Epothilone D affects cell cycle and microtubular pattern in plant cells

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Abstract

Epothilones, macrocyclic lactones from culture filtrates of the myxobacterium Sorangium cellulosum, are known as taxol-like microtubular drugs in human medicine. To date, nothing is known about the effect of epothilones on microtubules (MTs) in plant cells and/or on the plant cell cycle. As shown in this report, the treatment of tomato cell suspension cultures with epothilone D produced a continuous increase in the mitotic index. Dose–response curves revealed that epothilone D alters the mitotic index at concentrations as low as 1.5 μM. Mitotic arrest was already visible after only 2 h of treatment, and 55% of the cells were arrested after 24 h. As shown by immunocytological methods, abnormal spindles are formed during metaphase, which leads to a random distribution of chromosomes in the whole cell and prevents the formation of a metaphase plate. The process of chromosome decondensation does not seem to be affected, because micronuclei form at the same place with the distributed chromosomes. This suggests that epothilone D influences the stability of plant MTs mainly during metaphase of the mitotic cycle. In metaphase, the effects of epothilone D seem to be irreversible, because cells with an abnormal spindle could not be recovered after removal of the drug.

Key words: Cell cycle, epothilone, immunofluorescence, Lycopersicon esculentum, microtubuli, mitosis, mitotic arrest.

Introduction

The cytoskeleton is involved in a wide range of cellular functions connected to cell shape, movement of organelles and cell division. It consists of three classes of proteins: actin filaments, microtubules (MTs), and intermediate filaments. MTs play an important role in morphogenesis and the cell cycle (Kost et al., 1999). Significant differences in the MT cytoskeleton during the cell cycle of animals and higher plants can be observed. On one hand, angiosperm cells lack centrosomes as microtubule organizing centres (MTOCs) and are missing the contractile ring which serves as a tool for cytokinesis in animal cells. On the other hand, plant cells exhibit special patterns of microtubular organization during the cell cycle (Goddard et al., 1994): (i) during interphase MTs are arranged in parallel bundles (cortical MTs) near the plasma membrane perpendicularly orientated to the axis of elongation; (ii) at the onset of mitosis a dense ring of MTs builds up the so-called preprophase band (PPB), which defines the orientation of the new cell wall following karyokinesis (Gunning and Wick, 1985); and (iii) at the end of mitosis MTs form the phragmoplast to transport and orient vesicles in the plane of cell wall formation.

A prerequisite for living organisms is the regulated dynamics of the cytoskeletal elements. If these dynamics are disturbed by environmental stress or chemical compounds, typical life processes are drastically influenced. This can lead to changes in the cell cycle, disturbances of cell differentiation, and even cell death. These features are used to treat living cells for academic as well as for medical purposes. The stability and dynamics of MTs are influenced by natural compounds, which can inhibit the polymerization (e.g. colchicin) or the depolymerization of MTs. The most prominent example of a depolymerization inhibitor is Taxol® (Paclitaxel), which causes spontaneous assembling of MTs (Nicolaou et al., 1994). In animal cells, Taxol® increases the number of microtubular bundles during

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interphase, leading to a disturbed spindle formation followed by the arrest of mitosis and apoptosis (Flörshheimer and Altmann, 2001). Therefore, Taxol® is widely used in chemotherapy. These effects of Taxol® on the cell cycle are known not only for animal and human cells (Schiff and Horwitz, 1980; Rowinsky, 1997), but also for plant cells (Falconer and Seagull, 1985; Yasuhara et al., 1993).

Since 1995, additional natural compounds exhibiting a taxol-like effect have been described. Among them are epothilones and its derivatives, which resemble a new class of potent tubulin-polymerizing and microtubule-stabilizing compounds (Bollag et al., 1995). Epothilones, 16-membered macrolides, are produced by the myxobacterium Sorangium cellulolum (Geth et al., 1996; Höffe et al., 1996) or can be chemically synthesized (for reviews, see Wessjohann and Scheid, 1999, 16.10.2000; Wessjohann and Scheid, 1999, 13.07.2001, 16.10.2001) by Dr Eichelberger (Leibniz Institute of Plant Biochemistry, Halle, Germany). Treatment with epothilone D and Taxol® (Sigma, Teichenhofen, Germany) was performed by adding the compound at the appropriate concentration to a 24-h-old culture. In order to remove epothilone D from the cell culture, cells were collected by centrifugation at 800 g for 5 min, washed twice with fresh cultivation medium, and transferred into cell-free medium from a 24-h-old culture. As a control, non-treated cells were subjected to the same procedure.

**Materials and methods**

**Plant material and treatments with Taxol® and epothilone D**

A tomato (Lycopersicum esculentum cv. Lukullus) cell culture established and described by Tewes et al. (1984) was used, except that amino acids were not added to the culture medium. The cells were cultivated at 28 °C in the dark and were transferred to fresh medium twice per week. Erlenmeyer flasks (500 ml) containing 100 ml medium were placed on a rotator (160 rpm).

Epothilone D was synthesized following Wessjohann et al. (unpublished data; for partial syntheses along this route see Scheid et al., 2004; Wessjohann and Scheid, 1999, 16.10.2000; Wessjohann et al., 13.07.2001, 16.10.2001) by Dr Eichelberger (Leibniz Institute of Plant Biochemistry, Halle, Germany). Treatment with epothilone D and Taxol® (Sigma, Teichenhofen, Germany) was performed by adding the compound at the appropriate concentration to a 24-h-old culture. In order to remove epothilone D from the cell culture, cells were collected by centrifugation at 800 g for 5 min, washed twice with fresh cultivation medium, and transferred into cell-free medium from a 24-h-old culture. As a control, non-treated cells were subjected to the same procedure.

**Determination of mitotic activity**

At the time points indicated, 0.5 ml of cell suspension was harvested and washed in microtubule-stabilizing buffer (MSB, 0.1 M PIPES, 10 mM EGTA, 10 mM MgSO4, pH 6.9) containing 2% (w/v) sucrose, fixed with 4% paraformaldehyde (PFA, VWR International, Darmstadt, Germany) in MSB and stained with 2.8 mM 4,6-diamidino-2-phenylindole (DAPI, Sigma) in a buffer containing 0.1 M PIPES, 0.1 mM EGTA, 0.05% (v/v) Nonidet NP-40, 5% (v/v) DMSO, and 10% (w/v) sucrose (pH 7.2) for 15 min. Cells were analysed by fluorescence microscopy using an epifluorescence microscope (Axioskop, Zeiss, Jena, Germany) and the proper filter combination for DAPI. Mitotic activity was determined by counting at least 500 cells in triplicate. All cells showing a clear division pattern were evaluated as mitotic cells. The mitotic index was calculated as the percentage of mitotic cells in the total number of cells.

**Immunolabelling**

Suspension cells of tomato treated for various periods were collected in sieves, washed with MSB containing 2% (w/v) sucrose, and fixed with 3% PFA in MSB. After immobilization with 3% agar/1% gelatin in MSB and dehydration in a graded series of ethanol, cells were embedded in PEG and sectioned as described by Hause et al. (1993). Sections of 2 μm thickness were immunolabelled with the monoclonal mouse-anti-α-tubulin antibody DM1A (Sigma) diluted 1:200 in phosphate buffered saline (PBS) containing 5% (w/v) BSA. Subsequently, an anti-mouse-IgG antibody conjugated with AlexaFluor488 (Molecular Probes, Leiden, The Netherlands) was used according to the supplier’s instructions. After immunodecoration the sections were stained with 2.8 μM DAPI in PBS. Immunodecorated sections were analysed by confocal laser scanning microscopy with an LSM510 META (Zeiss) equipped with an Argon laser (488 nm) and a UV-laser (354 nm). Micrographs were processed using the programs LSM-Examiner (Zeiss) and Photoshop 8.0.1. (Adobe, Seattle, USA).

**Results**

**Changes in the mitotic cycle**

Tomato suspension cells were stained with DAPI to visualize the nuclei as well as chromosomal structures,
which are indicative of cell division. The stages of the cell cycle in an untreated, 24-h-old culture are shown in Fig. 2A. Nuclear or chromosomal structures typical for interphase (Fig. 2A, 1), and for all phases of cell division (Fig. 2A, 2–6) could be detected. Condensation of chromosomes starts in the prophase, followed by the arrangement of chromosomes in the metaphase plate in the metaphase. In the anaphase, chromatides move to two poles, where they start to decondense in the telophase/cytokinesis stage.

Addition of 10 μM epothilone D, however, caused severe changes in the chromosomal arrangement during cell division after as little as 2 h of treatment (Fig. 2B). The shape of the nuclei in interphase and the chromosomes in prophase appeared similar to the control (Fig. 2B, 1 and 2B, 2, respectively). However, from metaphase onwards the cells appeared completely different compared with the untreated ones. In the majority of cells in metaphase, chromosomes arranged in a metaphase plate could not be identified. Moreover, cells in anaphase and telophase were rarely detectable. Sometimes the chromosomes were arranged in a bright mass (Fig. 2B, 3), but usually the highly condensed chromosomes were randomly spread over the entire cell (Fig. 2B, 4). The chromatides were apparently not transported to the poles. For the determination of the mitotic index those cells obviously in mitotic arrest were counted as mitotic cells. After more than 8 h of treatment, it was observed that some cells did not contain condensed chromosomes, but contained weakly stained, swollen structures instead (Fig. 2B, 5). From 12 h onwards such cells contained numerous structures that looked like single small nuclei, each of them containing a structure like a nucleolus (Fig. 2B, 6). These structures possibly represent ‘micronuclei’ formed from randomly distributed and subsequently decondensed chromosomes.

Concentration and time dependency of epothilone D effects
To identify the lowest concentration of epothilone D leading to the cellular changes described above different concentrations of the substance in a range from 0.25 μM to 5.0 μM (Fig. 3A) were tested. After 4 h of treatment, cells were harvested and the mitotic index was determined. The lowest concentration causing a significant increase in mitotic cells was 1.5 μM (producing about 12% mitotic cells). A much stronger increase was visible at 2.5 μM and 5.0 μM leading to nearly 30% mitotic cells after 4 h of treatment.

To analyse the time-course of the effects of epothilone D on the plant cell cycle, cells were treated with epothilone D and the mitotic index was determined every 2 h over a period of 24 h. A general toxic effect of high concentrations of epothilone D on tomato cells could not be excluded. Therefore, 1.5 μM epothilone D was chosen for the determination of the time-course. The culture without epothilone D treatment exhibited the normal division pattern as shown in Fig. 2A. The mitotic index in this culture is about 5% (Fig. 3A) and this remained constant over 24 h. Treatment with 10 μM Taxol® led to a slight increase in mitotic division (up to 9% after 6 h of treatment, Fig. 3B). Upon treatment with epothilone D, however, a nearly linear increase in mitotic cells or cells in mitotic arrest was observed (Fig. 3B) reaching 55% at 22 h and 24 h of treatment.

Reversibility of epothilone D effects
The strong effect of epothilone D on the cell cycle of tomato cells raised the question of whether this effect is reversible. To check this, epothilone D was removed from the culture medium after different treatment times and

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**Fig. 2.** Cell division in tomato cells without treatment (A) and with treatment with 10 μM epothilone D for different times (B). Whole cells were fixed and stained with DAPI to visualize nuclei and chromosomes. In non-treated cells the following stages are visible: 1, interphase; 2, prophase; 3, prometaphase; 4, metaphase; 5, anaphase; 6, telophase. Upon treatment with epothilone D for 4 h the chromosomal pattern in the metaphase changed (3–5), whereas the interphase (1) and the prophase (2) seem to be unaffected. After 24 h of treatment, randomly distributed chromosomes have formed micronuclei (6). Bar in (A) represents 5 μm for all micrographs.
the mitotic indices were determined 24 h after removal (Table 1). At all time points analysed, the mitotic arrest seemed to be diminished by removal of the compound. Also the number of cells exhibiting abnormal metaphases (and ‘micronuclei’) decreased. The determination of the viability of cells at the same time points, however, showed that the number of dead cells increased in proportion to the treatment time with epothilone D (Table 1). This led to the conclusion that the reduction in the number of mitotic cells after removal of epothilone D seemed to be due not to the reversibility of the effect but rather to the higher death rate of arrested cells.

Changes in the MT pattern caused by epothilone D

The structure of nuclei in interphase and of chromosomes in prophase was not changed in epothilone D-treated cells compared with non-treated cells (Fig. 2A, 1–3 versus Fig. 2B, 1, 2). In the metaphase, however, treatment with epothilone D caused a random distribution of chromosomes throughout the cell, without formation of a metaphase plate (Fig. 2B, 4). Moreover, the later phases of cell division such as anaphase and telophase could not be observed. This suggests that epothilone D might influence the stability of plant MTs primarily during the mitotic cycle. To analyse this, an immunocytological approach was used to investigate the microtubular pattern in tomato cells (Fig. 4). Non-treated cells exhibited the typical pattern of MTs. All the plant-specific configurations were visible: cortical MTs (Fig. 4A), the preprophase band (Fig. 4B), the acentriolar spindle (Fig. 4C), and the phragmoplast (Fig. 4D). Short-term treatments with 1.5 μM epothilone D (2 h) led to some changes in these microtubular patterns (Fig. 4E–H). All of the configurations were visible, but the MTs appeared thicker. In contrast to the control, the spindle apparatus frequently exhibited a pattern like an ‘animal spindle’ which has centrosomes (Fig. 4G). The relative number of spindles containing focused poles changed from about 10% in the untreated cells (the total number of spindles was 206) to about 65% in cells treated with 1.5 μM epothilone D (the total number of spindles was 348). During cytokinesis, the phragmoplast and remnants of the mitotic spindle appeared thicker (Fig. 4H). After longer treatments with 1.5 μM epothilone D, however, the MT cytoskeleton changed drastically. As shown in Fig. 4I–L the spindle apparatus was primarily affected, forming completely new patterns. The MTs seemed still connected with the randomly distributed chromosomes. These effects were concentration-dependent, as shown by the spindle apparatuses from cells treated with increasing concentrations of epothilone D (Fig. 4M). The cortical MTs and the preprophase band

Table 1. Effects of removal of epothilone D on mitotic indices and viability of tomato cells

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Mitotic cells at the end of treatment (%)</th>
<th>Mitotic cells at 24 h after removal of epothilone D (%)</th>
<th>Dead cells at 24 h after removal of epothilone D (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mitotic index</td>
<td>Abnormal metaphases</td>
<td>Mitotic index</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------</td>
<td>------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>0</td>
<td>5.2±0.20</td>
<td>0</td>
<td>4.8±0.31</td>
</tr>
<tr>
<td>6</td>
<td>16.7±0.81</td>
<td>13.3±0.72</td>
<td>5.9±0.19</td>
</tr>
<tr>
<td>10</td>
<td>25.4±1.27</td>
<td>23.0±1.07</td>
<td>8.9±0.42</td>
</tr>
<tr>
<td>20</td>
<td>53.4±2.86</td>
<td>52.0±2.55</td>
<td>31.3±0.96</td>
</tr>
</tbody>
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were not affected (not shown) and phragmoplasts were not detected after incubation times longer than 4 h.

Discussion

MTs are heterodimeric polymers of the globular proteins α- and β-tubulin which assemble into protofilaments forming tubular structures. Cellular processes such as cell division depend on the ratio of assembling and disassembling of MTs. Currently, the regulation of this process is poorly understood (Baskin, 2000). The stability and dynamics of MTs, however, are also influenced by the application of natural compounds, which can inhibit polymerization (e.g. colchicin) or inhibit depolymerization (e.g. Taxol® and epothilones).

These experiments clearly show that the treatment of plant cells with epothilone D has stronger effects on the
cell cycle than Taxol®. An arrest of the cell cycle in the metaphase of mitosis was observed, leading to a random distribution of chromosomes in the entire cell. Epothilone D is not primary cytotoxic because cells in interphase were not affected and entered the prophase of mitosis even after several h of treatment. Furthermore, it was found that, without separation of the chromatides, the randomly dispersed single chromosomes start to form micronuclei. This suggests that processes such as chromatin decondensation and nucleus formation, which normally proceed during or after cytokinesis, are still initiated and at least partially completed in epothilone D-treated cells. Dead cells arise after a long arrest in metaphase possibly because of the long-lasting irregular processes in such cells.

In human carcinoma cells epothilones inhibit cell growth at low nanomolar concentrations (Bollag et al., 1995; Altmann et al., 2000). Compared with that, 1000-fold higher concentrations of epothilone D are necessary to influence the cell cycle of plant cells. In tomato cell cultures, significant changes could be identified at a concentration of 1.5 μM. This could be due to the plant cell wall, which may produce an accumulation of epothilone D in the cell wall or less effective penetration of the drug into the cytoplasm. It is also possible that animal cells could accumulate epothilones in the cytoplasm, leading to higher local concentrations acting on animal MTs. This is supported by the fact that the concentration of epothilones that inhibits the assembly of MTs in vitro is also in the range of 10⁻⁶ to 10⁻⁴ M (Altmann et al., 2000).

Studies of living plant cells after the injection of fluorescing tubulin (Zhang et al., 1993) and after the expression of GFP-fusions with tubulin (Shaw et al., 2003) or with MT-associated proteins (Dhonukshe and Gadella, 2003; Vos et al., 2004), respectively, demonstrated that all MT configurations during the plant cell cycle are characterized by continuous dynamic changes. These changes are a prerequisite for the progress of the cell cycle, but are disturbed or even stopped by epothilone in animal cells (Bollag et al., 1995). For human cells, Altmann et al. (2000) described a bundling of MTs after epothilone treatment. To obtain more information concerning the action of epothilone D as a MT-stabilizing and thus cell cycle-arresting drug in plant cells, the structure of the MT cytoskeleton was investigated using immunocytochemical methods. Considerable changes of the MT cytoskeleton were detectable after treatment with epothilone D. After as little as 2 h of treatment the ‘stabilizing’ effects could be observed. That means that cortical MTs appeared thicker, MTs in the spindle seemed to be bundled, and thick remnants of the spindle beside the prominent phragmoplast could be observed during cytokinesis. Furthermore, short-term treatments (2–4 h) with 1.0–1.5 μM epothilone D led to the formation of mitotic spindles similar to the spindles in animal cells. Two-thirds of the spindles do not have the typical plant barrel-like structure (Fig. 4C), but they appear similar to the spindles of animal cells with centromeres at the poles (Fig. 4G, M; 1.0 μM). In general, plants do not have centromeres. In plant cells, MTOCs for spindle microtubules are distributed in the cytoplasm or located at endomembranes (Baskin, 2000). According to Fant et al. (2004) the molecular components involved in the formation of the centriolar spindle are also present in centosome free cells. Whether epothilone D influences proteins at spindle poles or leads to a spontaneous bundling of MT ends could not be clarified in these studies.

The arrest in metaphase due to abnormal spindle formation seems to be irreversible. This was elucidated by measuring the mitotic index and the viability of the cells after the removal of epothilone D. Observations made 24 h after the removal of epothilone D show that the number of abnormal mitotic spindles decreased, and the number of dead cells increased, which corresponds to the number of abnormal metaphase cells at the end of the treatment. Therefore, the decrease in abnormal mitotic spindles does not represent a recovery effect caused by displacement of epothilone D. The MT-stabilizing effect of epothilone D, however, is obviously not irreversible in other phases of the cell cycles. Cortical MTs, preprophase bands as well as phragmoplasts did not seem to be stabilized irreversibly. On one hand, the linear increase in mitotic cells during epothilone D treatment points to the entry of cells into metaphase, thereby completing prophase. On the other hand, phragmoplast structures could not be observed after 6 h or more of treatment. That means that phragmoplasts which could be observed 2 or 4 h after the start of treatment disappeared. If epothilone D stabilizes all MTs irreversibly, cells arrested in all phases of the cell cycle should be visible at all the time points investigated.

To summarize, it can be concluded that epothilone D arrests the cell cycle of plant cells. The drug is not cytotoxic, but it affects the mitotic spindle irreversibly causing an irregular distribution of non-separated chromosomes within the cell and, subsequently, a mitotic arrest. Whether epothilone D could be used as a tool for biotechnological approaches (e.g. synchronization of cell cultures) or plant breeding (e.g. dihaploidization of haploid plants) can be determined in future experiments.

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References


