Usnic acid: a non-genotoxic compound with anti-cancer properties

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The majority of human tumors bear inactive p53 or cellular factors that down-regulate the expression and activity of the p53 network. Therefore, finding therapies that are effective in such tumors is of great interest. Usnic acid, a normal component of lichens, showed activity against the wild-type p53 breast cancer cell line MCF7 as well as the non-functional p53 breast cancer cell line MDA-MB-231 and the lung cancer cell line H1299 (null for p53). In MCF7 cells treated with usnic acid, although there was an accumulation of p53 and p21 proteins, the transcriptional activity of p53 remained unaffected. We also found that there was no phosphorylation of p53 at Ser15 after treatment of MCF7 cells with usnic acid, suggesting that the oxidative stress and disruption of the normal metabolic processes of cells triggered by usnic acid does not involve DNA damage. The property of usnic acid as a non-genotoxic anti-cancer agent that works in a p53-independent manner makes it a potential candidate for novel cancer therapy. ***Anti-Cancer Drugs*** **16:805–809 © 2005 Lippincott Williams & Wilkins.**

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Introduction

Lichens are a symbiotic association between fungi and photosynthetic partners. Their metabolites, such as the dibenzofuran derivative usnic acid [1], exert a wide variety of biological activities, i.e. anti-bacterial, anti-viral and anti-inflammatory [2–6]. *In vitro* anti-cancer effects of usnic acid were shown for the first time by Kupchan and Kopperman [7] against Lewis lung carcinoma. Since then, many other researchers reported anti-proliferative [8–10] and mitochondrial depressive effects [11,12] of usnic acid against *in vitro* malignant cells, suggesting its potential use as a chemotherapeutic agent.

Many current cancer therapies are based on agents that have DNA-damaging effects including alkylating agents and γ-irradiation. In response to DNA damage the protein level of p53 accumulates and its transcriptional activity is also increased [13]. Activation of the p53 tumor suppressor protein leads to inhibition of cellular proliferation by inducing cell cycle arrest or apoptosis [14]. Wild-type p53 is known to act as a transcription factor, up-regulating the expression of p21, mdm2 and Bax genes amongst others [15].

Although the cytotoxicity of usnic acid has been extensively reviewed [16–19] and recent studies suggested that usnic acid acts by inhibiting RNA transcription [20] or mitochondrial function [21], the involvement of p53 in its mechanism of action is unknown. The objective of this work was therefore to investigate the relationship between the anti-neoplastic activity of usnic acid and p53 activation.

Materials and methods

Unless otherwise stated, reagents were acquired from Sigma-Aldrich (Poole, UK).

Cell lines

The breast cancer cell lines MCF7 (estrogen-dependent, wild-type p53) and MDA-MB-231 (estrogen independent, mutant p53), and the lung cancer cell line H1299 (null for p53) were obtained from ATCC (Manassas, Virginia, USA). Cells were cultured in 5% CO_2 at 37°C using DMEM supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.

Cytotoxicity of usnic acid

Stock solutions of usnic acid (14.5 mM) were freshly prepared in DMSO and further diluted with culture medium to obtain the desired concentrations. A concentration range of 1–60 μM of usnic acid was tested. Subconfluent cells were trypsinized and seeded into 96-well tissue culture plates in 100 μl of medium at a density of 1×10^3 cells/well. After overnight incubation,

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the medium was aspirated off the adherent cells and fresh medium with varied drug concentrations was added. After drug exposure for 72 h, cell survival was determined in quadruple wells for each drug concentration using the MTT assay as follows. An aliquot of 50 µl of a solution in phosphate-buffered saline (2 mg/ml) was added to each well. The plates were incubated at 37°C at 5% CO_2 for 4 h. Then the medium was removed from each well and 50 µl DMSO added. The OD_{540} was determined using a microplate reader (Spectra Max GeminiXS; Molecular Devices, Wokingham, UK) containing an equivalent amount of DMSO to each drug concentration as controls. Each experiment was repeated in triplicate.

p53/p21 Induction and Ser15 phosphorylation studies in MCF7 cells

Cells were seeded into 150-mm plates and grown under standard conditions. Based on the MTT results, subconfluent MCF7 cultures were exposed to 29 µM usnic acid for 24 h. Negative controls were treated with media containing an appropriate amount of DMSO. For phosphorylation studies, a positive control was prepared by treating subconfluent MCF7 cultures with 20 µM mitomycin. After treatment the medium was aspirated, and the cells washed with ice-cold PBS, scraped off and lysed for 15 min on 4°C denaturing urea buffer (6.4 M urea, 0.1 M DTT, 0.05% Triton X-100, 25 mM NaCl and 200 mM HEPES, pH 7.6). Lysates were clarified by centrifugation at 13 000 *g* for 5 min. Protein concentration was determined by the method of Bradford (protein assay kit from Bio-Rad, Munich, Germany) and aliquots stored at −70°C until required. Equivalent amounts of protein (20 µg) were separated on Novex high-performance pre-cast gels and blotted onto nitrocellulose membranes (Protran, Dassel, Germany) following the specifications for the NuPAGE Bis-Tris System from Invitrogen (Paisley, UK). Proteins were detected with the following antibodies: DO-12 (monoclonal, specific for the core domain of p53; obtained as described by Vojtesek [22]), WAF1 (monoclonal, specific for p21; Oncogene Research Products, Darmstadt, Germany) and FPS15 (polyclonal, specific for phospho-Ser15; Cell Signaling Technology, Hitchin, UK). Secondary anti-mouse antibody was purchased from Dako (Oxford, UK). Secondary polyclonal anti-rabbit antibodies were acquired from Bio-Rad. The detection of the protein-antibody complex was carried out with enhanced chemiluminescence (ECL Western blotting detection reagents; Amersham Biosciences, Little Chalfont, UK). Equal loading was confirmed with actin (Stratech Scientific, Luton, UK).

p53 Activation studies

To determine if the transcriptional activity of p53 correlated with its protein level, the β-Gal assay was performed as follows. T22 mouse fibroblast cells bearing the reporter plasmid RGÄFos-LacZ (containing a 36-bp p53-binding site and its corresponding control plasmid, which lacks the p53-binding site) were trypsinized and seeded into 96-well tissue culture plates in 90 µl of medium at a density of 1×10^4 cells/well. After 48 h incubation, usnic acid at the concentrations of 3.6, 7.25, 14.5 and 29 µM was added. Negative controls were cells treated with media containing DMSO. Positive controls were cells treated with actinomycin D (an anti-neoplastic antibiotic that inhibits cell proliferation by forming a stable complex with DNA and blocking the movement of RNA polymerase, which interferes with DNA-dependent RNA synthesis, leading to an increase in the levels of active p53) at 6 and 60 ng/ml, and the nuclear export inhibitor, non-genotoxic activator of p53 leptomycin B at the concentrations of 2 and 20 nM. After 16 h medium was aspirated off, 50 µl of lysis buffer (Promega, Southampton, UK) added to each well and plates shaken for 1–2 h at room temperature. Then, 150 µl of CPRG reaction mix (0.1 M phosphate buffer containing 80 µg/ml CPRG, 0.1 M β-mercaptoethanol and 4.5 M $MgCl_2$) was added and plates incubated for 4 h at 37°C. (A color change from yellow to pink indicates p53 activity.) Then, 100 µl from each well was transferred to a new 96-well plate and the OD_{570} was determined using a microplate reader (Spectra Max GeminiXS).

Results

Cell sensitivity to usnic acid treatment

The dose-responses of the breast cancer cell lines MCF7 (estrogen-positive, wild-type for p53) and MDA-MB-231 (estrogen-negative, non-functional p53), and the lung cancer cell line H1299 (p53 null) to usnic acid were analyzed by determining the relative viability of the treated cells by the MTT assay. The three cell lines were sensitive to usnic acid with IC_{50} values of 18.9 (MCF7) and 22.3 µM (MDA-MB-231 and H1299) (Fig. 1).

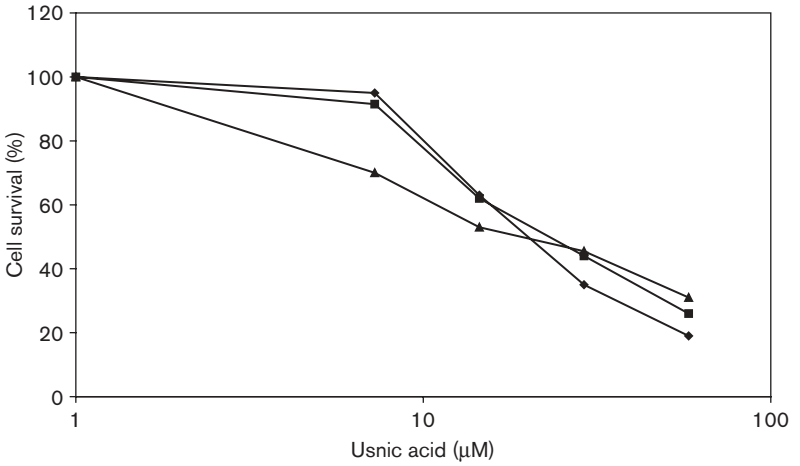
p53 and p21 Protein levels following usnic acid exposure

MCF7 cells were exposed to 29 µM usnic acid for 24 h as described in Materials and methods. The monoclonal antibody DO-12 was used as a probe for p53. This antibody is specific for the core domain of p53 and allows absolute quantitation of denatured p53 protein. p21 levels were detected with the monoclonal antibody WAF1. Figure 2(lanes 1 and 2) shows that MCF7 cells exhibited elevated p53 and p21 protein levels compared with vehicle controls after treatment with 29 µM usnic acid. Actin showed equal loading of proteins.

Ser15 phosphorylation of human p53 following usnic acid treatment

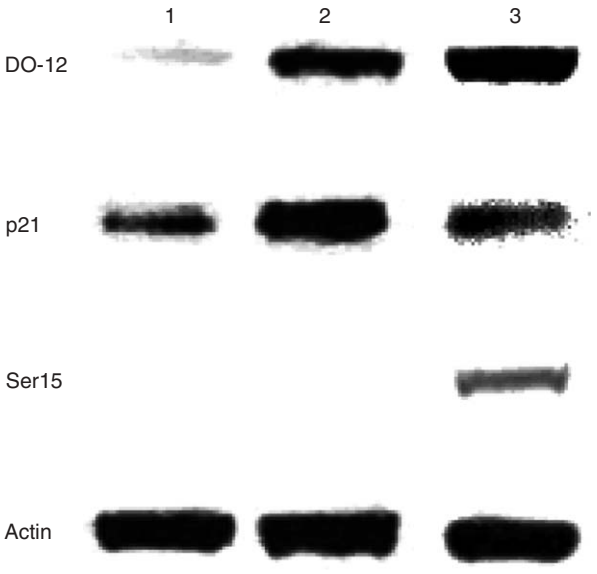
MCF7 cells treated with mitomycin C showed increased levels of Ser15 phosphorylation compared with the cells treated with vehicle control. Cells treated with usnic acid, however, showed no increase in Ser15 phosphorylation (Fig. 2, lanes 1–3).

Fig. 1



In vitro usnic acid dose–response assay. Subconfluent MCF7 (diamonds), MDA-MB-231 (squares) and H1299 (triangles) cells were trypsinized and seeded into 96-well tissue culture plates in 100 μl medium. After overnight incubation, the medium was aspirated off the adherent cells and fresh medium with varied drug concentrations was added. Cells were treated with usnic acid concentrations ranging from 1 to 60 μM for 72 h. Cell survival was determined in quadruple wells for each drug concentration using the MTT assay.

Fig. 2



Effect of usnic acid on the induction of p53/p21 and Ser15 phosphorylation. Subconfluent cultures in 150-mm plates were exposed for 29 μM usnic acid and 20 μM mitomycin for 24 h. After treatment, medium was aspirated off, and cells washed with ice-cold PBS, scraped off and lysed for 15 min on 4°C denaturing urea buffer. Equivalent amounts of protein were separated on Novex high-performance pre-cast gels and blotted onto nitrocellulose membranes (Protran). Proteins were detected with DO-12 (specific for the core domain of p53), WAF1 (Ab-1) (for p21) and FPS15 (specific for phospho-Ser15). Equal protein loading was confirmed with actin. Lane 1, vehicle control (24 h); lane 2, 29 μM usnic acid; lane 3, 20 μM mitomycin.

Table 1 β-Gal assay for evaluating the effect of usnic acid on p53 transcriptional activity

Treatment	Fold activity
Usnic acid (μM)	
3.6	0.89 ± 0.20
7.25	0.91 ± 0.25
14.5	0.58 ± 0.09
29	0.49 ± 0.05
Actinomycin D (ng/ml)	
6	3.88 ± 0.05
60	9.25 ± 1.51
Leptomycin (nM)	
2	2.52 ± 0.78
20	5.89 ± 0.32

Transcriptional activity of p53 following usnic acid exposure

Table 1 shows the effects of usnic acid at the concentrations of 3.6, 7.25, 14.5 and 29 μM, and 6 and 60 ng/ml actinomycin D, and 2 and 20 nM leptomycin B on T22 mouse fibroblast cells bearing the reporter plasmid RGΔFos-LacZ. Results are expressed as fold activity compared to cells treated with media containing DMSO (negative control). As expected, treatment with actinomycin D and leptomycin B, established activators of p53, resulted in increased activity related to the control. Cells exposed to usnic acid, however, showed no significant change, indicating that despite accumulation of p53 protein, there was no increase in p53 transcriptional activity.

Discussion

Finding therapies that are non-genotoxic and effective in tumors lacking p53 poses a great challenge for researchers.

DNA damage induced by many of the current cancer therapies may result in the development of second malignancies later in life [23], and the majority of human tumors bear inactive p53 or cellular factors that regulate the expression and activity of p53 [24].

Usnic acid is a common component of lichen cells found to provide anti-mitotic and anti-proliferative effects in a variety of biological systems [12,25]. Usnic acid has also showed anti-proliferative activity against normal cell lines, such as skin keratinocytes [9], fibroblasts, peripheral blood lymphocytes [8] and rat hepatocytes [26].

In the present study the toxicity of usnic acid against the breast cancer cell lines MCF7 (estrogen-positive and wild-type for p53) and MDA-MB-231 (estrogen-negative with a non-functional p53), and the lung cancer cell line H1299 (p53 null) was examined. All three cell lines showed sensitivity to usnic acid with IC_{50} values of about 20 μM (Fig. 1). The involvement of p53 and DNA damage occurrence in the mechanism of action of usnic acid were also investigated in MCF7 cells. DNA damage induced by physical or chemical stress is followed by a rapid increase in the level of the tumor suppressor p53 with activation of its transcriptional function. A network of signaling pathways is activated, which leads to arrest of the cell cycle progression in the late G_1 phase [27] or apoptosis [28] in proliferating cells. A key target for transcriptional activation by p53 is the cyclin-dependent kinase inhibitor p21, which suppresses cyclin E and A associated to cdk2 activities [14]. In fact, an increase in p21 expression is commonly accepted as an indication of p53 activation. This study showed that although there was an increase in p53 and p21 expression (Fig. 2), the transcriptional activity of p53 remained unaffected (Table 1). This fact suggests that the accumulation of p21 was not secondary to p53 transactivation. Under ordinary conditions the proteasome controlled degradation of intracellular proteins occurs in an ATP-dependent manner [29]. It has been shown that some anti-cancer agents such as rotenone and MPP^+ (1-methyl-4-phenylpyridinium) induce the increase of free radicals and reduce proteasomal activity via ATP depletion [30]. It was recently reported that usnic acid directly inhibits mitochondria function, which causes an increase in reactive oxygen species and a drop in cellular ATP levels [21]. It is reasonable to speculate, therefore, that activation of p53 is not involved in the action of usnic acid, and the increase in p53 and p21 levels, as observed in our experiments, was the result of the accumulation of inactive proteins due to proteasome inhibition.

In mammalian cells, ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) play crucial roles in early signal transmission after DNA damage. These two phosphoinositide 3-kinase kinases mediate phosphorylation of p53 at Ser15 [31]. It is thought that phosphorylation at this site reduces mdm2 protein binding (and subsequent ubiquitin-dependent p53 degradation) while promoting interaction of p53 with components of the transcriptional machinery (by stimulating CBP/p300 binding to and acetylation of p53). Moreover, a single mutation of Ser15 to alanine on full-length p53 can alter its apoptotic activity [32]. Our results showed that no phosphorylation of p53 at Ser15 after treatment of MCF7 cells with 29 μM usnic acid was observed (Fig. 2), indicating that DNA damage is not involved in the oxidative stress and disruption of the normal metabolic processes of cells triggered by usnic acid [21].

In conclusion, usnic acid has anti-proliferative activity against the wild-type p53 (MCF7) as well as the non-functional p53 (MDA-MB-231) breast cancer cell lines, and the lung cancer cell line H1299, which is null for p53. The properties of usnic acid as a non-genotoxic anti-cancer agent that works in a p53-independent manner support the need for further studies in order to establish a safe therapeutic range *in vivo*. Usnic acid thus has potential as either a systemic therapy or as a topical agent for the treatment of tumors.

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