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Usnic acid and atranorin exert selective cytostatic and anti-invasive effects on human prostate and melanoma cancer cells

Agnieszka Galanty [a,*], Paulina Koczurkiewicz [b,c], Dawid Wnuk [c], Milena Paw [c], Elżbieta Karnas [c], Irma Podolak [a], Michał Węgrzyn [d], Magdalena Borusiewicz [a,c], Zbigniew Madeja [c], Jarosław Czyż [c], Marta Michalik [c]

[a] Department of Pharmacognosy, Pharmaceutical Faculty, Medical College, Jagiellonian University, Medyczna 9, 30-688 Cracow, Poland
[b] Department of Pharmaceutical Biochemistry, Pharmaceutical Faculty, Medical College, Jagiellonian University, Medyczna 9, 30-688 Cracow, Poland
[c] Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Cracow, Poland
[d] Prof. Z. Czeppe Department of Polar Research and Documentation, Institute of Botany, Jagiellonian University, Kopernika 27, 31-501 Cracow, Poland

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ABSTRACT

Objectives and methods: Lichens are an interesting source of potential anti-tumor compounds, among which usnic acid and atranorin seem to be the most promising, but their impact on invasive potential of tumor cells has not yet been comprehensively addressed. The aim of the study was focused on the impact of the two lichen metabolites, on the viability (by Trypan blue test and fluoresceine diacetate and ethidium bromide assay), proliferation (cell counting in a Bürker's chamber), apoptosis (flow cytometry analysis and Western blot) and motile activity (cell movement recording and image analysis) and actin cytoskeleton organization (immunofluorescent staining) of melanoma HTB-140, prostate cancers DU-145 and PC-3, normal human skin fibroblasts and prostate epithelial PNT2 cells, with special emphasis to their selectivity and versatility.
Results: Both compounds exerted strong inhibitory effects on cancer cell proliferation, migration and actin cytoskeleton organization, while their effect on apoptosis process was less relevant. The impact of usnic acid on the examined cancer cells was found more efficient in comparison to atranorin. Also, selective effect of both agents on tumor cells was observed.
Significance: The ability of usnic acid and atranorin to inhibit cancer cells motility may have future implications for development of new therapeutic strategies targeted at the interference with the metastatic cascade.

1. Introduction

Cancer is the most severe health problem worldwide, with its invasive forms being the leading cause of death in world. Nowadays, it accounts for nearly 15% of all deaths recorded annually (WHO Statistics, 2013) and a continuous increase of cancer incidence is observed worldwide (Nguyen et al., 2014). One of the most frequently diagnosed human malignancies is prostate cancer which due to the predilection for metastasis to the bone, brain and lymph nodes, is a dominant cause of cancer-related deaths of elderly men (Lewińska et al., 2015). Also, the incidence of melanoma is increasing worldwide, with a growing fraction of patients with advanced drug-resistant tumors (Garbe et al., 2011). Although significant progress in oncology has been made, no effective therapy has been elaborated that would guarantee therapeutic success in the treatment of melanoma and prostate cancer.

Despite the enormous diversity of tumor types, most tumor cells share some common features. Hanahan and Weiberg (2000); Hanahan and Weinberg (2011) suggested that all cancers have acquired the same set of functional capabilities during their growth and development, although they can undertake different strategies of promotion and progression. Generally, tumor cells are insensitive to anti-growth signals, can efficiently avoid apoptosis and have the ability to invade surrounding tissues and metastasize (Hanahan and Weiberg, 2000; Hanahan and Weinberg, 2011). Phenotypic heterogeneity of tumor cells underlies their resistance to currently used, relatively non-selective chemotherapeutics and account for tumor relapses and treatment failures. Accordingly, chemotherapeutics affect only certain cells within the tumor while the growth of chemo-resistant cells remains unhindered by these agents. Thus, elaboration of new potential anticancer agents that would multidirectionally but selectively target tumor cells is necessary. This priority goal could be pursued by isolating novel, efficient compounds from natural resources. They should be characterized by comprehensive but selective activity, potentially leading to the inhibition of tumor progression at its terminal stages.

Lichens are an exceptionally interesting source of potential anti-tumor compounds. A number of bio-active substances are produced by lichens as a result of the symbiosis between fungi and algae or cyanobacteria. Lichen–specific secondary metabolites are a unique class of compounds, which are synthesized via the polyketide pathway

* Corresponding author.
E-mail address: mfgalant@cyf-kr.edu.pl (A. Galanty).

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and are reported to possess antibacterial, antifungal, antiviral, anti-inflammatory and analgesic activity (Molnár and Farkas, 2010). Accordingly, cytotoxic (Bézivin et al., 2004; Koparal et al., 2006; Bazin et al., 2008; Einarsdottir et al., 2010; Correche et al., 2004; Burlando et al., 2009; Bačkorová et al., 2011; Kristmundsdottir et al., 2005) and antiproliferative effects (Kumar and Müller, 1999; Burlando et al., 2009; Einarsdottir et al., 2010) of lichen compounds have been widely studied. Whereas usnic acid and atranorin seem to be the most promising agents belonging to this group, relatively few data describe proapoptotic properties of these metabolites. The obtained results are often contradictory (Correche et al., 2004; Bačkorová et al., 2011; Russo et al., 2012; Kosanić et al., 2014), while their impact on invasive potential of tumor cells has not yet been comprehensively addressed (Nguyen et al., 2014; Yang et al., 2016).

In the present study, we investigated the influence of usnic acid and atranorin on melanoma HTB-140 and prostate cancer DU-145 and PC-3 (derived from prostate cancer metastasis to brain and bone, respectively) cell properties. To evaluate the potential selectivity of these compounds, their effects on normal human skin fibroblasts HSFs and prostate epithelial PNT2 cells were also studied. Limited data referring to the cellular mechanism of usnic acid and atranorin urged us to focus on their combined impact on cell viability, proliferation, apoptosis and motile activity, with special emphasis to their selectivity and versatility.

2. Materials and methods

2.1. Secondary lichen metabolites

Two secondary lichen metabolites were analyzed in this study, namely dibenzofuran derivative: usnic acid (UA) and β-orcinol derivative: didepside atranorin (ATR). Atranorin (CAS no: 479-20-9) was purchased from Chromadex. Usnic acid (CAS no 7562-61-0) was isolated from *Cladonia arbuscula* (Wallr.). Ruoss subsp. *squarrosa*, collected in northern Poland in the dry, non-coastal European scots pine forests in July 2008. The lichen species was identified by one of the authors (MW). Voucher specimen has been deposited in the herbarium of Department of Pharmacognosy, Jagiellonian University (Ref. No. KFg/2008/L1). Isolation of usnic acid was performed according to the protocol by Bézivin et al. (2004). Briefly, lichen thalli were extracted consecutively with hexane and chloroform in a Soxhlet apparatus. Crystals of the compound, which had been obtained during extract cooling, were purified by recrystallization. The identity of the compound was assessed by measurement of melting point and by TLC and HPLC analyses, by comparing Rf and Rt values with those of standard usnic acid (Sigma-Aldrich).

2.2. Identification of usnic acid

A standardized TLC method was used for the identification of usnic acid. Briefly, the isolated compound, dissolved in chloroform was applied to TLC silica gel plates (Merck) with a glass pipette and the plates were developed in three solvent systems: A (toluene – 1, 4-dioxane – acetic acid 180:45:5), B (toluene – acetic acid 170:30) and C (toluene – ethylacetate – formic acid 139:83:8) (Orange et al., 2001). Usnic acid was visualized by spraying with 25% sulfuric acid and heating at 110 °C. Chloroform solution of usnic acid was also analyzed by HPLC under following conditions: Dionex HPLC system (PDA detector, Hypersil Gold C-18 column (250 × 4.6 mm), mobile phase consisting of methanol-water-phosphoric acid 80:20:0.9, flow rate: 1.0 mL/min, detection: 240 nm, according to Manojlović et al. (2010).

2.3. Cell culture and experimental conditions

Experiments were performed on three cancer cell lines obtained from American Type Culture Collection: HTB-140 (Hs 294T)- human melanoma derived from metastasis to lymph node; DU 145 (HTB-81)– human carcinoma derived from brain metastasis and PC-3 (CRL-1435) - human adenocarcinoma derived from bone metastasis) and two normal types of cells: human skin fibroblasts (HSF) and normal human prostate epithelium (PNT2). The cells were cultured in Dulbecco's Modified Eagle's Medium F12 HAM (DU-145, PC-3), RPMI-1640 Medium (PNT2), Dulbecco's Modified Eagle's Medium with low (1000 mg/L) and high (4500 mg/L) glucose (HSFs and HTB-140, respectively). The media were supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 10 μg/mL streptomycin. The cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. Usnic acid (UA) and atranorin (ATR) were diluted in the culture media from freshly made stock solution (ATR: 3.5 mg/mL; UA: 6.5 mg/mL in DMSO; Sigma-Aldrich) to the working concentrations (from 0 to 40 μg/mL). Medium containing DMSO at the concentration equivalent to 40 μg/mL ATR and UA was used as a solvent control.

2.4. Viability assay

For cell viability assays, the cells were seeded onto 24-well plates (2×10^4 cells/cm^2) and cultured for 24 h. Then, the culture medium was replaced with the same medium containing different concentrations of ATR or UA (0 μg/mL to 40 μg/mL). After 24 and 48 h of incubation, medium was collected to centrifuge tube, cells were harvested from the plate wells by trypsinization, centrifuged and suspended in the fresh medium. Subsequently, cell viability was determined by the Trypan blue exclusion dye and by the fluorescein diacetate (FDA) and ethidium bromide (EtBr) assays, as described previously (Galanty et al., 2008). Viable cells were counted using light (trypan blue exclusion) and fluorescent microscopy (FDA/EtBr test).

2.5. Proliferation assay

For proliferation assays, the cells were seeded onto 12-well plates (5×10^3 cells/cm^2) and incubated for 24 h. After various times (24, 48 and 72 h) of incubation with or w/o ATR and UA, cell numbers was determined using a Bürker's hemocytometer. Data were expressed as an EC_{50} value (concentration at which the proliferation is inhibited by 50%).

2.6. Analysis of apoptosis

2.6.1. Flow cytometry analysis

Cells were seeded to Corning flasks (25 cm^2) at the density of 9×10^3 cells/cm^2. After 24 h, the medium was replaced with the fresh one, containing DMSO (equivalent to 10 μg/mL ATR or UA concentration), ATR or UA (both at 10 μg/m), respectively. The cells were incubated in the presence of these compounds for 48 h. Afterwards, the medium was harvested into centrifuge tubes, the cells were washed with PBS without Ca^{2+} and Mg^{2+} trypsinized, centrifuged (1000 rpm; 5 min) and counted with Bürker's chamber. For apoptosis assessment, the cells were stained using Annexin V Apoptosis Detection Kit (BD Bioscience), according to modified manufacturer's protocol. Briefly, 1×10^5 cells were resuspended in 1× Binding Buffer and incubated in the presence of FITC-conjugated Annexin V (Ann V) and viability dye 7-aminoactinomycin D (7-AAD) for 15 min at room temperature. Subsequently, stained cells were analyzed with BD LSRFortessa flow cytometer and BD FACSDiva Software ver. 8.1 (BD Bioscience).

2.6.2. Proteins isolation and immunoblotting

After the preincubation in culture medium, the cells were incubated in the presence of 10 μg/mL ATR and UA at concentration for 24 and 48 h. Next, the medium was harvested into centrifuge tubes, cells were washed with PBS without Ca^{2+} and Mg^{2+} ions, trypsinized, centrifuged (1000 rpm; 5 min), suspended with PBS without Ca^{2+} and Mg^{2+} ions and centrifuged again. Afterwards, total cell protein extracts

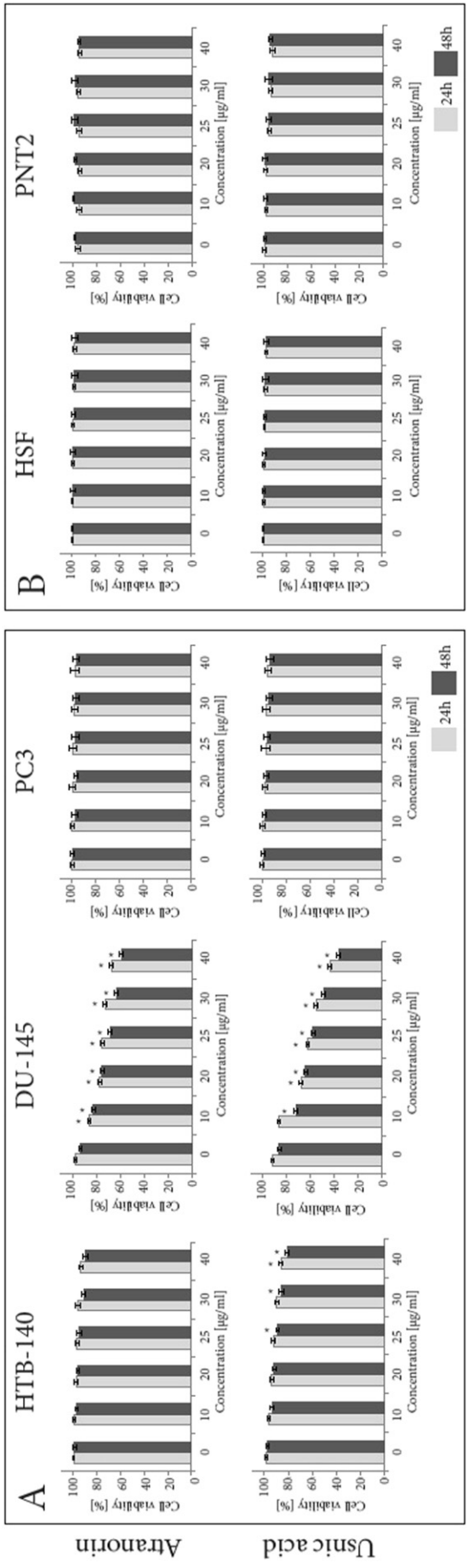

Fig. 1. Cytotoxic activity of atranorin and usnic acid on melanoma HTB-140, prostate cancer DU-145 and PC-3 cells (A), normal human skin fibroblasts HSF and normal prostate cells PNT2 (B). Cells were cultured in medium supplemented with 10% FBS in the absence (0 μg/mL) or presence (10, 20, 25, 30 or 40 μg/mL) of atranorin or usnic acid. Cell viability was assessed by fluoresceine diacetate and ethidium bromide assay. Values represent means ± SEM of three independent experiments; each experiment was done in triplicate. (*) statistically significant vs. control, $p < 0.05$.

were collected by suspending the cell pellet in the lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 2% Triton X-100; 0.02% NaN3) with protease and phosphatase inhibitors. The protein concentration was determined using the Bradford method, aliquots containing 15 μg of protein were separated by 15% SDS-polyacrylamide gel electrophoresis under reducing conditions and then transferred onto a polyvinylidene difluoride membrane (BioRad). After blocking in PBS-T (0.1% Tween 80 in PBS) containing 5% skimmed milk at room temperature, the membranes were incubated with rabbit monoclonal anti-Bax, anti-Caspase 3, 7, 9, anti-Cleaved-Caspase 3, 7, 9, anti-PARP and anti-Cleaved-PARP antibody (all from Cell Signaling Technology, 1:500) and with mouse monoclonal anti-GAPDH (Sigma 1:3000). After washing, the membrane was incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated antibody (1:3000, Invitrogen, Carlsbad, CA). After extensive washing, membranes were incubated with the chemiluminescent reagent Super Signal West Pico Substrate (Pierce, Rockford, IL) and analyzed with MicroChemi (DNR Bio Imaging System).

2.7. Staining of actin cytoskeleton.

To visualize the actin cytoskeleton (microfilaments and focal adhesions), the cells were seeded on glass coverslips inserted into 12-well plates at density of 8×10^3 cells/cm^2, and cultivated in the absence (DMSO control) or presence of ATR and UA (10 and 25 μg/mL) for 72 h. Then, the cells were washed with PBS with Ca^{2+} and Mg^{2+} ions, fixed in 3.7% paraformaldehyde/PBS at room temperature and permeabilized with 0.1% Triton X-100/PBS for 8 min. The cells were then incubated with 1% BSA/PBS for 1 h, followed by the overnight incubation with mouse monoclonal antibody against human vinculin (Sigma-Aldrich) at 4 °C. Afterwards, cells were washed with PBS, incubated with corresponding Alexa Fluor 488 conjugated goat IgG antibody (clone A11001, Sigma-Aldrich), and counterstained with Hoechst 33,342 (Sigma-Aldrich) and phalloidin conjugated with Alexa Fluor 563 for 45 min at room temperature. After 5 washes with distilled water, the coverslips were mounted using Fluorescent mounting medium (DAKO). The immunofluorescence images were obtained using a Leica DMI6000B microscope.

2.8. Cell migration assay

Cell movement was observed with an inverted Leica DMI 6000 B microscope equipped with time-lapse system, IMC contrast optics and incubation chamber (37 °C, 5% CO_2). Cells were plated to 6-well plates at the density of 20×10^4 cells/cm^2 and incubated in culture medium for 24 h. Then the medium was replaced with the fresh one containing DMSO (control) or the tested compounds (10 μg/mL) and cell movement was recorded for 8 h. The tracks of individual cells were generated as described previously (Koczurkiewicz et al., 2013). Cell trajectories were constructed from 88 cell centroid positions recorded over 440 min at 5-min time's intervals immediately after the addition of atranorin or usnic acid or after 24 h preincubation in their presence. Analysis of the parameters characterizing cell migration was performed with the "Mathematica" software (by Stephen Wolfram). The following parameters were computed for each cell (Galanty et al., 2008):

- the total length of cell trajectory (μm)
- the total length of cell displacement (μm)
- the average speed of cell movement (μm/min)
- the average speed of cell displacement (μm/min)

2.9. Statistical analysis

The statistical significance was determined using the Student's *t*-test (viability, proliferation and cytometry analyses) or the non-parametric Mann-Whitney *U* test (time-lapse analyses) with $p < 0.05$ considered

Table 1
Antiproliferative activity of atranorin and usnic acid against melanoma HTB 140 cells, prostate cancer cells: DU145 and PC3, normal human skin fibroblasts (HSF) and normal human prostate epitelium cells PNT-2. Cells were cultured in medium supplemented with 10% FBS in the absence (0 μg/mL) or presence (5, 10 or 30 μg/mL) of atranorin or usnic acid for 24, 48 and 72 h. After incubation the cells were counted using a Bürker's hemocytometer. Values represents mean of the experiments done in triplicate.

EC_{50} [μg/mL]															
	Cancer cells									Normal cells					
	HTB-140			DU-145			PC-3			HSF			PNT2		
Time	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
ATR	>30	>30	>30	>30	27.2	26.5	>30	29	21.5	>30	>30	>30	>30	>30	3.6
UA	22.1	16.6	13.7	23.3	8.6	8.4	7.05	2.67	2.23	22.4	20.5	19.3	26.2	18.2	3.9

to indicate significant differences. Each parameter was calculated as the mean (±SEM). At least three independent experiments (each in duplicate) were performed for every different assay.

3. Results

3.1. Identification of the isolated usnic acid

Yellow crystals of the compound isolated from the hexane and chloroform extracts from *Cladonia arbuscula* were identified as usnic acid, by measurement of its melting point and by HPLC and TLC analyses. The data obtained for the isolated substance were compared with those for a standard usnic acid: melting point 202–204 and 203–204 °C, Rt 8.85 and 8.82 min, respectively (Fig. 1S). Rf factors for the isolated compound and standard usnic acid were 0.69 and 0.70 in solvent system A, 0.71 and 0.71 in solvent system B, 0.87 and 0.88 in solvent system C, respectively.

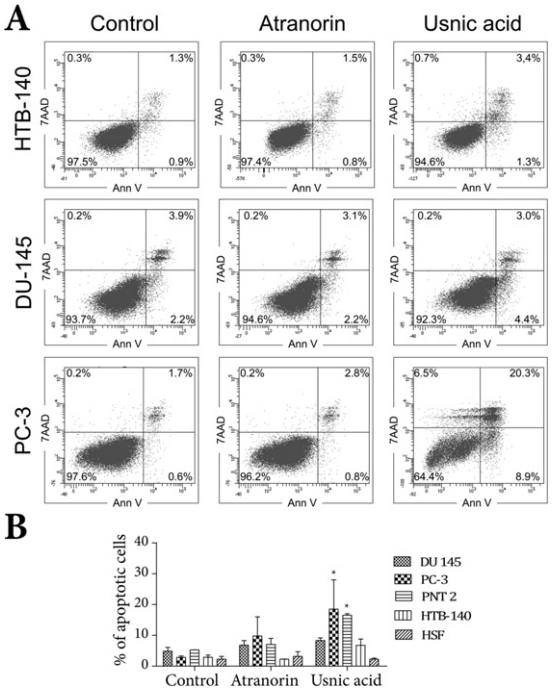

Fig. 2. The effect of atranorin and usnic acid on apoptosis induction in melanoma HTB-140 cells, prostate cancer DU-145 and PC-3 cells, normal human skin fibroblasts HSF and normal prostate cells PNT2. Cells were cultured for 48 h in control and atranorin- or usnic acid- supplemented medium (both at 10 μg/mL) and then apoptotic cells in analyzed cell populations were identified by flow cytometry. (A) Representative dot plots of Ann V/7-AAD-stained HTB-140, DU-145 and PC-3 cells. The percentage of cells in each quadrant is indicated. (B) Summarized quantitative results for all analyzed cell types, expressed as a % of apoptotic cells (sum of early apoptotic Ann V+ 7-AAD- and late apoptotic Ann V+ 7-AAD+). At least 50,000 cells were counted for each experiment. Each experiment was done three times. (*) Statistically significant vs. control, $p < 0.05$.

3.2. Usnic acid and atranorin selectively affect the viability of prostate cancer DU-145 cells

Atranorin and usnic acid showed some differences in activity towards DU-145 cells (Fig. 1). Although both compounds affected the viability of DU-145 prostate cancer cells in a dose- dependent manner (Fig. 1A), stronger cytotoxic effect of usnic acid could be observed (Fig. 1B), whereas no significant cytotoxic effects of both agents on normal

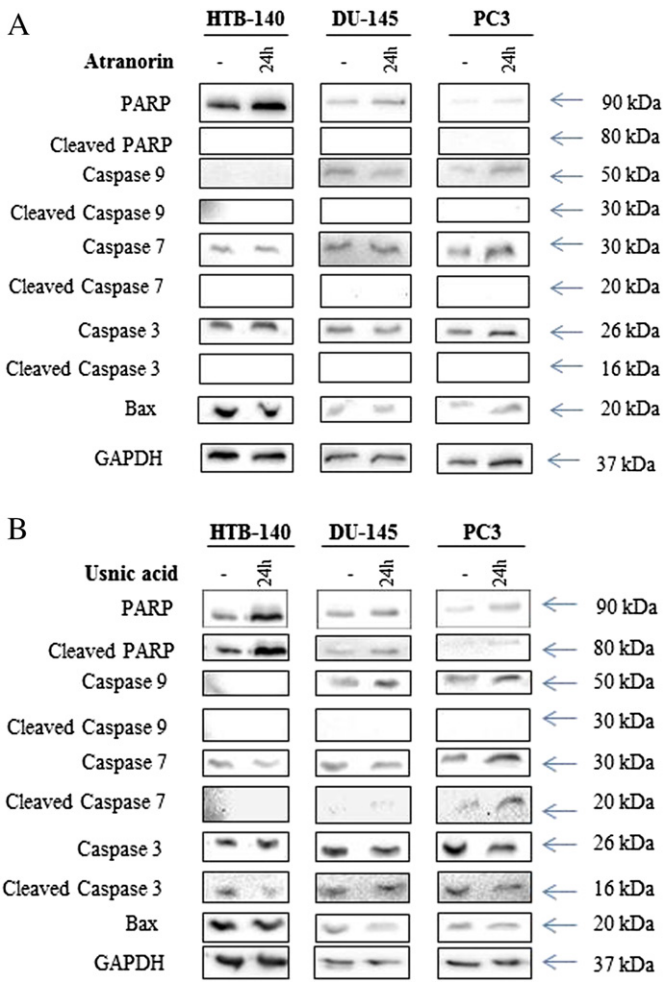

Fig. 3. A. Effect of atranorin on the expression of protein involved in apoptosis signal transduction in melanoma HTB-140 cells, prostate cancer DU-145 and PC- 3 cells. Cells were incubated with 10 μg/mL of atranorin for 24 h and protein levels was assessed by Western blot analysis as described in Materials and methods. GAPDH was used as the internal control. B. Effect of usnic acid on the expression of protein involved in apoptosis signal transduction in melanoma HTB-140 cells, prostate cancer DU-145 and PC- 3 cells. Cells were incubated with 10 μg/mL of usnic acid for 24 h and protein levels was assessed by Western blot analysis as described in Materials and methods. GAPDH was used as the internal control.

prostate PNT2 cells could be seen (Fig. 1B). At the concentrations tested, none of the compounds had significant influence on the viability of PC-3 prostate cancer cells (Fig. 1A). A slight cytotoxic effect was observed in HTB-140 melanoma cells exposed to usnic acid, but not to atranorin (Fig. 1A), while normal human skin fibroblasts were insensitive to both agents (Fig. 1B).

3.3. Usnic acid and atranorin exert differential cytostatic effects on human prostate cancer and melanoma cells

EC_{50} values revealed vast differences between the effects of two lichen metabolites on cell proliferation (Table 1). All tested cell lines were much more resistant to atranorin than to usnic acid. Prostate cancer cell lines appeared to be more sensitive to atranorin than melanoma cells, with EC_{50} value 26.2 and 21.5 after 72 h of incubation, for DU-145 and PC-3 cells, respectively. On the other hand, normal skin fibroblasts were not affected by atranorin, while the inhibition of prostate epithelial cells proliferation was only observed after 72 h-long incubation, with EC_{50} value equal to 3.6 μg/mL.

When applied at the non-cytotoxic concentrations, usnic acid showed a significant and time-dependent inhibitory effect on cancer cell proliferation (Table 1). PC-3 cells were most vulnerable to usnic acid (EC_{50} value 7.05, 2.67, 2.23 after 24, 48 and 72 h of incubation, respectively). The proliferation of DU-145 cells was also significantly inhibited, but the effect was more pronounced after 48 and 72 h only. The growth of normal prostate epithelium cells was less affected by this agent; a strong inhibition of their proliferation was observed only after 72 h of incubation in its presence. The cytostatic effect of usnic acid on normal skin fibroblasts was observed only after longer incubation time. Interestingly, no significant effects of usnic acid on melanoma HTB-140 cell proliferation were seen.

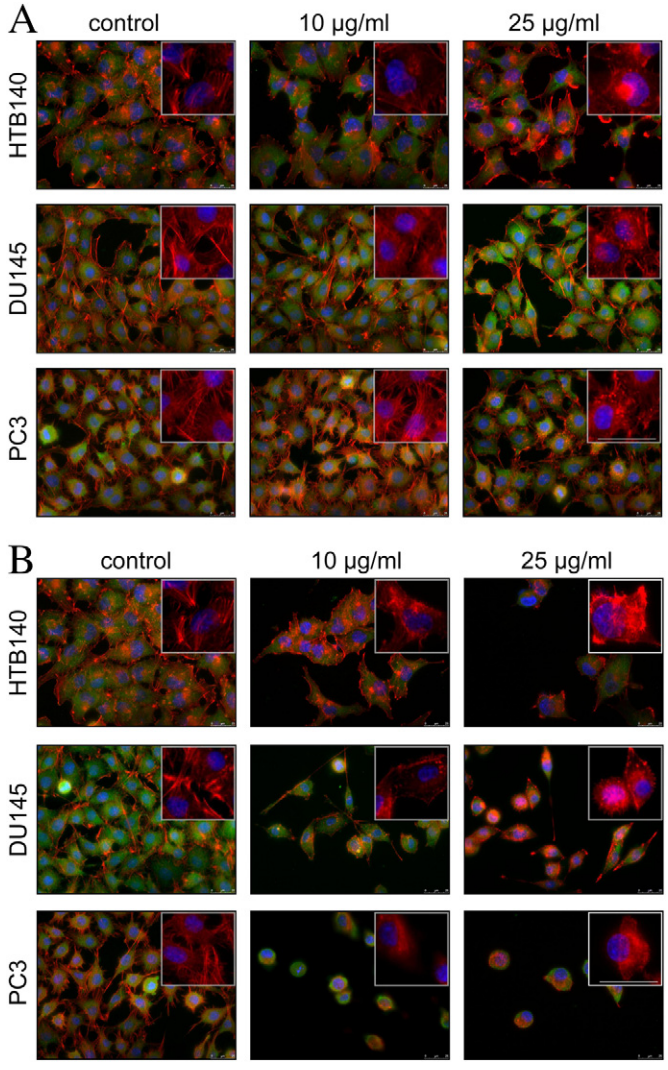

Fig. 4. A. Effect of atranorin on actin cytoskeleton (F-actin, vinculin and cell nuclei are stained with red, green and blue, respectively) in melanoma HTB-140 cells, prostate cancer DU-145 and PC-3 cells. Cells were incubated in medium supplemented with 10% FBS in the absence (control: 0 μg/mL) or presence (10 or 25 μg/mL) of atranorin for 72 h. Immunocytochemical analysis of actin cytoskeleton organization was performed as described in Materials and methods section. Bar = 25 μm. B. Effect of usnic acid on actin cytoskeleton (F-actin, vinculin and cell nuclei are stained with red, green and blue, respectively) in melanoma HTB-140 cells, prostate cancer DU-145 and PC- 3 cells. Cells were incubated in medium supplemented with 10% FBS in the absence (control: 0 μg/mL) or presence (10 or 25 μg/mL) of usnic acid for 72 h. Immunocytochemical analysis of actin cytoskeleton organization was performed as described in Materials and methods section. Bar = 25 μm.

3.4. Usnic acid induces slight apoptotic response of cancer cells

To evaluate whether atranorin and usnic acid induce apoptosis in the tested cancer cells, we performed double staining with Ann V (which binds to the phosphatydilserine expressed on the surface of apoptotic cells) and viability dye 7-AAD, followed by a subsequent flow cytometry analyses. The obtained results demonstrated different pro-apoptotic activity of atranorin or usnic acid (Fig. 2). The treatment of HTB-140, DU-145 or PC-3 cells with atranorin (10 μg/mL) only slightly decreased the percentage of alive (Ann V^- $7AAD^-$) cells, comparing to the control medium, indicating that this compound did not induce apoptosis in the analyzed cancer cells (Fig. 2A,B). Normal cells (HSFs, PNT2) were resistant to atranorin as well (Figs. 2B and 2S). Interestingly, usnic acid increased the percentage of the apoptotic Ann V-positive cells in HTB-140 and PC-3 populations (Fig. 2), with the strongest effect for the latter (19% of apoptotic PC-3 cells after 48 h). No pro-apoptotic effect of usnic acid was observed in human skin fibroblast populations, while this compound slightly induced the apoptosis of normal prostate cells (Fig. 2S).

Western blot analysis performed to detect the changes in the levels of proteins associated with the apoptotic signaling pathway showed increase PARP protein levels in HTB-140, DU-145 and PC-3 cells treated with atranorin, while we could not detect cleaved PARP, Bax, caspase-3, 7 or 9 in these cells (Fig. 3A). Increased expression of PARP was also observed in usnic acid-treated HTB-140, DU-145 or PC-3 cells, accompanied by elevated levels of cleaved PARP and caspase-3, while the levels of cleaved caspase-7 was increased only in prostate cancer cells (Fig. 3B). On the other hand, no changes in the levels of the tested proapoptotic proteins were observed in human skin fibroblasts HSFs and human normal prostate epithelial cells PNT2 undergone the treatment with both agents (Fig. 3SA,B).

3.5. Atranorin and usnic acid induce cytoskeletal rearrangements in prostate cancer and melanoma cells

To complete the data on the activity of the two tested lichen metabolites we examined their impact on actin cytoskeleton organization. Both compounds, when administered at the concentrations of 10 and 25 μg/mL, induced significant rearrangements of actin cytoskeleton in prostate cancer and melanoma cells in a dose-dependent manner (Fig. 4A,B), however much more pronounced effect of usnic acid on this parameter could be seen. Disintegration of microfilament bundles in the cells treated with the tested compounds was observed, accompanied by the reorganization of F-actin network into sub-membraneously or perinuclearly-located aggregates seen upon cell exposition to atranorin (Fig. 4A) and usnic acid (Fig. 4B). A significant decrease of vinculin-positive focal adhesion plaques could also be observed (Fig. 4A,B). Noteworthy, usnic acid exerted much stronger effects on actin cytoskeleton than atranorin. It induced cell detachment resulting in decreased cell numbers, which is in accordance with the cytostatic activity of usnic

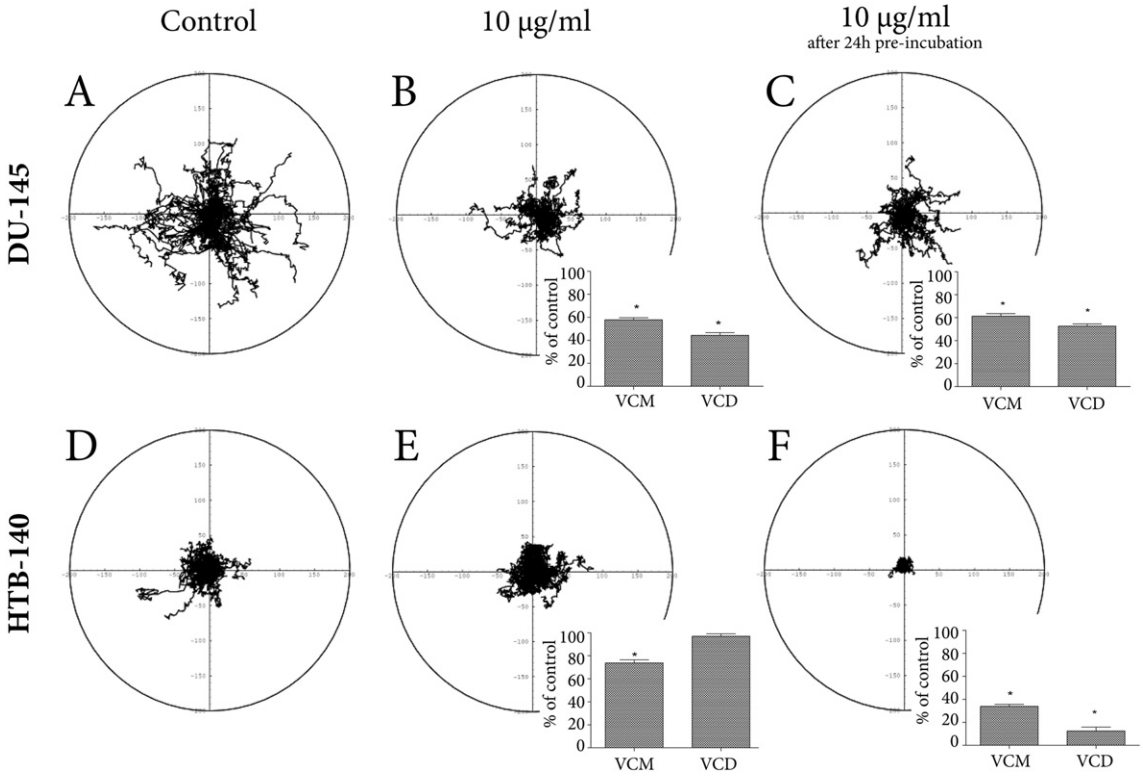

Fig. 5. Effect of atranorin on the motility of human prostate cancer DU-145 and melanoma HTB-140 cells. Cell movement was time-lapse recorded in the absence (control: A, D) of atranorin, directly after the addition (B, E) and after 24-h preincubation (C, F) of 10 μg/mL of atranorin. Cell trajectories are presented in circular diagrams, with the starting point of each trajectory at the plot centre. Cells were seeded at a density of 20×10^4 cells/cm^3. The panels show the trajectories of 50 individual cells. Axis scale in μm. Average VCM (total length of cell movement) and VCD (total length of cell displacement), counted as % of control, for Du-145 and HTB-140 cells movement recorded directly after the addition and after 24-h preincubation of 10 μg/mL of atranorin are presented in the graphs below (B, E) and (C, F), respectively. Results are presented as means ± SEM. Each experiment was done three times. *Statistically significant; $p < 0.05$.

acid (see above). HTB-140 melanoma cells seemed to be the most sensitive to atranorin, while prostate cancer cells, particularly PC-3 cells, were more vulnerable to usnic acid.

3.6. Usnic acid and atranorin interferes with the invasive potential of prostate and melanoma cancer cells

Changes in the organization of actin filaments observed in atranorin- and usnic acid-treated cells inspired us to examine the influence of both agents on the migration of cancer cells. Usnic acid and atranorin significantly inhibited the motility of prostate cancer and melanoma cells, as demonstrated on the circular diagrams illustrating trajectories of individual cells moving in the presence or absence of the tested compounds (Figs. 5, 6). Interesting differences were observed between the short-term and long-term effects of 10 μg/mL atranorin on the movement of HTB-140 and DU-145 cells. Similar displacement rates of HTB-140 cells were observed in control conditions and directly after the administration of 10 μg/mL of atranorin, whereas a significant (ca. 3-fold) decrease of this parameter was seen 24 h after agent administration (Fig. 5D,E,F). However, the exposure of DU-145 cells to 10 μg/mL of atranorin caused an immediate decrease of their movement parameters (Fig. 5B; Table 2), while DU145 cell preincubation with this compound did not affect cell movement (Fig. 5C). These differential effects may probably be explained by phenotypic differences between both types of tumor.

Noteworthy, a considerably higher sensitivity of HTB-140 cells to usnic acid than to atranorin was observed. A significant decrease of cell movement parameters was observed directly after the administration of 10 μg/mL of usnic acid (55 and 70% decrease in total lengths of cell trajectory and displacement, respectively; Fig. 6D,E, Table 2). 24-h preincubation of the cells with usnic acid resulted in a total loss of cell motility (data not shown). Usnic acid also inhibited the motile activity of DU-145 cells during the first 8 h of treatment. A pronounced decrease in the total length of cell trajectories and the total cell displacement was observed, to 63 and 56% of control, respectively (Fig. 6B). Exposing cells to 10 μg/mL of usnic acid for a longer time (24 h preincubation) caused further decrease of these parameters to 68 and 70% of control, respectively (Fig. 6C).

4. Discussion

Although many reports concern the impact of lichen-derived compounds on the physiology of cancer cells, little is known on the mechanisms of their action (Correche et al., 2004, Einarsdottir et al., 2010, Koparal et al., 2006, Bézivin et al., 2004, Bazin et al., 2008, Mayer et al., 2005, O'Neill et al., 2010, Bačkorová et al., 2011) and the data on this topic are still ambiguous. Therefore, we determined not only the cytotoxic and antiproliferative effects of two lichen acids - atranorin and usnic acid, but also focused on their impact on other cellular functions crucial for tumor development, such as apoptosis, cytoskeleton architecture and cell migration. To better address the specificity of their effects, and also the potential selectivity, we have chosen melanoma HTB-140, prostate cancer cell lines DU-145 and PC-3, together with normal human skin fibroblasts and prostate epithelial cells.

So far, usnic acid and atranorin were predominantly investigated for their cytotoxic and antiproliferative activity. However, only two literature reports described the antiproliferative potential of usnic acid on human cancer cell lines: pancreatic adenocarcinoma Capan-2 (Einarsdottir et al., 2010) and colon carcinoma HCT-116 (Bačkorová et al., 2011). The influence of atranorin on cell proliferation has so far been described on normal human keratinocytes and human melanoma

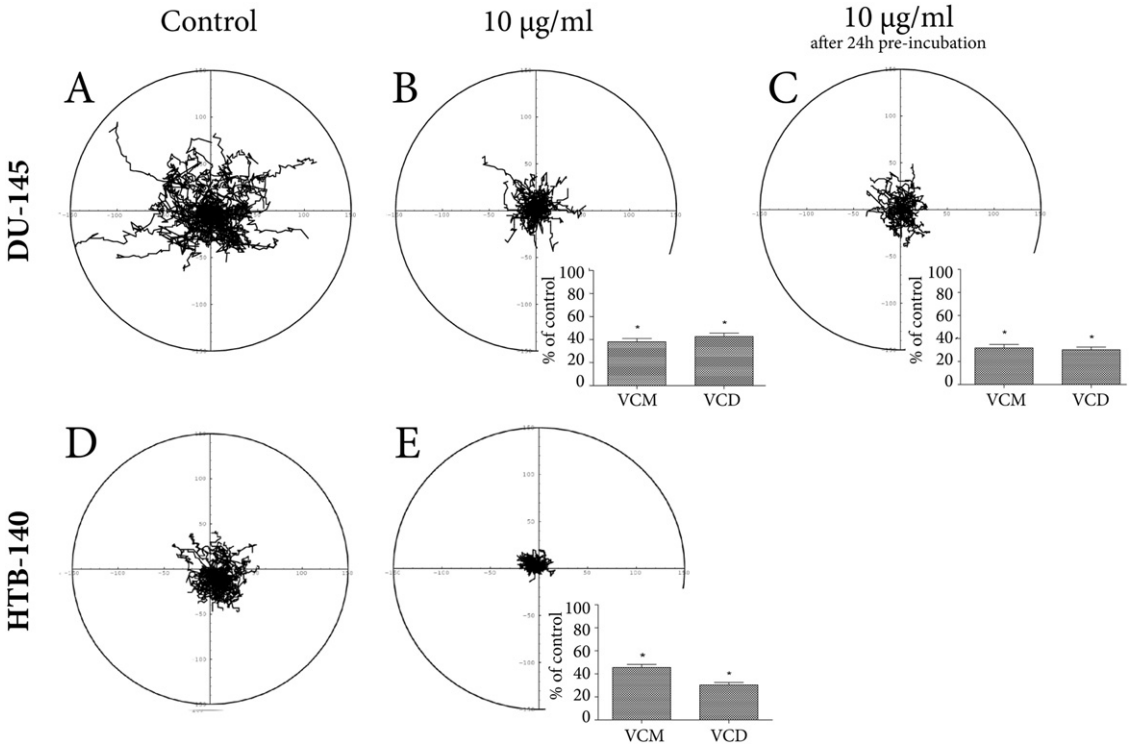

Fig. 6. Effects of usnic acid on the motility of human prostate cancer cells DU-145 and human melanoma HTB-140. Cell movement was time-lapse recorded in the absence (control: A, D) of usnic acid, directly after the addition (B, E) and after 24-h preincubation (C) of 10 μg/mL of usnic acid. Cell trajectories are presented in circular diagrams, with the starting point of each trajectory at the plot centre. Cells were seeded at a density of 20×10^4 cells/cm^3.The panels show the trajectories of 50 individual cells. Axis scale in μm. Average VCM (total length of cell movement) and VCD (total length of cell displacement), counted as % of control, for Du-145 and HTB-140 cells movement recorded directly after the addition and after 24-h preincubation of 10 μg/mL of usnic acid are presented in the graphs below (B, E) and (C), respectively. Results are presented as means ± SEM. Each experiment was done three times. *Statistically significant; $p < 0.05$.

UACC-62 and also on murine B16-F10 melanoma and murine fibroblasts (Kumar and Müller, 1999, Brandão et al., 2013), whereas growth inhibition was observed only on UACC-62 melanoma cells (Brandão et al., 2013). Only in two cases, recently described by Ranković et al. (2014), atranorin revealed significant cytotoxic activity against human melanoma FemX and human colon carcinoma LS 174, with IC_{50} values about 20 μg/mL. Usnic acid was previously tested against prostate cancer DU-145 cell line by Bazin et al. (2008), with the activity described as high (IC_{50} 57.4 ± 2.1 μM, which is about 20 μg/mL). The effect of usnic acid and atranorin on cancer cells have never been determined so far on the HTB-140 melanoma cell line, only two reports describe the impact of these two compounds on any other human melanoma cell lines (Manojlović et al., 2012; Ranković et al., 2014). The impact of the two lichen metabolites on HSF and PNT2 cells has been determined in this study for the first time and the results of atranorin effect on PC-3 cells are novel as well.

In our hands, both examined compounds displayed cytotoxicity against DU-145 prostate cancer cells, while the other two cancer cell lines were unaffected. Noteworthy, almost no toxic effect on normal cells was observed. Cytotoxic potential of atranorin, described by other authors, was rather low (Perry et al., 1999, Kumar and Müller, 1999 a, Kristmundsdottir et al., 2005), which is similar to the effect obtained in our study.

Atranorin exerted moderate, time-dependent antiproliferative effect on both prostate cancer cell lines, while the growth of normal epithelial prostate cells was almost not affected by this compound. No growth inhibition was observed for melanoma HTB-140 cells and also for normal skin fibroblasts. Usnic acid exerted considerably higher anti-proliferative effects on cancer cells than atranorin, especially against prostate cancer cell lines PC-3 and DU-145. It is noteworthy, that normal prostate cells PNT2 were less sensitive than cancer cells. Rather moderate, in comparison to DU-145 cells, impact of usnic acid on melanoma HTB-140 cells and human skin fibroblasts was observed. Data regarding anti-proliferative activity of usnic acid on any human melanoma or prostate cancer cell line obtained in our study, are novel and might be of great importance when taking into account that usnic acid strongly inhibited growth of cancer cells while being not cytotoxic in the same concentration range. These results also show that the effect of both metabolites on cell proliferation profoundly depends on the cell type.

Evaluation of anticancer properties of any compound involves not only the assessment of its cytotoxicity and antiproliferative activity, but also the potential to induce apoptosis. Our results indicate that atranorin shows a weak pro-apoptotic effect against the tested cancer cells. A slight increase of Ann V positive cells population after 48 h incubation in the presence of this compound was observed, in comparison to control conditions. Similarly, the levels of pro-apoptotic proteins in the

Table 2
Quantitative data showing the movement parameters of human melanoma HTB-140 and human prostate cancer cells Du 145 in control conditions (without the tested agents).

	Total length of cell trajectory (μm)	Average speed of cell movement (μm/min)	Total length of cell displacement (μm)	Average rate of cell displacement (μm/min)
HTB-140	144.92 ± 4.53	0.34 ± 0.01	33.50 ± 1.66	0.08 ± 0.01
DU-145	243.28 ± 7.75	0.57 ± 0.01	58.78 ± 5.97	0.14 ± 0.06

cells incubated in the presence of atranorin were increased only to a small extent. The results obtained by Russo et al. (2012) showed that atranorin did not affect the expression of proteins involved in apoptotic process (Bcl-2, Bax, Hsp70 etc.), and also the activity of caspase-3 was not increased in androgen-sensitive (LNCaP) and androgen-insensitive (DU-145) human prostate cancer cells, which is in accordance with our results. The only report on proapoptotic ability of atranorin in human cancer cells, namely A2780 ovarian cancer, HCT-116 colon carcinoma and HL-60 leukemia, was described by Bačkorová et al. (2011).

Our results revealed the prominent pro-apoptotic activity of usnic acid, which was most significant in the case of malignant PC-3 cells. This conclusion was confirmed by increased levels of PARP protein, caspase-3, 7 and 9 and the presence of cleaved PARP and caspase-7 forms in these cells. It suggests that intrinsic pathway of apoptosis is induced by usnic acid in these cells. Till now, pro-apoptotic effect of usnic acid on A2780 ovarian cancer, HCT-116 colon carcinoma, HL-60 leukemia (Bačkorová et al., 2011), A549 lung cancer (Koparal et al., 2006; Nguyen et al., 2014), FemX melanoma (Manojlović et al., 2012), HT29 colon cancer, AGS gastric cancer and CWR22Rv-1 prostate cancer cells (Nguyen et al., 2014) was described, with the increase of cleaved PARP, caspase-3 and Bax levels in the latter. On the other hand, no proapoptotic effect was seen in T-47D breast cancer, Capan2 pancreatic adenocarcinoma (Einarsdottir et al., 2010) and MCF7 breast cancer (Mayer et al., 2005).

We have proved so far that both tested compounds exert cytostatic effects on prostate cancer and melanoma cells. Because cell proliferation is regulated by actin cytoskeleton (McBeath et al., 2004, Christopher et al., 1997), and also a well documented relationship between actin cytoskeleton and the motile ability of the cell has been reported (Mitchison and Cramer, 1996; Olson and Sahai, 2009). We further focused on the impact of atranorin and usnic acid on actin cytoskeleton organization and the motile activity of the tested cancer cells. Which may be of great importance regarding the ability of cancer cells to metastasis. Both atranorin and usnic acid caused significant disassembly of actin filaments. Also, a decline in focal adhesion contacts in the tested cancer cell lines was seen, though with an impact dependent on the type of cell line – atranorin presented the strongest effect on HTB-140 melanoma cells, while PC-3 cells were most sensitive to usnic acid. What is noteworthy, these effects were observed at non-cytotoxic concentrations of both compounds. Our study is probably the first to demonstrate the impact of any lichen metabolites on the actin cytoskeleton organization. The only report concerning the influence of usnic acid on cell cytoskeleton elements is the study of O'Neill et al. (2010), with no observed effect on microtubules.

However, the studies indicating the effect of atranorin and usnic acid on other basic features crucial for cancer cell invasion are rather limited. In this context, we proved the impact of the tested compounds on the motile activity of cancer cells. Atranorin was a strong inhibitor of cell movement, with more profound impact on melanoma cells; however its effect was weaker in comparison to usnic acid, which strongly inhibited migration of DU-145 prostate cancer and HTB-140 melanoma cells. It is worth noting that such strong inhibitory effect on cell movement was observed within the low concentration range of both compounds. Suppression of the motile activity after treatment of usnic acid was described only twice so far, by Nguyen et al. (2014) and Yang et al. (2016), by means of transwell system, on human A549 lung cancer and AGS gastric cancer cell lines, and both authors reported strong motility inhibition. To our best knowledge, this is the first report concerning the influence of atranorin on cell movement.

In summary, we conclude that both tested lichen metabolites revealed significant impact of human cancer cells functioning. The results of our study indicate a strong ability of atranorin to influence actin cytoskeleton architecture and the motility of cancer cells, whereas this agent has rather weak cytotoxic and antiproliferative activity. Usnic acid strongly inhibits proliferation of both prostate cancer cell lines and stimulates the apoptotic process in malignant prostate cancer PC-3 cells, while affecting cancer cells motility. At the same time, it remains relatively neutral to normal cells, which indicates an impressive selectivity of the compound. Our results expand the knowledge on the wide range of lichen compounds activities in general, and in particular atranorin and usnic acid impact on prostate cancer and melanoma cells in culture. The differences in the influence of both agents on the tested cancer cells emphasize their specificity of action, depending on cell type. The ability of usnic acid and atranorin to inhibit cancer cells motility may have future implications for development of new therapeutic strategies targeted at the interference with the metastatic cascade. Thus, further in vitro and in vivo studies should be continued to address mechanistic aspects of potential anti-tumor activity of usnic acid and atranorin.

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Transparency document

The Transparency document associated with this article can be found, in the online version.

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References

Bačkorová, M., Bačkor, M., Mikeš, J., Jendzelovsky, R., Fedoročko, P., 2011. Variable responses of different human cancer cells to the lichen compounds parietin, atranorin, usnic acid and gyrophoric acid. Toxicol. in Vitro 25, 37–44.

Bazin, M.A., Le Lamer, A.C., Delcros, J.G., Rouaud, I., Uriac, P., Boustie, J., Corbel, J.C., Tomasi, S., 2008. Synthesis and cytotoxic activities of usnic acid derivatives. Bioorg. Med. Chem. 16, 6860–6866.

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.

Brandão, L.F.G., Alcantara, G.B., Matos, M.F.C., Bogo, D., dos Santos Freitas, D., Oyama, N.M., Honda, N.K., 2013. Cytotoxic evaluation of phenolic compounds from lichens against melanoma cells. Chem. Pharm. Bull. 61, 176–183.

Burlando, B., Ranzato, E., Volante, A., Appendino, G., Pollastro, F., Verotta, L., 2009. Antiproliferative effects on tumor cells and promotion of keratinocytes wound healing by different lichen compounds. Planta Med. 75, 607–613.

Christopher, Chen S., Mrksich, Milan, Huang, Sui, Whitesides, George M., Ingber, Donald E., 1997. Geometric Control of Cell Life and Death. SCIENCE 276, 30 MAY 1997.

Correche, E.R., Enriz, R.D., Piovano, M., Garbarino, J., Gomez Lechon, M.J., 2004. Cytotoxic and apoptotic effects on hepatocytes of secondary metabolites obtained from lichens. ATLA 32, 605–615.

Einarsdottir, E., Groeneweg, J., Björnsdottir, G.G., Harðardottir, G., Omarsdottir, S., Ingolfsdottir, K., Ögmundsdottir, H.M., 2010. Cellular mechanisms of the anticancer effects of the lichen compound usnic acid. Planta Med. 76, 1–6.

Galanty, A., Michalik, M., Sędek, Ł., Podolak, I., 2008. The influence of LTS-4, a saponoside from *Lysimachia thyrsiflora* L., on human skin fibroblasts and human melanoma cells. Cell. Mol. Biol. Lett. 13, 585–598.

Garbe, C., Eigentler, T.K., Keilholz, U., Hauschild, A., Kirkwood, J.M., 2011. Systematic review of medical treatment in melanoma: current status and future prospects. Oncologist 16, 5–24.

Hanahan, D., Weiberg, R.A., 2000. The hallmarks of cancer. Cell 100, 57–70.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.

Koczurkiewicz, P., Podolak, I., Wójcik, K.A., Galanty, A., Madeja, Z., Michalik, M., Czyż, J., 2013. Lclet 4 enhances pro-apoptotic and anti-invasive effects of mitoxantrone on human prostate cancer cells — *in vitro* study. Acta Biochim. Pol. 60, 331–338.

Koparal, A.T., Ayaz Tüylü, B., Türk, H., 2006. In vitro cytotoxic activities of (+)usnic and (−) usnic acid on V79, A549 and human lymphocyte cells and their non-genotoxicity on human lymphocytes. Nat. Prod. Res. 20, 1300–1307.

Kosanić, M., Ranković, B., Stanojković, T., Rančić, A., Manojlović, N., 2014. Cladonia lichens and their major metabolites as possible natural antioxidant, antimicrobial and anticancer agents. LWT Food Sci. Technol. 59, 518–525.

Kristmundsdottir, T., Jonsdottir, E., Ogmundsdottir, H.M., Ingolfsdottir, K., 2005. Solubilization of poorly soluble lichen metabolites for biological testing on cell lines. Eur. J. Pharm. Sci. 24, 539–543.

Kumar, S.K.C., Müller, K., 1999. Lichen metabolites 2: antiproliferative and cytotoxic activity of gyrophoric, usnic and diffractic acid on human keratinocytes growth. J. Nat. Prod. 62, 821–823.
Lewińska, A., Siwak, J., Rzeszutek, I., Wnuk, M., 2015. Diosmin induces genotoxicity and apoptosis in DU145 prostate cancer cell line. Toxicol. in Vitro 29, 417–425.
Manojlović, N., Ranković, B., Kosanić, M., Vasiljević, P., Stanojković, T., 2012. Chemical composition of three Parmelia lichens and antioxidant, antimicrobial and cytotoxic activities of some their major metabolites. Phytomedicine 19, 1166–1172.
Manojlović, N., Vasiljević, P., Jusković, M., Najman, S., Janković, S., Milenković-Andjelković, A., 2010. HPLC analysis and cytotoxic potential of extracts from the lichen, *Thamnolia vermicularis* var. *subuliformis*. J. Med. Plants Res. 4 (9), 817–823.
Mayer, M., O'Neill, M.A., Murray, K.E., Santos-Magalhaes, N.S., Carneiro-Leao, A.M.A., Thompson, A.M., Appleyard, A.C.M., 2005. Usnic acid: a non-genotoxic compound with *anti*-cancer properties. Anti-Cancer Drugs 16, 805–809.
McBeath, R., Pirone, D.M., Nelson, C.M., Bhadriraju, K., Chen, C.S., 2004 Apr. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell. 6 (4), 483–495.
Mitchison, T.J., Cramer, L.P., 1996. Actin-based cell motility and cell locomotion. Cell 84, 371–379.
Molnár, K., Farkas, E., 2010. Current results on biological activities of lichen secondary metabolites: a review. Z. Naturforsch. 65 (c), 157–173.
Nguyen, T.T., Yoon, S., Yang, Y., Lee, H.-B., Oh, S., Jeong, M.H., Kim, J.J., Yee, S.T., Crisan, F., Moon, C., Lee, K.Y., Kim, K.K., Hur, J.S., Kim, H., 2014. Lichen secondary metabolites in Flavocetraria cucullata exhibit anti-cancer effects on human cancer cells through the induction of apoptosis and suppression of tumorigenic potentials. PLoS ONE 9 (10), e111575. http://dx.doi.org/10.1371/journal.pone.0111575.
Olson, M.F., Sahai, E., 2009. The actin cytoskeleton in cancer cell motility. Clin. Exp. Metastasis 26, 273–287.
O'Neill, M.A., Mayer, M., Murray, K.E., Rolim-Santos, H.M.L., Santos-Magalhães, N.S., Thompson, A.M., Appleyard, V.C.L., 2010. Does usnic acid affects microtubules in human cancer cells? Braz. J. Biol. 70. http://dx.doi.org/10.1590/S1519-69842010005000013.
Orange, A., James, P.W., White, F.J., 2001. Microchemical Methods for the Identification of Lichens. British Lichen Society.
Perry, D.P.G., Benn, N.B., Brennan, M.H., Burgess, N.J., Ellis, E.J., Galloway, G., Lorimer, S.D., Tangney, R.S., 1999. Antimicrobial, antiviral and cytotoxic activity of New Zealand lichens. Lichenologist 31, 627–636.
Ranković, B., Kosanić, M., Manojlović, N., Rančić, A., Stanojković, T., 2014. Chemical composition of *Hypogymnia physodes* lichen and biological activities of some its major metabolites. Med. Chem. Res. 23, 408–416.
Russo, A., Caggia, S., Piovano, M., Garbarino, J., Cardile, V., 2012. Effect of vicanicin and protolichesterinic acid on human prostate cancer cells: role of Hsp70 protein. Chem. Biol. Interact. 195, 1–10.
WHO World Health Statistics 2013.
Yang, Y., Nguyen, T.T., Jeong, M.-H., Crişan, F., Yu, Y.H., Ha, H.-H., Choi, K.H., Jeong, H.G., Lee, K.Y., Kim, K.K., Hur, J.-S., Kim, H., 2016. Inhibitory activity of (+)-usnic acid against non-small cell lung cancer cell motility. PLoS ONE 11 (1), e0146575. http://dx.doi.org/10.1371/journal.pone.0146575.