Toxicology in Vitro 25 (2011) 37–44

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Variable responses of different human cancer cells to the lichen compounds parietin, atranorin, usnic acid and gyrophoric acid

M. Bačkorová [a], M. Bačkor [b], J. Mikeš [a], R. Jendželovský [a], P. Fedoročko [a,*]

[a] Institute of Biology and Ecology, Department of Cellular Biology, Pavol Jozef Šafárik University in Košice, Slovakia
[b] Institute of Biology and Ecology, Department of Botany, Pavol Jozef Šafárik University in Košice, Slovakia

ARTICLE INFO

Article history:
Received 31 May 2010
Accepted 7 September 2010
Available online 17 September 2010

Keywords:
Lichen metabolites
Cytotoxicity
Cell cycle
Cancer cell

ABSTRACT

One of the ways for searching for potentially new anti-cancer drugs is the testing of various naturally synthesized compounds. Lichens are a source of unique chemical agents of which some have already been proved to be effective against various cancer *in vitro* models. Our study reports on the sensitivity of up to nine human cancer cell lines (A2780, HeLa, MCF-7, SK-BR-3, HT-29, HCT-116 $p53^{+/+}$, HCT-116 $p53^{-/-}$, HL-60 and Jurkat) to the anti-proliferative/cytotoxic effects of four typical secondary metabolites of lichens (parietin, atranorin, usnic acid and gyrophoric acid). Variations in the dynamics of tumour cell line populations were evaluated by the MTT, clonogenic and viability assays, cell proliferation and detachment, cell cycle transition and apoptotic nuclear morphology, thereby confirming their concentration- and time-dependent cytotoxicity. However, in comparison with parietin and gyrophoric acid, the suppression of viability and cell proliferation by usnic acid or atranorin was found to be more efficient at equitoxic doses and correlated more strongly with an increased number of floating cells or a higher apoptotic index. Moreover, the analysis of cell cycle distribution also revealed an accumulation of cells in S-phase. This study has confirmed a differential sensitivity of cancer cell lines to lichen secondary metabolites.

1. Introduction

Cancer is a major cause of death worldwide. For this reason, different kinds of cancer therapies have been developed, including the application of a wide range of various anti-cancer agents with known effects on cancer cells. In the nature, we can find various examples of control and regulation of one organism's viability and cell division through the production of specific chemical substances by another organism. For example, the interactions between photoautotrophic algae (photobionts) and heterotrophic fungi (mycobionts) that began approximately 400–600 million years ago (Yuan et al., 2005) and led to the formation of stable and self-supporting symbiotic associations called lichens. The beneficial effects of lichenization resulted in the ability of lichens to be the dominant organisms in environments characterized by extreme ecological conditions.

The presence of photobionts in lichens has stimulated fungi into producing novel metabolites resulting from the symbiosis between the two partners (Ahmadjian, 1993). These novel metabolites are secreted by the fungal partner and are deposited on the surface of the symbiont body (called thallus), typically constituting 0.1–5.0% (w/w) of thallus dry weight (Fashelt, 1994). Thus far, nearly a thousand secondary metabolites of lichens have been discovered. The distribution of the secondary metabolites is restricted mostly to lichens. These compounds are considered to have important biological and ecological roles, including the regulation of cell division of photobionts, antimicrobial activity, allelopathy, antiherbivory, chelation of heavy metals and light screening (Lawrey, 1986; Pöykkö et al., 2005; Budová et al., 2006; Latkowska et al., 2006; Hauck et al., 2009; Solhaug et al., 2009).

Lichens and the cytotoxicity of their secondary metabolites have already been studied for more than three decades (Kupchan and Kopperman, 1975; Takai et al., 1979; Cardarelli et al., 1997; Kumar and Müller, 1999; Haraldsdóttir et al., 2004; Russo et al., 2008; Ren et al., 2009). Yet, only a small group of lichen compounds have been tested. Usnic acid, one of the most studied lichen compounds, was found to be effective against Lewis lung carcinoma, murine P388 leukaemia (Kupchan and Kopperman, 1975; Takai et al., 1979) and many other forms of cancer. Although the mechanisms of usnic acid toxicity are still mostly unknown, Cardarelli et al. (1997) found that this compound has an anti-mitotic effect on cancer cells, which was later confirmed as well in the cells of lichen algae (Budová et al., 2006). These studies support the

* Corresponding author. Address: Institute of Biology and Ecology, Pavol Jozef Šafárik University in Košice, Moyzesova 11, 040 01 Kosice, Slovakia. Tel.: +421 55 6228310; fax: +421 55 6222124.
E-mail address: peter.fedorocko@upjs.sk (P. Fedoročko).

0887-2333/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2010.09.004

hypothesis concerning the regulatory activity of usnic acid in nature. Moreover, Bézivin et al. (2004) confirmed that usnic acid also initiates apoptosis in cancer cells. Although some other secondary metabolites of lichens have been tested for their potential anti-proliferative effects against human cancer cells (e.g. Kumar and Müller, 1999; Russo et al., 2006; Burlando et al., 2009), the available sources of data are scarce. Therefore it is difficult to make broad conclusions on the anti-proliferative effects of all lichen compounds. This is mostly due to the limited number of compounds tested (specific compounds or mixtures of compounds in water, methanol or acetone extracts that are not chemically well defined) as well as the limited number of cancer cell lines tested.

Therefore the aim of the present study is focused on the testing of the sensitivity of nine human cancer cell lines with different histological origins to the cytotoxic effects of four secondary lichen metabolites from different chemical classes. For this purpose, two different assays evaluating direct toxicity (MTT assay) and long-term survival (human tumour clonogenic assay – HTCA) were chosen. Both approaches are considered as being useful tools for the evaluation of drug sensitivity in tumour cell lines and tumour tissues (Kawada et al., 2002). Analyses of cell cycle distribution and apoptotic morphology of selected cancer cell lines after application of lichen metabolites were employed as well.

2. Materials and methods

2.1. Secondary metabolites of lichens

Four secondary lichen metabolites were selected (Fig. 1). Dibenzofuran derivative (+)-usnic acid (UA) (CAS No.: 7562-61-0; purity – 99.9%) and didepside atranorin (A) (CAS No.: 479-20-9; purity – 96%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Antraquinone parietin (P) (CAS No.: 521-61-9) was isolated from the lichen *Xanthoria parietina*. This lichen was collected from the trunks of *Juglans regia* near Zemplínsky Branč, eastern Slovakia. Tridepside gyrophoric acid (GA) (CAS No.: 548-89-0) was isolated from the lichen *Umbilicaria hirsuta*. This lichen was collected from rocks near Harmanec, central Slovakia. Lichens were identified by Dr. Martin Bačkor. Voucher specimens of each lichen species were sent to our laboratory for future reference.

Parietin and gyrophoric acid were isolated from lichens following the protocols of Asahina and Shibata (1954) and Solhaug and Gauslaa (1996). Crystals of the compounds were finally dissolved in pure acetone and recrystallized. The identity and purity of the compounds was assessed using TLC and HPLC analyses.

2.2. TLC and HPLC analyses of lichen substances

A standardized TLC method for the identification of lichen products was used. Acetone extracts of previously isolated compounds were applied in successive steps using 5 µl glass pipettes at 1 cm intervals along the short side of 10 cm × 20 cm pre-coated thin-layer plates of Merck silica gel 60 F-254. Three solvent systems: A (toluene/1,4-dioxane /acetic acid, 180:45:5), B (hexane/methyl *tert*-butyl ether/formic acid, 140:72:18) and C (toluene/acetic acid, 170:30) were used. Compounds were visualized by spraying with 10% sulfuric acid and heating for 30 min at 110 °C. Filtered acetone extracts were analyzed by gradient HPLC (Pöykkö et al., 2005), under following conditions: column Tessek SGX C18 5 µm (4 × 250 mm), flow rate 0.7 ml min^{-1}. Mobile phase: A = H_2O acetonitrile:H_3PO_4 (80:19:1) and B = 95% acetonitrile. Gradient programme: 0 min 25% B, 5 min 50% B, 20 min 100% B and 25 min 25% B. Detection was performed at a wavelength of 245 nm (detector Ecom LCD 2084). Final purity of compounds used was 98.5% (parietin) and 97% (gyrophoric acid).

2.3. Cell culture

Nine different human cancer cell lines were used for this experiment: A2780, human ovarian carcinoma; MCF-7, human breast adenocarcinoma; HT-29, human colon adenocarcinoma; HL-60, human promyelocytic leukaemia and Jurkat, human T cells lymphocyte leukaemia (all ATCC, Rockville, MD) were grown in RPMI 1640 medium with 10% fetal calf serum (FCS) (Gibco Invitrogen Corp., Carlsbad, CA, USA) and antibiotics (penicillin 100 U ml^{-1}, streptomycin 100 µg ml^{-1} and amphotericin 25 µg ml^{-1}; Invitrogen Co., Carlsbad, CA, USA). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

HeLa, human cervix adenocarcinoma; SK-BR-3, human breast adenocarcinoma (both ATCC); HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$, human colon carcinoma wild-type p53 as well as p53-null HCT-116 subline, respectively (Bunz et al., 1998); both kindly provided by Dr. Alois Kozubík, Institute of Biophysics, Brno, Czech Republic were grown in McCoy's 5A medium (Sigma–Aldrich) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin 100 U ml^{-1}, streptomycin 100 µg ml^{-1} and amphotericin

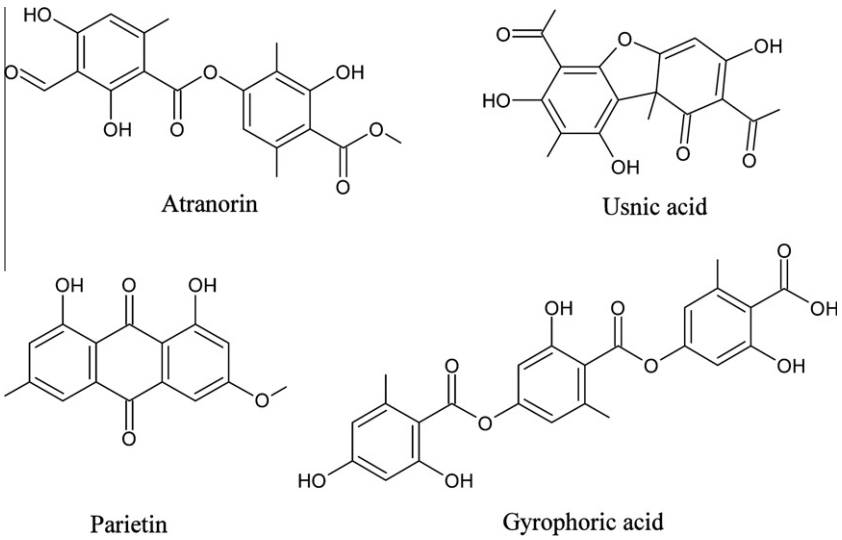

Fig. 1. Chemical structures of atranorin, usnic acid, parietin and gyrophoric acid.

25 μg ml^{-1}; Invitrogen Co.). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cell viability was analyzed by staining of cells with 0.15% eosin via light microscopy.

2.4. Cytotoxicity assays

2.4.1. MTT assay

Cancer cell lines were trypsinized (except for floating cell lines HL-60 and Jurkat) and seeded into 96-well cell culture plates (TPP, Trasadingen, Switzerland) at initial densities (cells/well) as follows: 7.5×10^3 for A2780, HL-60 and Jurkat, 1×10^4 for HeLa and MCF-7, 1.5×10^4 for SK-BR-3, 2×10^4 for HT-29, 7×10^3 for HCT-116 p53$^{+/+}$ and HCT-116 p53$^{-/-}$. After 24 h incubation, the cells were treated for 24, 48 and 72 h by lichen secondary metabolites at final concentrations of 50, 100, 150 and 200 μM. Metabolites were freshly prepared in DMSO (stock solutions of 14.5 mM) and further diluted with culture medium to obtain the desired concentrations. All tests included controls with equivalent concentrations of media DMSO corresponding to the relevant dilutions of the test metabolites. Then 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich) was added in the final concentration of 0.2 mg ml^{-1}. After 4 h incubation at 37 °C the MTT-formazan product was solubilized using 10% SDS (Sigma–Aldrich). The absorbance measurements were carried out using a universal microplate reader FluorStar Optima (BMG Labtech GmbH, Offenburg, Germany) at 584 nm. The blank value was subtracted and the results are expressed as a percentage of the dye extracted from untreated control cells ([OD value of treated cells/mean OD value of control cells] × 100%).

2.4.2. Clonogenic assay

Adherent cancer cell lines (A2780, HeLa, MCF-7, SK-BR-3, HT-29, HCT-116 p53$^{+/+}$ and HCT-116 p53$^{-/-}$) were also tested using the clonogenic assay. Cells were trypsinized and seeded into 6-well tissue culture plates at initial culture densities (cells/well) as follows: 3×10^5 for A2780, 5×10^5 for HeLa, 2×10^5 for MCF-7 and SK-BR-3, 4.5×10^5 for HT-29 and HCT-116 p53$^{-/-}$ and 3.5×10^5 for HCT-116 p53$^{+/+}$. After 24 h incubation, cells were treated by lichen secondary metabolites at final concentrations of 100 and 200 μM for 48 h. Metabolites were freshly prepared in DMSO (stock solutions of 14.5 mM) and further diluted with culture medium to obtain the desired concentrations. All test included controls with equivalent concentrations of media corresponding to the relevant dilutions of the test metabolites. After 48 h treatment by metabolites, cells were harvested by trypsinization and 500 viable cells were seeded in 6-well plates and allowed to grow in culture conditions. Ten days later, the plates were stained with methylene blue dye (0.08% w/v) and scanned. The colonies were counted using Clono-Counter software (Niyazi et al., 2007). Results are expressed as number of colonies per well.

2.4.3. Cell number, floating cell quantification and viability

For the experiment, three adherent (A2780 7.5×10^5, HCT-116 p53$^{+/+}$ 1×10^6 and HCT-116 p53$^{-/-}$ 1×10^6) and one suspension cell line (HL-60 5×10^5) were seeded in Petri dishes (TPP) and treated according to standard protocol. Later, 48 or 72 h after treatment, the adherent and floating cells were counted separately using a Coulter Counter (Model ZF, Coulter Electronics Ltd., Luton, BEDS, UK). Total cell number (cellularity) was expressed as a percentage of the untreated control. The number of floating cells was expressed as a percentage of total cell number. Adherent and floating cells (total cell viability) were analyzed by staining with 0.15% eosin using light microscopy and evaluated as the percentage of unstained (viable) cells in a total number of 100 cells.

2.4.4. Cell cycle analysis

Adherent and floating cells were harvested together 48 or 72 h after treatment, washed in cold PBS, fixed in cold 70% ethanol and kept at −20 °C overnight. Fixed cells were centrifuged, washed with PBS and stained with staining solution (20 μg ml^{-1} propidium iodide, 137 μg ml^{-1} RNAse A and 0.1% Triton X-100 in PBS). Then the samples were kept in dark conditions for 30 min and measured with the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The ModFit 3.0 (Verity Software House) software was used to quantify the number of cells in individual phases of cell cycle.

2.4.5. Fluorescence microscopy of apoptotic morphology

Total cell population was harvested by trypsinisation of adherent cells and collection of medium with floating cells, washed in PBS, fixed in 70% ethanol and stored at −20 °C overnight. Subsequently, the samples were stained in fixative solution by addition of 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; 2 μg ml^{-1}) and incubated for 30 min at room temperature. The cells were washed twice, resuspended in PBS, mounted into MOWIOL 4-88 (Sigma–Aldrich) and left to harden for at least 24 h at 4 °C. The slides were analyzed using a Nikon Eclipse 400 (Nikon Instech Co., Ltd., Kawasaki, Japan) fluorescent microscope and evaluated as the percentage of cells with fragmented nuclei from a total number of minimum 300 cells.

2.5. Statistical analysis

One-way analysis of variance and Tukey's pairwise comparisons (MINITAB Release 11, 1996) were applied to determine the significance ($P < 0.05$) of differences in all measured parameters. All data are expressed as means ± standard deviation (SD) of three independent experiments.

3. Results

3.1. MTT assay

The testing of all four secondary metabolites on the various cell lines proved a time- and concentration-dependent action as evaluated by the MTT cytotoxicity assay (Table 1). Vast differences among tested cell lines as well as different sensitivities to tested metabolites were demonstrated by calculating of the half maximal inhibitory concentrations (IC_{50}).

The effect of parietin proved to be significant in some cell lines (e.g. A2780, Jurkat or HT-29) already at 50 μM. However noticeable differences among cell lines were observed. Whereas Jurkat and A2780 cells were found to be markedly sensitive, others (e.g. HL-60, HCT-116 p53$^{+/+}$ or MCF-7) were rather resistant (Suppl. Table 1a.).

It should be noted that already at the lowest treatment level of 50 μM atranorin proved to be effective against HL-60 cells 24 h after administration (Suppl. Table 1b). Other tested cell lines were mostly resistant at this concentration, however higher atranorin doses also evoked cytotoxicity in the other seven cell lines. Only HeLa cells did not show any significant time- or concentration-dependent responses.

Usnic acid seems to be the most effective of the metabolites tested here, with already 50 μM proving to be effective against almost all cell lines (except for HeLa cells and HCT-116 p53$^{+/+}$). In addition, higher concentrations of usnic acid further increased the effects the metabolite had on the cell line responses (Suppl. Table 1c).

Gyrophoric acid seemed to be mostly ineffective at the lowest concentration. However 100 μM gyrophoric acid induced a strong

Table 1
Cytotoxic effects of parietin, atranorin, usnic acid and gyrophoric acid on nine human cancer cell lines assessed by MTT assay after 72 h exposition.

IC_{50}										
	Time (h)	A2780	HeLa	MCF-7	SK-BR-3	HT-29	HCT-116 $p53^{+/+}$	HCT-116 $p53^{-/-}$	HL-60	Jurkat
Parietin	72	139.7 ± 13.5	>200	>200	>200	>200	>200	>200	>200	129.5 ± 11.5
Atranorin	72	197.9 ± 12.0	>200	>200	>200	>200	>200	197.5 ± 3.4	93.5 ± 3.8	181.6 ± 4.4
Usnic acid	72	75.9 ± 2.0	178.3 ± 9.7	94.6 ± 7.9	199.2 ± 18.8	99.7 ± 8.4	157.2 ± 4.0	143.1 ± 11.3	48.5 ± 9.1	76.3 ± 8.2
Gyrophoric acid	72	198.3 ± 5.3	>200	>200	>200	>200	>200	>200	146.7 ± 6.0	>200

Table 2
Effect of atranorin, parietin, usnic acid and gyrophoric acid on human cancer cell lines assessed by clonogenic assay. Values in horizontal lines followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

Time	Cell line	Metabolites	0 μM	100 μM	200 μM	*F* value	*P* value
48 h	A2780	Parietin	184 ± 61.6a	160 ± 25.5a	138 ± 15.3a	1.03	0.411
		Atranorin	277 ± 63.6a	18.7 ± 4.0b	1.3 ± 0.6c	52.74	<0.001
		Usnic acid	186 ± 51.6a	23.0 ± 10.1b	0.6 ± 1.1c	32.03	0.001
		Gyrophoric acid	228 ± 45.6a	239 ± 78.0a	187 ± 69.8a	0.52	0.616
	HeLa	Parietin	191 ± 81.4a	67.3 ± 9.1b	18.3 ± 3.5c	10.56	0.011
		Atranorin	255 ± 47.6a	34.3 ± 5.5b	34.0 ± 2.6b	63.44	<0.001
		Usnic acid	254 ± 61.0a	33.7 ± 6.1b	14.0 ± 7.0c	41.94	<0.001
		Gyrophoric acid	262 ± 20.0a	328 ± 90.2a	225 ± 14.9a	2.79	0.139
	MCF-7	Parietin	76.0 ± 6.2a	60.0 ± 6.1a	30.2 ± 11.3b	23.71	0.001
		Atranorin	63.3 ± 12.2a	34.0 ± 2.6b	0.3 ± 0.6c	57.09	<0.001
		Usnic acid	68.3 ± 5.5a	21.0 ± 2.0b	5.3 ± 2.5c	238.09	<0.001
		Gyrophoric acid	72.7 ± 4.2ab	57.7 ± 5.1b	94.7 ± 23.5a	5.23	0.048
	SK-BR-3	Parietin	299 ± 49.3a	305 ± 39.5a	303 ± 42.6a	0.00	0.966
		Atranorin	235 ± 83.6a	28.3 ± 5.0b	0.3 ± 0.6c	21.06	0.002
		Usnic acid	299 ± 10.7a	11.7 ± 6.0b	3.0 ± 1.0b	1848.87	<0.001
		Gyrophoric acid	261 ± 88.5a	116 ± 11.4b	120 ± 15.1b	7.52	0.023
	HT-29	Parietin	184 ± 29.5a	182 ± 28.2a	175 ± 65.8a	0.03	0.966
		Atranorin	111 ± 39.1a	73.3 ± 10.1a	0.0 ± 0.0b	17.49	0.003
		Usnic acid	239 ± 86.9a	31.3 ± 4.0b	12.3 ± 8.5b	18.63	0.003
		Gyrophoric acid	183 ± 67.0a	132 ± 12.1a	166 ± 49.7a	0.85	0.475
	HCT-116 $p53^{+/+}$	Parietin	221 ± 32.0a	194 ± 34.7a	225 ± 12.5a	2.17	0.196
		Atranorin	182 ± 33.2a	164 ± 23.0a	0.3 ± 0.6b	55.35	<0.001
		Usnic acid	190 ± 96.6a	20.3 ± 9.7b	0.3 ± 0.6c	10.41	0.011
		Gyrophoric acid	184 ± 31.7a	180 ± 83.3a	177 ± 44.8a	0.01	<0.990
	HCT-116 $p53^{-/-}$	Parietin	201 ± 42.4a	240 ± 44.4a	180 ± 11.3a	2.85	0.135
		Atranorin	265 ± 99.3a	151 ± 17.0a	2.3 ± 1.5b	14.98	0.005
		Usnic acid	212 ± 53.2a	17.0 ± 4.5b	0.0 ± 0.0c	43.71	<0.001
		Gyrophoric acid	180 ± 32.9a	272 ± 72.1a	211 ± 27.0a	2.82	0.137

Table 3
Viability and cellularity (%) of four selected cancer cell lines treated by selected concentrations of parietin, atranorin, usnic acid and gyrophoric acid. Values in horizontal lines followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

Cell line	Time (h)	Metabolites (μM)							
		Control	Parietin		Atranorin		Usnic acid		Gyrophoric acid
			50	100	100	200	50	100	200
Viability									
A2780	48	95.1 ± 2.7a	91.7 ± 3.9a	94.4 ± 5.1a	63.4 ± 19.4ab	9.3 ± 6.2b	22.7 ± 9.5b	11.0 ± 19.0b	34.0 ± 19.7b
	72	98.2 ± 0.5a	97.0 ± 2.0a	96.7 ± 4.2a	16.2 ± 10.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	16.3 ± 16.5b
HCT-116 $53^{+/+}$	48	98.7 ± 2.3a	97.7 ± 3.2a	99.3 ± 1.2a	33.7 ± 7.0c	0.7 ± 1.2d	25.0 ± 7.5c	20.0 ± 4.0c	78.7 ± 4.2b
	72	98.7 ± 2.3a	96.0 ± 3.6a	98.3 ± 1.5a	24.3 ± 6.5c	0.0 ± 0.0d	24.3 ± 8.5c	19.0 ± 7.2c	71.3 ± 11.4b
HCT-116 $p53^{-/-}$	48	98.7 ± 1.1a	98.7 ± 2.3a	99.3 ± 1.2a	94.3 ± 8.1a	40.0 ± 3.5c	80.7 ± 6.8b	82.7 ± 7.0b	88.3 ± 4.7ab
	72	97.3 ± 2.3a	96.7 ± 3.1a	98.3 ± 2.1a	81.5 ± 6.3b	5.3 ± 5.5d	17.8 ± 3.9c	19.5 ± 3.2c	82.5 ± 4.7b
HL60	48	97.0 ± 2.0a	96.0 ± 3.5a	96.7 ± 1.5a	58.3 ± 8.7b	24.7 ± 6.1c	25.0 ± 16.0c	22.0 ± 5.3c	27.3 ± 6.0c
	72	96.7 ± 1.1a	93.3 ± 3.1a	93.3 ± 2.1a	61.7 ± 14.2b	15.7 ± 4.7c	6.3 ± 4.2c	12.3 ± 6.5c	8.4 ± 4.5c
Cellularity									
A2780	48	100 ± 0.0a	61.3 ± 11.9b	69.0 ± 19.5b	44.7 ± 8.0b	43.7 ± 10.3b	36.0 ± 5.3b	44.0 ± 10.4b	42.2 ± 3.2b
	72	100 ± 0.0a	51.7 ± 11.5b	70.0 ± 14.1b	22.3 ± 4.0c	18.1 ± 2.2c	25.0 ± 1.7c	22.7 ± 1.6c	20.7 ± 3.8c
HCT-116 $p53^{+/+}$	48	100 ± 0.0a	90.0 ± 32.0a	79.0 ± 30.8a	78.3 ± 22.2a	69.0 ± 12.7a	82.0 ± 15.1a	56.8 ± 17.2a	66.3 ± 14.2a
	72	100 ± 0.0a	104.7 ± 30.0a	83.7 ± 22.0a	76.7 ± 22.1a	67.7 ± 15.6a	71.3 ± 14.4a	57.7 ± 16.2a	64.0 ± 8.5a
HCT-116 $p53^{-/-}$	48	100 ± 0.0a	94.3 ± 16.2a	70.3 ± 20.6ab	53.0 ± 3.6b	49.7 ± 6.4b	55.0 ± 19.3b	40.0 ± 7.5b	70.3 ± 12.9ab
	72	100 ± 0.0a	98.3 ± 7.4a	69.3 ± 8.0b	58.0 ± 6.1b	55.7 ± 11.7b	51.7 ± 9.5bc	33.7 ± 5.5c	72.7 ± 4.2b
HL-60	48	100 ± 0.0ab	125.0 ± 19.5a	86.3 ± 10.5ab	12.8 ± 7.4c	23.5 ± 11.8c	6.2 ± 2.2c	3.9 ± 2.6c	6.1 ± 4.8c
	72	100 ± 0.0a	87.7 ± 1.5b	92.0 ± 4.6ab	7.3 ± 5.8c	13.6 ± 2.2c	5.1 ± 5.9c	5.8 ± 5.0c	4.6 ± 3.8c

effect in HL-60 cells as quickly as only 24 h after incubation (Suppl. Table 1d). These effects are very similar to the ones evoked by equimolar usnic acid in the same cells. Gyrophoric acid was mainly observed to be effective against A2780, HL-60 and Jurkat cells.

Summing up the results of MTT assay we can conclude that each of the tested compounds demonstrated some anti-cancer action, however the usnic acid proved to be the most effective when considering the action of metabolites against the whole spectrum of cell lines. Even the most resistant ones (SK-Br-3 and HT-29) showed IC_{50} lower than 200 μM 72 h after usnic acid administration.

Scoring the sensitivity of cell lines to all four tested compounds we can create a "gross sensitivity list" of cell lines going from the most resistant to the most sensitive as follows: SK-Br-3 > HeLa > HCT-116 $p53^{-/-}$ > HCT-116 $p53^{+/+}$ > HT-29 > MCF-7 > A2780 > Jurkat ≫ HL-60. As the HL-60 cells were the most sensitive ones and the A2780 cells were again the most sensitive among adherent cell lines, those were chosen for further analysis. To consider the role of protein p53, the adherent colon adenocarcinoma cells HCT-116 $p53^{-/-}$ and HCT-116 $p53^{+/+}$ were chosen and tested as well.

3.2. Clonogenic assay

The tendency of cells to create colonies demonstrates their ability for clonal expansion (Table 2). Since only a small number of viable cells are seeded, virtually every viable cell may generate a colony. In reality, however, the number of created colonies is smaller even in untreated control groups. Still, equal numbers of viable cells are seeded in the controls as well as in the treated groups, therefore variations in clonogenic ability reflect the expansion rate and invasiveness of the surviving fraction. Based on MTT results, higher, 100 and 200 μM concentrations of lichen metabolites were chosen for this study.

Similar to the results of MTT assay, both atranorin and usnic acid were the most effective metabolites and proved to significantly inhibit clonogenic ability of all tested tumour cells (Table 2). The two metabolites even induced total eradication in some tests. On the other hand, parietin and gyrophoric acid were ineffective against colon adenocarcinomas (HT-29, HCT-116s) and ovarian carcinoma (A2780) but still demonstrated some action against cervix and breast tumour cells (HeLa, MCF-7 – parietin; SK-BR-3 – gyrophoric acid).

3.3. Cell number, floating cell quantification and viability assay

For further establishment of cytokinetical parameters four cell lines were chosen in order to include various histological origins and to test the involvement of p53 function (HCT-116 $p53^{-/-}$ *versus* HCT-116 $p53^{+/+}$). It should also be noted that only particular concentrations of tested metabolites were selected based on the results of the MTT test for A2780 and HL-60 cell lines assessed in screening tests at start of experiments.

Here again, the vast differences in the anti-tumour efficiency of tested metabolites were found to be obvious. Whereas already at 50 μM usnic acid totally damaged the A2780 cells, evoked their massive detachment and also seriously affected the HL-60 cell line (Table 3; Fig. 2). The action of 100 μM parietin was rather weak (Table 3). Although it did affect cell proliferation in some cases, the impact on cell viability and percentage of floating cells (Fig. 2) was insignificant in all four tested cell lines. Therefore, the action of parietin may be regarded as cytostatic. Contrariwise, atranorin and gyrophoric acid were found to be highly effective and cytotoxic at the given concentrations, but not to the extent as usnic acid, especially when considering the metabolite concentrations used. Testing the impact of the p53 protein functionality, the comparison of HCT-116 $p53^{+/+}$ and HCT-116 $p53^{-/-}$ cell lines proved to be valuable. Whereas cellularity seems to be more suppressed in p53-deficient cells, the viability (Table 3) and the percentage of floating cells (Fig. 2) reveal the tendency for better survival (atranorin, gyrophoric acid) or at least a delayed onset of cell death (usnic acid).

3.4. Cell cycle analysis

In an effort to get a greater picture on cell population dynamics, cell cycle distribution was studied in addition to viability, cellular-

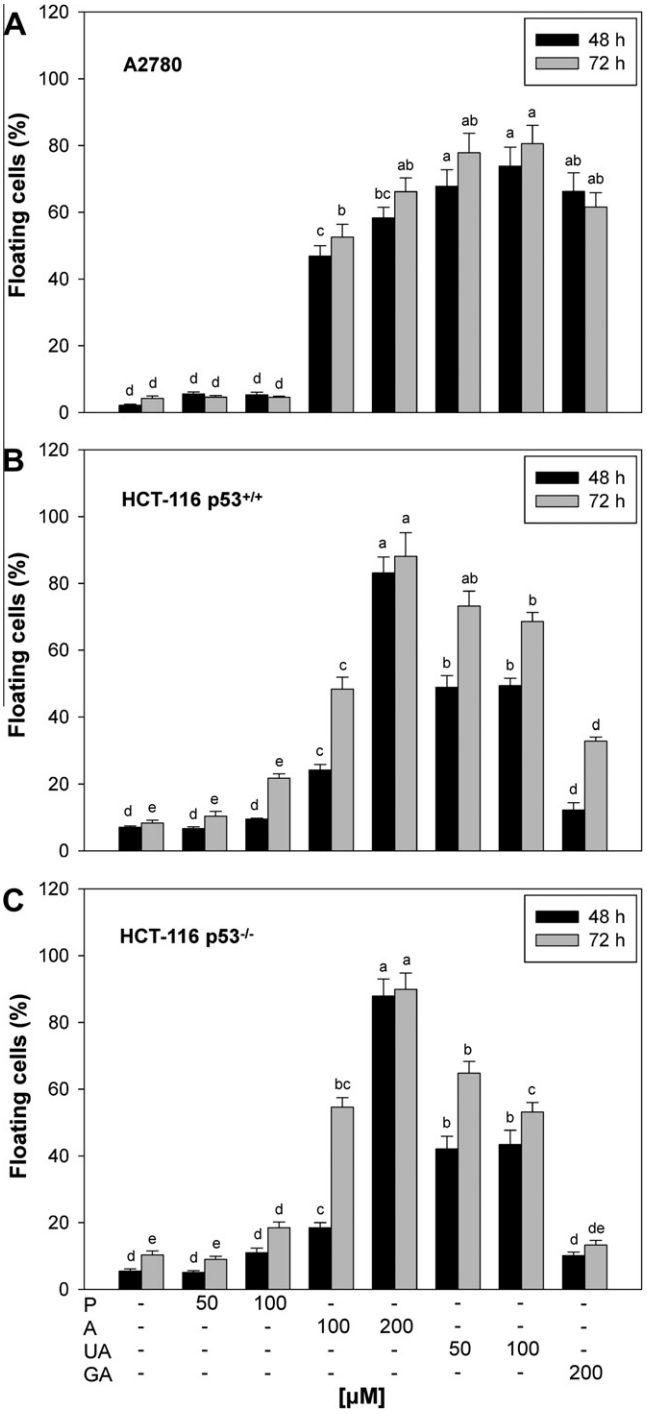

Fig. 2. Effect of parietin (50 and 100 μM), atranorin (100 and 200 μM), usnic acid (50 and 100 μM) and gyrophoric acid (200 μM) on floating cells percentage of selected cancer cell lines after 48 and 78 h. Values in vertical columns followed by the same letter(s) do not differ significantly at $P < 0.05$ by Tukey's test.

Table 4
Cell cycle analysis of four selected cancer cell lines treated by parietin, atranorin, usnic acid and gyrophoric acid. Values in horizontal lines followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

Cell line	Cell cycle	Time (h)	Metabolites (μM) Control	Parietin		Atranorin		Usnic acid		Gyrophoric acid
				50	100	100	200	50	100	200
A2780	G0/G1	48	56.7 ± 2.0a	58.1 ± 1.6a	57.9 ± 0.9a	53.1 ± 5.6a	24.9 ± 4.3b	57.3 ± 8.2a	50.9 ± 3.6a	53.1 ± 5.2a
		72	66.1 ± 2.2a	65.9 ± 3.6a	66.2 ± 2.9a	53.3 ± 6.5ab	48.7 ± 7.8b	57.5 ± 5.7ab	23.1 ± 6.1c	20.7 ± 7.8c
	S	48	29.5 ± 1.1 cd	28.0 ± 0.8d	28.3 ± 0.9d	27.4 ± 2.8d	64.6 ± 3.7a	41.4 ± 3.4b	34.1 ± 4.3bc	36.7 ± 3.3bc
		72	24.7 ± 1.4e	24.2 ± 3.1de	23.5 ± 2.7e	38.8 ± 7.9 cd	56.7 ± 5.2b	41.5 ± 4.5c	75.2 ± 4.5a	77.3 ± 7.0a
	G2/M	48	13.8 ± 0.8a	13.9 ± 1.4a	13.8 ± 0.5a	19.5 ± 3.9a	7.1 ± 6.2a	4.7 ± 4.8a	15.0 ± 3.6a	6.9 ± 1.6a
		72	9.3 ± 1.0a	10.0 ± 1.3a	10.3 ± 0.6a	7.2 ± 2.6ab	4.6 ± 4.0bc	1.0 ± 1.6c	1.7 ± 1.7c	0.4 ± 0.7c
HCT-116 $p53^{+/+}$	G0/G1	48	76.1 ± 4.0a	78.1 ± 4.0a	79.4 ± 4.5a	80.1 ± 4.7a	47.3 ± 1.0b	46.3 ± 5.9b	55.8 ± 7.1b	74.5 ± 7.9a
		72	81.9 ± 7.1a	81.4 ± 7.2a	81.1 ± 6.8a	79.4 ± 7.5a	42.3 ± 6.6b	49.3 ± 4.1b	52.9 ± 6.6b	73.2 ± 2.7a
	S	48	15.0 ± 4.6c	12.6 ± 3.5c	12.7 ± 4.0c	9.8 ± 2.2c	53.1 ± 7.3a	31.7 ± 0.5b	30.9 ± 3.6b	13.6 ± 2.4c
		72	9.7 ± 3.1b	9.6 ± 3.1b	10.1 ± 3.9b	11.9 ± 5.6b	37.3 ± 7.3a	30.2 ± 1.3a	33.9 ± 6.4a	17.9 ± 3.2b
	G2/M	48	14.6 ± 4.8a	9.5 ± 0.7a	14.7 ± 7.5a	15.1 ± 6.4a	18.0 ± 3.8a	20.6 ± 1.5a	1.7 ± 2.4b	8.9 ± 0.1a
		72	9.4 ± 5.3a	9.6 ± 5.7a	9.7 ± 3.6a	10.1 ± 0.8a	17.0 ± 5.4a	20.8 ± 5.3a	13.5 ± 0.3a	8.3 ± 0.4a
HCT-116 $p53^{-/-}$	G0/G1	48	67.5 ± 5.7ab	74.2 ± 4.3a	68.4 ± 6.3a	69.4 ± 6.0a	41.9 ± 2.7c	43.0 ± 6.9c	48.8 ± 3.4bc	64.8 ± 7.1a
		72	75.4 ± 3.1a	81.2 ± 1.6a	74.7 ± 4.1a	68.8 ± 7.0a	48.1 ± 3.9b	54.1 ± 5.7b	49.9 ± 1.2b	70.2 ± 3.9a
	S	48	16.6 ± 2.6c	11.8 ± 2.3c	19.8 ± 7.5c	15.7 ± 3.2c	33.0 ± 4.5ab	37.4 ± 7.9a	34.6 ± 2.7ab	20.7 ± 6.2bc
		72	19.4 ± 6.0b	11.0 ± 3.6b	13.5 ± 2.3b	13.5 ± 3.2b	39.9 ± 2.5a	40.8 ± 4.9a	34.0 ± 3.3a	18.9 ± 3.3b
	G2/M	48	16.2 ± 4.5a	14.5 ± 5.8a	16.5 ± 5.6a	15.8 ± 3.3a	24.3 ± 1.8a	23.9 ± 6.3a	17.9 ± 2.1a	14.8 ± 1.2a
		72	14.7 ± 4.0b	12.5 ± 4.1b	17.6 ± 6.6b	18.9 ± 0.4b	12.1 ± 3.0b	20.2 ± 7.1ab	21.9 ± 6.1a	10.7 ± 0.8b
HL-60	G0/G1	48	51.6 ± 2.2a	51.9 ± 3.7a	49.6 ± 1.3a	34.3 ± 4.7b	33.6 ± 4.2b	44.5 ± 4.4a	45.1 ± 6.0a	45.2 ± 1.8a
		72	63.9 ± 3.4a	68.8 ± 4.0a	62.5 ± 1.0a	29.0 ± 0.4b	22.7 ± 2.9b	56.6 ± 6.6a	58.2 ± 4.9a	56.3 ± 5.5a
	S	48	36.9 ± 1.9b	37.3 ± 1.3b	35.0 ± 4.1b	43.2 ± 4.2b	54.4 ± 4.6a	41.7 ± 4.0b	38.7 ± 3.0b	44.2 ± 3.5b
		72	29.9 ± 1.8c	25.9 ± 3.2c	30.1 ± 1.0c	57.0 ± 5.3a	68.3 ± 3.0a	36.9 ± 1.7bc	38.2 ± 2.5b	41.0 ± 1.1b
	G2/M	48	10.6 ± 0.1b	8.7 ± 1.8b	11.0 ± 2.3b	21.2 ± 0.4a	10.9 ± 1.3b	11.8 ± 0.6b	14.5 ± 2.7ab	9.6 ± 1.3b
		72	7.6 ± 1.4a	5.1 ± 1.0a	7.0 ± 0.1a	6.0 ± 1.8a	9.3 ± 0.8a	0.0 ± 0.1b	3.5 ± 3.0a	4.7 ± 4.4a

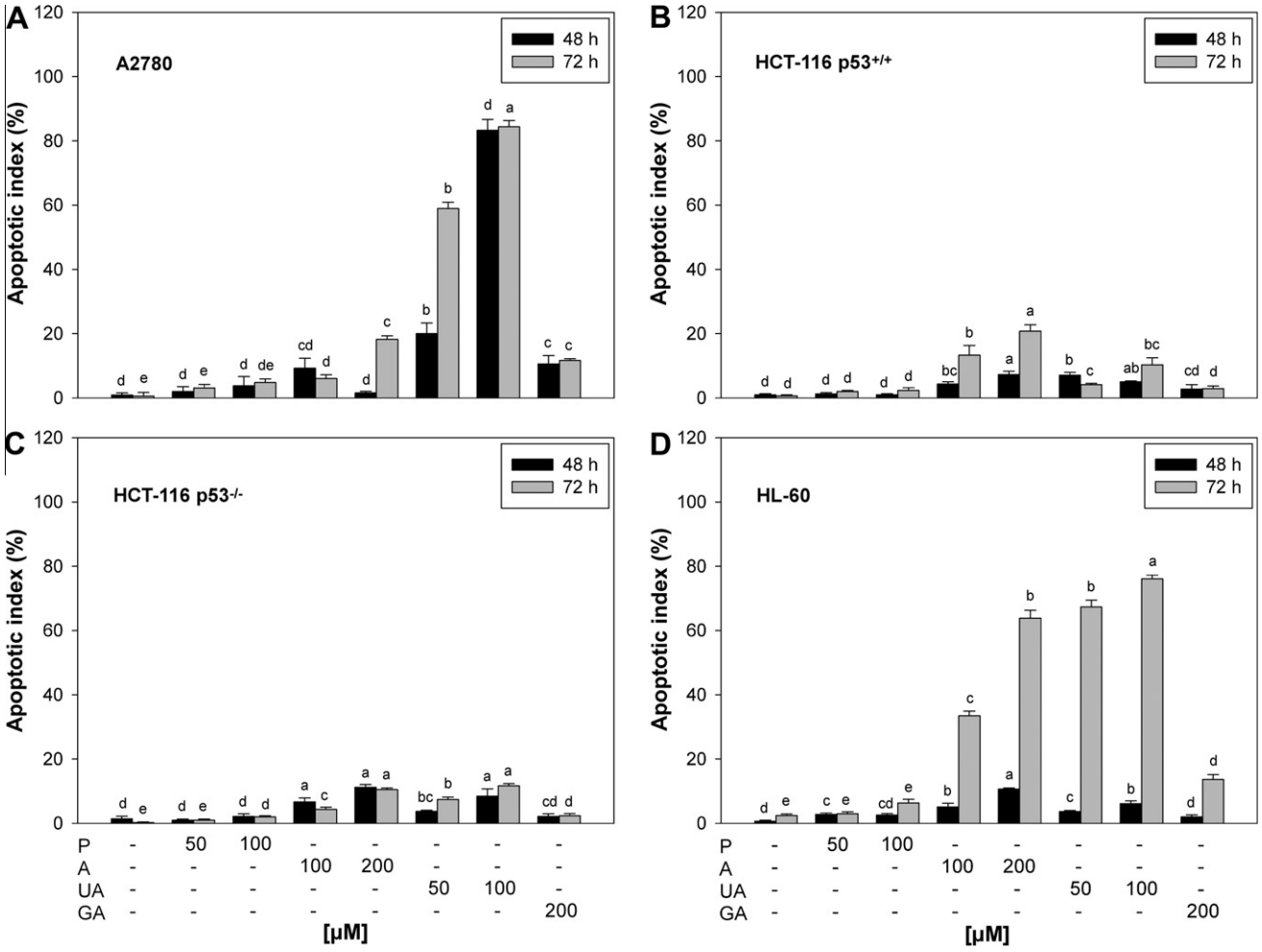

Fig. 3. Effect of parietin (50 and 100 μM), atranorin (100 and 200 μM), usnic acid (50 and 100 μM) and gyrophoric acid (200 μM) on apoptotic index of selected cancer cell lines after 48 and 78 h. Values in vertical columns followed by the same letter(s) do not differ significantly at $P < 0.05$ by Tukey's test.

ity (Table 3) and floating cells percentage (Fig. 2). The results of analyses accomplished 72 h after drug administration are presented (Table 4). In accord with previous conclusions, the presence of parietin at selected concentrations did not alter cell cycle distribution in any of tested cancer cell lines. This is in contrast with the other metabolites. Atranorin, usnic acid as well as gyrophoric acid achieved common effects as represented by the accumulation of cells in S-phase at the expense of the G_1/G_0-phase. However, the efficiency and spectrum of affected cell lines were observed to be different. Whereas atranorin did induce changes in all tested cell lines at 200 μM, it was effective only in HL-60 cells at the lower concentrations at 72 h. Usnic acid demonstrated its activity at both tested concentrations in adherent cell lines but not in the suspension cell lines. Gyrophoric acid proved to be effective only in the case of the A2780 cell line exposed for 72 h. In summary, we can conclude that the action of the tested metabolites might be linked to the modulation of cell cycle transition.

3.5. Analysis of nuclear morphology by fluorescence microscopy

Analysis of nuclear morphology was accomplished 48–72 h after drug administration by staining with DAPI (Fig. 3). In this analysis the cells with typically condensed fragmented nuclei were considered as being apoptotic.

The results clearly demonstrate the pro-apoptotic action of usnic acid, which we found to be a successful inductor of apoptosis in all four tested cell lines by calculating the half maximal inhibitory concentrations. Atranorin seems to also be an effective pro-apoptotic agent however not to the extent of usnic acid. In A2780 cells, however, the level of apoptosis was relatively low and in contrast with other analysis. This result may indicate occurrence of necrosis. The ability of parietin to induce apoptosis was rather limited, since the only significant results were found in HCT-116 $p53^{-/-}$ cells sampled after 72 h incubation at 100 μM concentration (Fig. 3). The influence of gyrophoric acid on the apoptotic index was also less pronounced when compared to atranorin and usnic acid. We only found a slightly significant increase of apoptotic index in A2780 (Fig. 3), HCT-116 $p53^{-/-}$ and HL-60 cells due to gyrophoric acid exposure (Fig. 3). In considering the p53-dependent action, only atranorin demonstrated conclusive differences, showing a significantly lower incidence in p53-deficient cells.

4. Discussion

There are some studies such as Haraldsdóttir et al. (2004) that demonstrated variable anti-proliferative effects of chemically different classes of lichen metabolites on a larger number of cancer cell lines. That study tested the secondary metabolites protolichesterinic, lobaric and baeomycesic acid against 12 different human cancer cell lines. Usnic acid, a well known and arguably the most tested secondary compound of lichens was found to be a very effective anti-cancer agent against a wide range of various cell lines originating from different tissues. For example Cardarelli et al. (1997) found an inhibiting capacity (concentration- and time-dependent) of usnic acid when tested on K-562, Ishikawa and HEC-50 cells. However usnic acid action was also confirmed in the present study.

Nevertheless, this represents only a small fraction of what nature has to offer. Considering the wide range of biological functions, the extensive family of lichen secondary compounds and broad spectrum of their chemical structures, lichen secondary metabolites represent a potentially rich source of biologically active compounds. These secondary metabolites deserve more detailed study using various cell lines to screen for the potential anti-cancer properties and pharmaceutical potential of the lichen compounds. To further knowledge on the potential anti-cancer action of these compounds, we present this study comparing the cytotoxicity of atranorin, parietin, usnic and gyrophoric acid on up to nine various cancer cell lines. To our knowledge as of time of publication there has yet to have been a study comparing these four compounds together.

Although the effect of all four tested compounds was found to be concentration- and time-dependent, it is also obvious that the cellular attributes of each particular cancer cell line participate in the overall outcome. To summarize our results, however, we can conclude in general that usnic acid and atranorin inhibit cell proliferation more efficiently and induce cell death to a markedly larger extent when comparing with parietin or gyrophoric acid. Similarly, it was demonstrated that gyrophoric acid was relatively less toxic to usnic acid when tested on MM98, A431 and HaCaT cells (Burlando et al., 2009). Based on MTT results, 100 and 200 μM concentrations of usnic acid were found to have significant anti-tumour and anti-bacterial activity (Dias and Urban, 2009). Likewise, our data demonstrate the prevailing efficacy of usnic acid in comparison to atranorin.

Since the majority of human tumours bear some malfunction of the p53 signalling pathway, this attribute of cancer cells was also covered by our study. The p53-independent action of usnic acid has been previously demonstrated once (Mayer et al., 2005); however various cell lines with different p53-status were used. To minimize the effect of other variables, the derived p53-deficient cell line HCT-116 was compared to its wild-type p53 bearing progenitor in our study where usnic acid proved to induce apoptosis independently of p53. The onset of cell death was still found to be delayed in p53-deficient cells (viability assay). Interestingly, in the cells exposed to 100 μM atranorin, higher viability and a lower contrariwise apoptotic index was found. Moreover, gyrophoric acid stimulated the clonogenic potential of the cells. Therefore we may perhaps conclude that the function of p53 protein affects the toxicity of atranorin and gyrophoric acid.

The exact molecular mechanisms of lichen secondary metabolite action are almost entirely unknown. Yet even if secondary metabolites are known to differ in cytotoxicity, when tested in various mammalian cancer cells, they have not been found to possess mutagenic properties (Zeytinoglu et al., 2008). A study focused on the anti-proliferative and pro-apoptotic mechanisms of acetone and methanolic extracts from the lichen *Lethariella zahlbruckneri* (Ren et al., 2009) proved that even if both kinds of extracts decreased viability of HT-29 cells in a dose- and time-dependent manner, the acetone extracts showed stronger cytotoxicity. The consequent apoptosis induced by the acetone extracts was associated with the activation of initiator caspases-8 and -9 as well as the effector caspase-3. Other well known lichen secondary compounds such as sphaerophorin and pannarin have been shown to inhibit the growth of melanoma cells (Russo et al., 2006, 2008). The induction of apoptosis in this case was demonstrated by fragmentation of the genomic DNA using COMET or TUNEL assays and by significant activation of caspase-3. However these results demonstrate the onset of apoptosis only. It seems that the mechanisms of cytotoxicity induced by these compounds are rather complex as they have also been found, for example, to inhibit 5- and 12-lipoxygenases (LOXs) (Haraldsdóttir et al., 2004). Since little is known about events up-stream of the apoptotic cascade or regulation of cell cycle, future study is required.

In summary, we proved a highly variable anti-proliferative and/or pro-apoptotic potential of the four presented lichen secondary compounds using analyses of nuclear morphology supported with percentage of floating cells, viability and proliferation. Moreover we demonstrated significant variations in cell cycle distribution with a tendency to accumulate in S-phase. In addition we presented the results of clonogenic assay which demonstrated the di-

verse ability of tumour cells to create colonies when exposed to various secondary compounds and concentrations. Atranorin and usnic acid proved to induce apoptosis and inhibit cell proliferation in all tested cell lines in a more efficient mode of action than the other lichen compounds. The action of parietin and gyrophoric acid was less pronounced at lower concentrations after 72 h and differed in dependence on the tested cell line. The differences in action against various cell lines indicate that some of their action might be invoked by target specific mechanisms.

Acknowledgments

This work was financially supported by the Slovak Research and Development Agency under the contract No. VVCE-0001-07 and by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic under contract VEGA 1/4337/07. The authors thank Kenneth Dvorsky (Ontario, Canada) and Ján Kovaľ for comments on the manuscript and Viera Balážová for assistance with technical procedures.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tiv.2010.09.004.

References

Ahmadjian, V., 1993. The Lichen Symbiosis. John Wiley and Sons, Inc., New York.
Asahina, Y., Shibata, S., 1954. Chemistry of Lichen Substances. Japan Society for the Promotion of Science, Tokyo. 240pp.
Bézivin, C., Tomasi, S., Rouaud, I., Delcros, J.G., Boustie, J., 2004. Cytotoxic activity of compounds from the lichen: *Cladonia convoluta*. Planta Med. 70, 874–877.
Buďová, J., Bačkor, M., Bačkorová, M., Židzik, J., 2006. Usnic acid and copper toxicity in aposymbiotically grown lichen photobiont *Trebouxia erici*. Symbiosis 42, 169–174.
Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J.P., Sedivy, J.M., Kinzler, K.W., Vogelstein, B., 1998. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 282, 1497–1501.
Burlando, B., Ranzato, E., Volante, A., Appendino, G., Pollastro, F., Verotta, L., 2009. Antiproliferative effects on tumour cells and promotion of keratinocyte wound healing by different lichen compounds. Planta Med. 75, 607–613.
Cardarelli, M., Serino, G., Campanella, L., Ercole, P., De Cicco Nardone, F., Alesiani, O., Rossiello, F., 1997. Antimitotic effects of usnic acid on different biological systems. CMLS 53, 667–672.
Dias, D.A., Urban, S., 2009. Phytochemical investigation of the Australian lichens *Ramalina glaucescens* and *Xanthoria parietina*. Nat. Prod. Commun. 4, 959–964.
Fashelt, D., 1994. Secondary biochemistry of lichens. Symbiosis 16, 117–165.
Haraldsdóttir, S., Gudlaugsdóttir, E., Ingolfsdóttir, K., Ogmundsdóttir, H.M., 2004. Anti-proliferative effects of lichen-derived lipoxygenase inhibitors on twelve human cancer cell lines of different tissue origin in vitro. Planta Med. 70, 1098–1100.
Hauck, M., Willenbruch, K., Leuschner, C., 2009. Lichen substances prevent lichens from nutritient deficiency. J. Chem. Ecol. 35, 71–73.
Kawada, K., Yonei, T., Ueoka, H., Kiura, K., Tabata, M., Takigawa, N., Harada, M., Tanimoto, M., 2002. Comparison of chemosensitivity tests: clonogenic assay versus MTT assay. Acta Med. Okayama 56, 129–134.
Kumar, S., Müller, K., 1999. Lichen metabolites. 2. Antiproliferative and cytotoxic activity of gyrophoric, usnic, and diffractaic acid on human keratinocyte growth. J. Nat. Prod. 62, 821–823.
Kupchan, S.M., Kopperman, H.L., 1975. L – usnic acid: tumor inhibitor isolated from lichens. Experientia 31, 625.
Latkowska, E., Lechowski, Z., Bialczyk, J., Pilarski, J., 2006. Photosynthesis and water relations in tomato plants cultivated long-term in media containing (+)-usnic acid. J. Chem. Ecol. 32, 2053–2066.
Lawrey, J.D., 1986. Biological role of lichen substances. Bryologist 89, 111–122.
Mayer, M., O' Neill, M.A., Murray, K.E., Santos-Magalhaes, N.S., Carneiro-Leao, A.M., Thompson, A.M., Appleyard, V.C.L., 2005. Usnic acid: a non-genotoxic compound with anti-cancer properties. Anti-Cancer Drugs 16, 805–809.
Niyazi, M., Niyazi, I., Belka, C., 2007. Counting colonies of clonogenic assays by using densitometric software. Radiation Oncology 2, 4.
Pöykkö, H., Hyvärinen, M., Bačkor, M., 2005. Removal of lichen secondary metabolites affects food choice and survival of lichenivorous moth larvae. Ecology 86, 2623–2632.
Ren, M.R., Hur, J.S., Kim, J.Y., Park, K.W., Park, S.C., Seong, C.N., Jeong, I.Y., Byun, M.W., Lee, M.K., Seo, K.I., 2009. Anti-proliferative effects of *Lethariella zahlbruckneri* extracts in human HT-29 human colon cancer cells. Food Chem. Toxicol. 47, 2157–2162.
Russo, A., Piovano, M., Lombardo, L., Vanella, L., Cardile, V., Garbarino, J., 2006. Pannarin inhibits cell growth and induces cell death in human prostate carcinoma DU-145 cells. Anti-Cancer Drugs 17, 1163–1169.
Russo, A., Piovano, M., Lombardo, L., Garbarino, J., Cardile, V., 2008. Lichen metabolites prevent UV light and nitric oxide-mediated plasmid DNA damage and induce apoptosis in human melanoma cells. Life Sci. 83, 468–474.
Solhaug, K.A., Gauslaa, Y., 1996. Parietin, a photoprotective secondary product of the lichen *Xanthoria parietina*. Oecologia 108, 412–418.
Solhaug, K.A., Lind, M., Nybakken, L., Gauslaa, Y., 2009. Possible functional roles of cortical depsides and medullary depsidones in the foliose lichen *Hypogymnia physodes*. Flora 204, 40–48.
Takai, M., Uehara, Y., Beisler, J.A., 1979. Usnic acid derivatives as potential antineoplastic agents. J. Med. Chem. 22, 1380–1384.
Yuan, X., Xiao, S., Taylor, T.N., 2005. Lichen-like symbiosis 600 million years ago. Science 308, 1017–1020.
Zeytinoglu, H., Incesu, Z., Tuylu, B.A., Turk, A.O., Barutca, B., 2008. Determination of genotoxic, antigenotoxic and cytotoxic potential of the extract from lichen *Cetraria aculeata* (Schreb.) Fr. in vitro. Phytother. Res. 22, 118–123.