Does usnic acid affect microtubules in human cancer cells?

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(With 2 figures)

Abstract

Usnic acid, a lichen metabolite, is known to exert antimitotic and antiproliferative activities against normal and malignant human cells. Many chemotherapy agents exert their activities by blocking cell cycle progression, inducing cell death through apoptosis. Microtubules, protein structure involved in the segregation of chromosomes during mitosis, serve as chemotherapeutical targets due to their key role in cellular division as well as apoptosis. The aim of this work was to investigate whether usnic acid affects the formation and/or stabilisation of microtubules by visualising microtubules and determining mitotic indices after treatment. The breast cancer cell line MCF7 and the lung cancer cell line H1299 were treated with usnic acid 29 μM for 24 hours and two positive controls: vincristine (which prevents the formation of microtubules) or taxol (which stabilizes microtubules). Treatment of MCF7 and H1299 cells with usnic acid did not result in any morphological changes in microtubules or increase in the mitotic index. These results suggest that the antineoplastic activity of usnic acid is not related to alterations in the formation and/or stabilisation of microtubules.

Keywords: usnic acid, microtubules, breast cancer, lung cancer.

O ácido úsnico pode afetar microtúbulos em células cancerosas humanas?

Resumo

O ácido úsnico, um metabólito de liquens, é conhecido por sua atividade antimitótica e antiproliferativa em células humanas normais e malignas. Muitos quimioterápicos exercem suas atividades bloqueando a progressão do ciclo celular e induzindo morte celular por apoptose. Os microtúbulos, estruturas protéicas envolvidas na segregação dos cromossomos durante a mitose, servem como alvo quimioterapêutico devido ao seu importante papel tanto na divisão celular quanto nos mecanismos de morte celular por apoptose. O objetivo deste trabalho foi investigar se o ácido úsnico afeta a formação e/ou estabilização dos microtúbulos, a partir da visualização de microtúbulos e determinação de índices mitóticos após o tratamento. Células de câncer de mama MCF7 e de câncer de pulmão H1299 foram tratadas por 24 horas com 29 μM de ácido úsnico e dois controles positivos: vincristina (que impede a formação de microtúbulos) e taxol (que estabiliza microtúbulos). O tratamento das células MCF7 e H1299 com o ácido úsnico não resultou em aumento do índice mitótico. Os resultados sugerem que a atividade antineoplásica do ácido úsnico não está relacionada a alterações na formação e/ou estabilização de microtúbulos.

Palavras-chave: ácido úsnico, microtúbulos, câncer de mama, câncer de pulmão.

1. Introduction

Lichens synthesize over eight hundred types of metabolites (Müller, 2001). Due to their therapeutic properties, many Cultures have utilised these compounds in traditional medicine for centuries (Cocchietto et al., 2002). Depsides, depsidones, dibenzofuranes, xanthenes, anthraquinones and usnic acids are amongst the more extensively studied lichen metabolites (Honda and Vilegas, 1998). Usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-dibenzofuran-1,3[2H,9bH]-dione; $C_{18}H_{16}O_7$) (Figure 1), is one of the most abundant secondary lichen

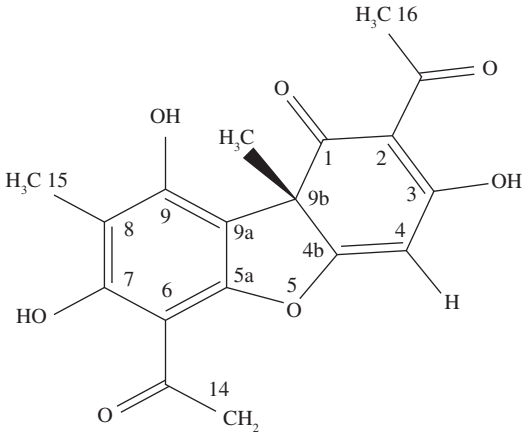

Figure 1. Chemical structure of (+)-usnic acid (Merck, 1989).

metabolites and can be found in nature in two enantiomeric forms (+) and (–) (Merck, 1989).

Usnic acid was initially used in the treatment of pulmonary tuberculosis (Plichet, 1955), and there are data regarding its biological activities as an antibiotic (Cocchietto et al., 2002), antiproliferative (Carderelli et al., 1997), analgesic and antipyretic (Okuyama et al., 1995), antiinflammatory (Vijayakumar et al., 2000), antiviral (Campanella et al., 2002; Scirpa et al., 1999), antifungal (Halama and Haluwin, 2004), against the parasite *Trypanosoma cruzi* (Carvalho et al., 2005) and as an immunologic modulator (Santos et al., 2004). Antitumor activity of usnic acid was shown for the first time by Kupchan and Kopperman (1975) against Lewis lung carcinoma in mice. Since then, many other researchers reported antiproliferative (Ögmundsdóttir et al., 1998; Kumar and Müller, 1999), mitochondrial depressive (Al-Bekairi et al., 1991) and antimitotic effects (Cardarelli et al., 1997). In addition, its mutagenic and cytotoxic activities have been determined against normal and malignant human cells lines (Bucar et al., 2004; Mayer et al., 2005; Perry et al., 1999; Takai et al., 1979; Santos et al., 2005, Santos et al., 2006; Bazin et al., 2008).

Although the cytotoxicity of usnic acid have been extensively reviewed (Müller, 2001; Cocchietto et al., 2002; Ingólfsdóttir, 2002) there is no data regarding the effects of usnic acid on microtubules. These intracellular structures composed of the protein tubulin are one of the components of the cytoskeleton. They are required for many cell functions, including cell division (mitosis), cell shape maintenance, intracellular transport, extracellular secretion, cell signalling, and cell motility (Yasuda et al., 2002). Microtubules are highly dynamic, with rapid changes occurring in their growth and length, particularly during cell division (Rusan et al., 2001).

In previous studies performed in our laboratory, H1299 lung cancer cells were exposed to 29 μM usnic acid for up to 48 hours (Mayer et al., 2005). It was observed that the growth rate was slower when compared to the untreated cultures. Moreover, although the cells did not lose adherence to the flask, they became deformed and elongated suggesting possible involvement of microtubules in the toxicity of the drug.

The aim of this work was to establish whether the antitumour effects of usnic acid could be related to the formation and/or stabilisation of microtubules.

2. Material and Methods

2.1. Cell lines

The breast cancer cell lines MCF7 (oestrogen-dependent, wild type p53) and the lung cancer cell line H1299 (null for p53) were obtained from American Tissue Culture Collection (Manassas, Virginia, USA). Cells were cultured in 5% CO_2 at 37 °C using Dulbecco's modified Eagle's medium supplemented with 10% FCS and 1% penicillin/streptomycin.

2.2. Effect of usnic acid on microtubule morphology

To study the effect of usnic acid on the formation and stabilisation of microtubules, cells were seeded in glass chamber slides from Lab-Tek (USA) at a density of 10^3 cells per chamber. After 24 hours incubation at 37 °C and 5% CO_2, the medium was aspirated off the adherent cells. Then, fresh medium containing 29 μM usnic acid, a concentration higher than the IC_{50} for both cell lines (Mayer et al., 2005), was added and cells exposed to the drug for 4 or 24 hours. Positive controls were prepared by treating cells with 1 μM vincristine or 1 μM taxol for 4 hours. At this concentration, vincristine and taxol are known to exert their activity depolymerising or stabilising microtubules respectively (Checci et al., 2003). Stock solutions of usnic acid (14.5 mM in 100% DMSO) taxol and vincristine (10 mM in 100% DMSO) were freshly prepared and further diluted with culture medium. Negative controls were prepared using 100% DMSO added to fresh medium at the same volume as used for the maximum concentration dose during 24 hours. Microtubule stabilising buffer (MTSB: 80 mM PIPES, 1 mM $MgCl_2$, 4 mM EGTA) was prepared immediately before use.

2.3. Microtubule visualisation

After treatment, cells were fixed in –20 °C methanol for 1-3 minutes and permeabilised in warm MTSB containing 0.5% TritonX-100 for 1 minutes. To minimise non-specific reactions, cells were blocked with PBS containing 0.1% TritonX-100 for 40 minutes. The slides were incubated with anti-α-tubulin antibody at a dilution of 1/2000 in blocking solution for 1 hour at 37 °C. Then they were incubated with FITC-conjugated donkey anti-mouse (Jackson Immuno Research Laboratories, Germany) at a 1/80 dilution for 45 minutes. Cells were

washed, drained, mounted and sealed. Microtubules were visualised with a Nikon E600 fluorescence microscope equipped with a Nikon coolpix 4500 digital camera.

2.4. Mitotic index

To evaluate if usnic acid behaves as a typical spindle poison, MCF7 and H1299 cells were seeded into 150 mm plates and grown under standard conditions. Sub-confluent cultures were treated with 29 μM usnic acid or 10 μM vincristine (positive control) for 4 and 8 hours. Negative controls were also included. After treatment, cells were trypsinised, washed in PBS and resuspended in 0.075 M KCl at 4 °C for 15 minutes. Cells were harvested by centrifugation at 1000 rpm for 10 minutes, fixed with freshly prepared Carnoy's fixative (MeOH:acetic acid at 3:1) and stored overnight at 4 °C. The following day, small amounts of preparation (app. 500 μL) were dropped onto microscope slides and heated for 2 seconds. Slides were stained with 5% Giemsa prepared in Gurrs buffer (pH 6.8 tablets from BDH, Leicestershire – UK). Cells were observed with a Zeiss® Axiovert 25 microscope using a 400× of magnification. The mitotic index was reported as the percentage of mitotic cells per total number of cells. The experiment was performed 3 times. An average of 1000 cells per treatment were counted. Results for the three different treatments were compared with Student's *t*-test with two-tailed distribution and two-sample equal variance.

3. Results

3.1. Effect of usnic acid on microtubule morphology

Figure 2 shows the results of the treatment of MCF7 cells (Panel 1) and H1299 cells (Panel 2) with usnic acid (1b and 2b), taxol (1c and 2c) and vincristine (1d and 2d). Cells treated with usnic acid (1b and 2b), were morphologically similar to the cells with no treatment (1a and 2a) even after 24 hours exposure to usnic acid.

3.2. Mitotic index

Table 1 shows the absolute number of analysed cells and mitotic indices for MCF7 and H1299 untreated cells and cells treated with usnic acid or vincristine as averages of three experiments. Treatment with vincristine resulted in accumulation of M phase cells compared to the negative control (Student's *t*-test $p < 0.05$), whereas untreated cells (negative control) and cells treated with usnic acid showed similar mitotic indices (Student's *t*-test $p > 0.05$).

4. Discussion

Although reports in the literature confirm the anticancer activity of usnic acid (Ögmundsdóttir et al., 1998; Kumar and Müller, 1999; Al-Bekairi et al., 1991; Bucar et al., 2004; Mayer et al., 2005; Perry et al., 1999; Takai et al., 1979), its mechanism of action has not yet

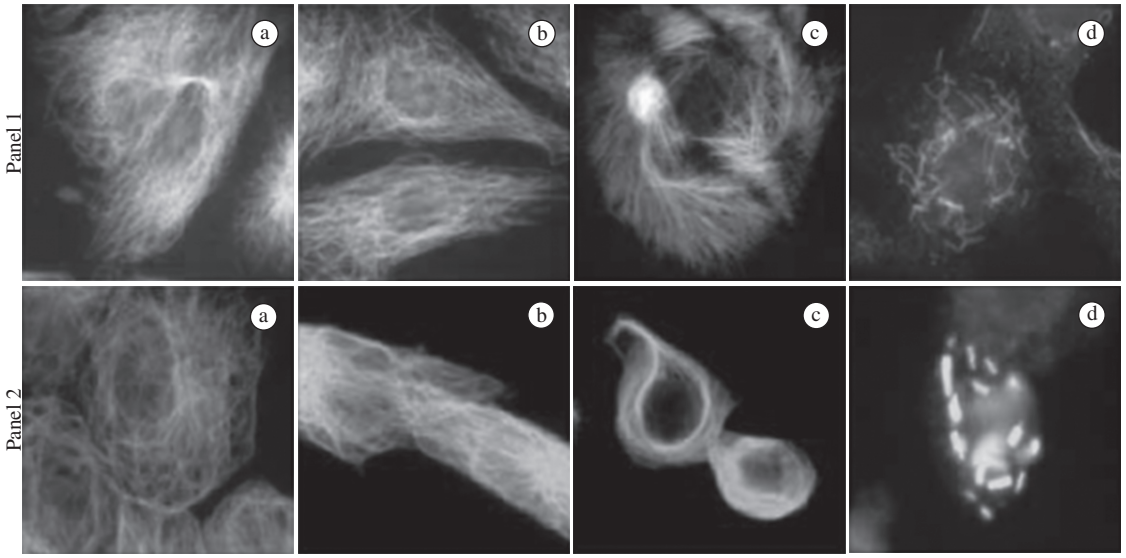

Figure 2. Effect of usnic acid on formation and stabilisation of microtubules in MCF7 (Panel 1) and H1299 (Panel 2) cells. Cell cultures in glass chamber slides from Lab-Tek (USA) were exposed to 29 μM usnic acid for 4 hours (not shown) and 24 hours. Positive controls were prepared by treating cells with 1 μM vincristine and 1 μM taxol for 4 hours. After treatment, cells were fixed in –20 °C methanol and stained with anti-β-tubulin antibody. MCF7 cells (1c) and H1299 cells (2c) treated with taxol showed stabilised microtubules with characteristic asters and bundles whereas MCF7 cells (1d) and H1299 cells (2d) treated with vincristine showed microtubule depolymerisation. However, cells treated with usnic acid MCF7 cells (1b) and H1299 cells (2b) were similar to untreated cells (1a and 1b) even after 24 hours exposure to the drug.

Table 1. Effects of usnic acid on the mitotic indices of MCF7 and H1299 cells.

Treatments	MCF7 cells				H1299 cells			
	4 hours		8 hours		4 hours		8 hours	
	Absolute No of cells analysed	Mitotic index (%)	Absolute No of cells analysed	Mitotic index (%)	Absolute No of cells analysed	Mitotic index (%)	Absolute No of cells analysed	Mitotic index (%)
Untreated	965	4.6	973	4.2	1028	2.6	1029	2.2
Vincristine	999	14	936	48	1059	12.7	1015	49.7
Usnic Acid	987	5.9	970	5.7	1023	3.1	1027	3

been completely understood. This compound has been shown to work as a phosphorylation chain uncoupling agent in rat hepatocyte mitochondria at concentrations in the range of 1 μM (Abo-Khatwa et al., 1996), as well as leading to oxidative stress and disruption of normal metabolic process in cells (Han et al., 2004). Recent studies also suggested that usnic acid acts by inhibiting RNA transcription (Campanella et al., 2002). According to Mayer et al. (2005), usnic acid showed antiproliferative activity against MCF7 breast cancer cells (oestrogen positive, wild type for p53) and the lung cancer cell line H1299 (p53 null), with IC_{50} of 18.9 and 22.3 μM respectively. The authors found that the antitumour activity of usnic acid did not involve DNA damage or p53 activation. These non-genotoxic and p53-independent features make usnic acid a potential candidate for either systemic or topical therapy for the treatment of tumours.

In the present work, after 4 hours exposure the effects of vincristine and taxol were observed in both MCF7 (Panel 1) and H1299 (Panel 2) cells. Panels 1c and 2c show asters and bundles characteristic of stabilised microtubules as a consequence of taxol treatment. Panels 1d and 2d show microtubule depolymerisation resulting from the treatment with vincristine. Cells treated with usnic acid (Panels 1b and 2b) however, did not show any differences when compared to the untreated cells (Panels 1a and 2a) after 24 hours exposure to the drug.

The majority of chemotherapy agents exert their activities by blocking cell cycle progression and unleashing cell death through apoptosis (Son et al., 2003). During mitosis, the DNA of a cell is replicated and the newly replicated chromosomes divided into the two forming cells along spindle fibres constructed with microtubules. The crucial role that microtubules play in cell division makes them a very suitable target for the development of therapeutic drugs against rapidly dividing cells such as cancer cells (Jordan and Wilson, 2004). The efficacy of microtubule targeting agents (MTAs) has been validated by their successful use for the treatment of a wide variety of human cancers (Rosolen et al., 2005). The MTAs bind to tubulin in a variety of ways (Hait et al., 2003; Dumontet and Sikic, 1999) in microtubules and prevent cancer cell proliferation by interfering with the microtubule formation required for cell division (Jordan, 2002).

Although the antiproliferative activities of MTAs have been thought to result from their actions on microtubule formation and stability, there is evidence that at low concentrations the antimitotic and anticancer effects of microtubule targeting agents may be largely due to their suppression of microtubule dynamics without affecting microtubule mass (Jordan et al., 1996). Indeed, the cytoplasmic motor protein dynein, a protein involved in the movement of chromosomes and positioning the mitotic spindles for cell division, is an early target for destruction during apoptosis (Karp, 2005). The concentration of usnic acid used in our experiments was above the IC_{50} established previously for both cell lines (Mayer et al., 2005). However, no changes were observed in the morphology of microtubules in MCF7 or H1299 even when cells were exposed to usnic acid for considerably longer periods (24 hours) than to vincristine or taxol (4 hours).

The Mitotic Index (MI), as the name suggests, is a count of the number of mitotic cells visible and expressed as a fraction of the total. For asynchronously growing cultures such as those used in these experiments, the mitotic index reflects the fraction of time that cells spend in mitosis versus the rest of the cell cycle. Increased mitotic index results from a lengthening of mitosis, usually an arrest (Golias et al., 2004). This approach revealed that vincristine, as a well established spindle poison, triggered a mitotic arrest state with about 50% mitotic index after 8 hours treatment. Usnic acid, however, did not appear to arrest the cell cycle at M phase (Table 1).

Collectively, our results indicate that the disruption of normal metabolic process in cells triggered by the action of usnic acid does not primarily involve depolymerisation or stabilisation of microtubules in breast or lung cancer cells.

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