmol/L **4**, (D) c-mitosis induced by 25 μmol/L **6**.

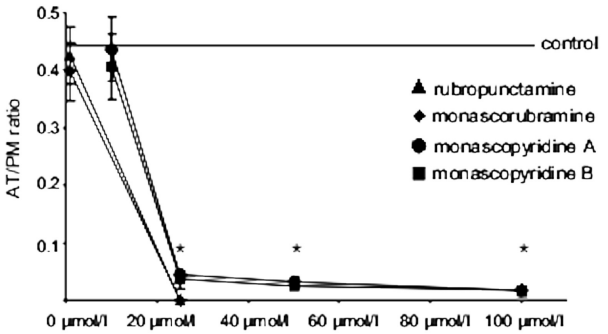

Figure 6. AT/PM ratio (proportion of anaphases plus telophases to prometa- and metaphases) evaluated after incubation of IHKE cells for 24 h. AT/PM ratio of the control: 0.48 ± 0.03 AT/PM ratio of the positive control colchicine at the concentration of 25 nmol/L: 0.00 ($n = 6$, mean ± SD). *, indicates significant difference from control ($p < 0.05$).

which also consisted mainly of c-mitoses; **2** and **5** did not cause multipolar spindles. The occurrence of c-mitosis is an indication that mitosis is arrested by the interference of chemicals with the mitotic spindle. This assumption was confirmed by staining the spindle tubulin immunologically with anti-α-tubulin CY3 conjugated antibodies (Fig. 8). The monascopyridines A and B (**4**, **6**) led to changed tubulin assembly (Fig. 8A). Some spindles were partly degraded (Fig. 8B) or broke down completely. This effect was also found after incubation with colchicine. The pigments (**2**, **5**) caused mainly decentralized spindles (Fig. 8C). Even with strongly condensed chromosomes, no spindle disassembly could be detected. Since the pigments (**2**, **5**) and the monas-

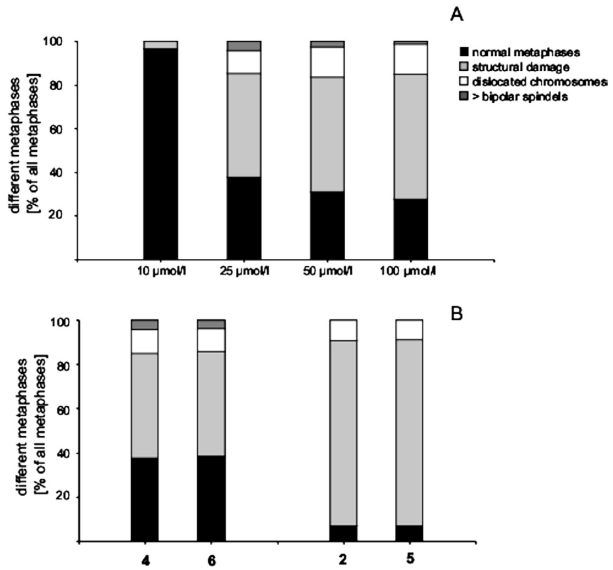

Figure 7. Percentage of normal and the different aberrant metaphases after 24 h incubation with (A) monascopyridine A (**4**) as a representative example for the effects of both monascopyridines; (B) 25 μmol/L of monascopyridine A (**4**), B (**6**), rubropunctamine (**2**) and monascorubramine (**5**). Percentage of abnormal metaphases of the control and 1 μmol/L **2** and **5**: 0%; percentage of abnormal metaphases of the positive control colchicine at the concentration of 25 nmoL/L: 95% ($n = 6$).

copyridines (**4**, **6**) cause different effects in IHKE cells, the 2,4-dihydropyridine and the pyridine ring, respectively, seem to play an important role for the interference with the mitotic spindle tubulin. The monascopyridines A and B led to the same effects as the recently isolated monascopyridines C and D, which caused low cytotoxicity detected with CCK8 assay and electronic cell counting [6].

4 Concluding remarks

The monascopyridines and the red pigments led to an increase of the mitotic index by accumulation of cells in the metaphase. The red pigments rubropunctamine and monascorubramine displayed stronger cytotoxicity and antimitotic effects, which is probably due to the higher reactivity of the dihydropyridine ring. All substances acted upon interference with the spindle tubulin. The monascopyridines led in some cases to a breakdown of the mitotic spindle. As stated before, substances that interact with the mitotic spindle can lead to aneuploidy [20, 21]. Although not directly proven, a lot of data suggest that aneuploidy is involved in cancer formation [22].

An antimitotic and cytotoxic potential has been shown for all compounds in cell culture studies with IHKE cells. Although the bioavailability of the tested substances is not

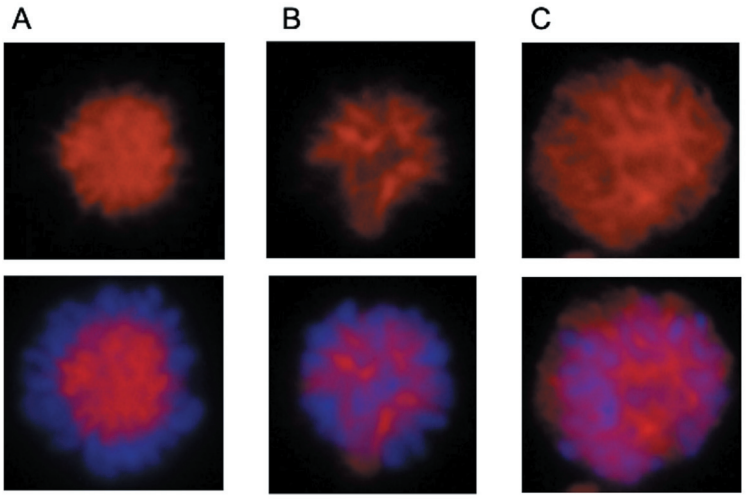

Figure 8. Fluorescent microscopic photography of CY3 immunologically stained tubulin spindles (above) and overlapping of tubulin and DNA stained with Hoechst 33258 (below). IHKE cells were incubated for 24 h with (A) 50 μmol **4**, (B) 25 μmol/L **6**, (C) 25 μmol/L **2**.

known yet, red yeast rice should be examined further, since the other main pigments have been shown to have even stronger effects than rubropunctamine and monascorubramine [10, 11].

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