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Lichen metabolites prevent UV light and nitric oxide-mediated plasmid DNA damage and induce apoptosis in human melanoma cells

A. Russo [a,*], M. Piovano [b], L. Lombardo [c], J. Garbarino [b], V. Cardile [c]

[a] Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, V.le A. Doria 6, 95125, Catania, Italy
[b] Department of Chemistry, University Técnica Federico Santa Maria, Casilla 110-V, Valparaìso, Chile
[c] Department of Physiological Sciences, University of Catania, V.le A. Doria 6, 95125, Catania, Italy

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ABSTRACT

In humans both UV-A and UV-B can cause gene mutations and suppress immunity, which leads to skin cancer, including melanoma. Inhibition of reactive oxygen species (ROS) and reactive nitrogen species (RNS) appears particularly promising as ROS and RNS production by both UV-A and UV-B contributes to inflammation, immunosuppression, gene mutation and carcinogenesis. We evaluated the effect of two lichen compounds, sphaerophorin (depside) and pannarin (depsidone) on pBR322 DNA cleavage induced by hydroxyl radicals (·OH), and by nitric oxide (NO), and their superoxide anion (O_2^-) scavenging capacity. In addition, we investigated the growth inhibitory activity of these compounds against human melanoma cells (M14 cell line). Sphaerophorin and pannarin showed a protective effect on plasmid DNA and exhibited a superoxide dismutase like effect. The data obtained in cell culture show that these lichen metabolites inhibit the growth of melanoma cells, inducing an apoptotic cell death, demonstrated by the fragmentation of genomic DNA (COMET and TUNEL Assays) and by a significant increase of caspase-3 activity, and correlated, at least in part, to the increase of ROS generation, These results confirm the promising biological properties of sphaerophorin and pannarin and encourage further investigations on their molecular mechanisms.

Introduction

Lichens are complex symbiotic organisms of fungi and algae. They are the earliest colonizers of terrestrial habitats on earth, and they show a worldwide distribution from arctic to tropical regions and from the plains to the highest mountains. In particular, they are the most conspicuous macroscopic organisms in Continental South America (Chile) and in Antarctica, in terms of species, biomass and distribution (Muller, 2001). Lichens and their metabolites have long been used by humans. Throughout the ages lichen extracts have been used for various purposes, in particular as dyes, perfumes and for various remedies in folk medicine since ancient Egyptian times (Huneck, 2001). Chemical studies on the secondary metabolites present in lichens have led to the isolation of many new substances, which by today number over 800 (Muller, 2001; Huneck, 2001). These compounds, which comprise aliphatic, cycloaliphatic, aromatic, and terpenic compounds, are unique with respect to those of higher plants and show interesting biological and pharmacological activities (Muller, 2001; Huneck, 2001). Usnic acid, the prototype of low-molecular weight compounds derived from lichens, has been the most extensively studied. It is used in pharmaceutical preparations against infections, bacterial eczema, mastitis, furunculosis and polydermy (Cocchietto et al., 2002). In addition, preclinical studies have permitted to hypothesize its possible use as an antineoplastic agent (Bezivin et al., 2004). Several well-characterized depsidones and depsides exhibit anti-inflammatory, analgesic, antipyretic, antibacterial, antifungal and anticancer properties (Muller, 2001; Piovano et al., 2002; Russo et al., 2006a).

Recently, substances extracted from lichens have been considered as potential sunscreens thanks to their absorption in the ultraviolet (UV) region and to their antioxidant power. Rancan et al. (2002) demonstrated that some compounds extracted from Chilean lichens are able to exhibit protection against ultraviolet radiation (UV-R). In fact, Chilean lichens live in regions where the UV-R is particularly intensive, due to the altitude and the ozone rarefaction. Under UV-R they are stimulated to synthesize metabolites with a strong absorption in the UV region, creating for themselves a protection against dangerous radiations.

UV-R is considered the major etiological factor in skin cancer. In humans both UV-B (280–320 nm) and UV-A (320–400 nm) can cause gene mutations and suppress immunity. These biological events could lead to skin cancer, including melanoma. Melanoma is an aggressive, therapy-resistant malignancy of melanocytes (Russo and Halliday, 2006). Its incidence has been steadily increasing worldwide, resulting in an increasing public health problem. Of the major forms of skin cancer, it carries the highest risk of mortality from metastasis. The prognosis for patients in the late stages of this disease remains very poor with average survival from 6 to 10 months (Armstrong and

* Corresponding author. Tel.: +39 095 7384073; fax: +39 095 7384220.
E-mail addresses: alrusso@unict.it, ales0303@libero.it (A. Russo).

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Kricker, 2001). Currently, there is no effective long-term treatment for patients suffering from the advanced stages of this cancer (Balch et al., 2001; Margolin, 2004; Sondak et al., 2006). Surgery in malignant melanoma often leads to substantial defects where reconstruction poses a difficult challenge. Chemotherapy is not giving significant benefits and it is often associated to severe toxicity, while immunotherapy and vaccines are promising, but still ineffective. It is therefore of primary interest to search for new therapeutic agents that are able to prevent and contrast this aggressive tumor.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) production by both UV-A and UV-B contributes to inflammation, immunosuppression, gene mutation and carcinogenesis. Therefore, substances able to inhibit these reactive species could be used in the prevention of skin cancer, including melanoma (Russo and Halliday, 2006). In view of these considerations using cell-free systems, we evaluated the effect of sphaerophorin (depside) and pannarin (depsidone) (Fig. 1) on pBR322 DNA cleavage induced by hydroxyl radicals (OH), generated from UV-photolysis of hydrogen peroxide (H_2O_2) and by nitric oxide (NO), and their superoxide anion ($O_2^{-\cdot}$) scavenging capacity. We also investigated the growth inhibitory activity of these compounds against the human melanoma cell line, M14.

Materials and methods

Chemicals

All reagents were of commercial quality and were used as received. pBR322 plasmid DNA, diethylenetriaminepentaacetic acid (DTPA), 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) and β-nicotinamide-adenine dinucleotide (NADH) were obtained from Sigma Aldrich Co (St. Louis, USA). All other chemicals were purchased from Sigma Aldrich Co (St. Louis, USA) and GIBCO BRL Life Technologies (Grand Island, NY, USA).

Plant material

The tested metabolites arise from diverse lichen species collected in different localities of continental and Antarctic Chile. Sphaerophorin and pannarin were isolated from *Sphaerophorus globosus*, and different species of the genus *Psoroma* (*Psoroma reticulatum*, *P. pulchrum*, *P. palladium*) respectively, as previously described (Quilhot et al., 1989a,b). The compounds, after extraction from all lichens, were isolated by chromatography using Si gel column, and identified by spectroscopic techniques (IR, ^1H and ^{13}C NMR and MS). The purity grade of sphaerophorin and pannarin were 99%.

Activity in cell-free systems

DNA cleavage induced by hydrogen peroxide UV-photolysis

The experiments were performed, as previously reported (Russo et al., 2004), in a volume of 20 μl containing 33 μM in bp of pBR322 plasmid DNA in 5 mM phosphate saline buffer (pH 7.4), and lichen compounds, sphaerophorin and pannarin at different concentrations. Immediately prior to irradiating the samples with UV light, H_2O_2 was added to a final concentration of 2.5 mM. The reaction volumes were held in caps of polyethylene microcentrifuge tubes, placed directly on the surface of a transilluminator (8000 μW cm^{-1}) at 300 nm. The samples were irradiated for 5 min at room temperature. After irradiation, 4.5 μl of a mixture, containing 0.25% bromophenol blue, 0.25% xylen cyanol FF, and 30% glycerol, was added to the irradiated solution. The samples were then analyzed by electrophoresis on a 1% agarose horizontal slab gel in Tris–borate buffer (45 mM Tris–borate, 1 mM EDTA). Untreated pBR322 plasmid was included as a control in each run of gel electrophoresis, conducted at 1.5 V/cm for 15 h. Gel was stained in ethidium bromide (1 μg/ml; 30 min) and photographed on Polaroid-Type 667 positive land film. The intensity of each scDNA band was quantified by means of densitometry.

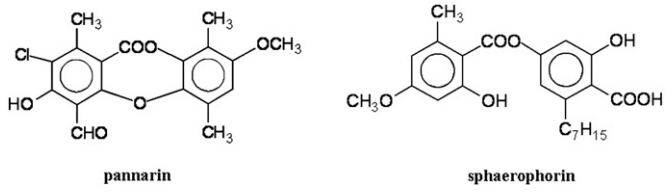

Fig. 1. Structure of lichen compounds, sphaerophorin (depside) and pannarin (depsidone).

Analysis of DNA single-strand breaks induced by Angeli's salt

The experiments were performed as previously reported (Russo et al., 2004) by incubating pBR322 plasmid DNA in 100 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM DTPA, 0.15 mM Angeli's salt (prepared in 0.01 N NaOH), an appropriate amount of HCl to neutralize the NaOH present in the solution of Angeli's salt, and lichen compounds at different concentrations at 37 °C for 1 h (final volume 10 μl, final pH 7.5). After the reaction, 2 μl of electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylen cyanol FF, and 30% glycerol) was added to the reaction mixture and an aliquot (8 μl) was loaded onto a 0.7% agarose gel. Untreated pBR322 plasmid was included as a control in each run of gel electrophoresis, conducted at 1.5 V/cm for 15 h. Gel was stained in ethidium bromide (1 μg/ml; 30 min) and photographed on Polaroid-Type 667 positive land film. The intensity of each scDNA band was quantified by means of densitometry.

Scavenger effect on superoxide anion

Superoxide anion was generated in vitro as previously reported (Russo et al., 2006b). The assay mixture contained in a total volume of 1 ml, 100 mM triethanolamine–diethanolamine buffer, pH 7.4, 3 mM NADH, 25 mM/12.5 mM EDTA/$MnCl_2$, 10 mM β-mercapto-ethanol; some samples contained lichen compounds at different concentrations. After 20 min incubation at 25 °C, the decrease in absorbance was measured at λ = 340 nm. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

Study on human tumor cell line

Cell culture and treatments

M14 human melanoma cells were grown in RPMI containing 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml fungizone. The cells were plated at a constant density to obtain identical experimental conditions in the different tests, thus to achieve a high accuracy of the measurements. After 24 h incubation at 37 °C under a humidified 5% carbon dioxide atmosphere to allow cell attachment, the cells were treated with different concentrations of lichen compounds and incubated for 72 h under the same conditions. Stock solution of the natural compounds was prepared in DMSO and the final concentration of this solvent was kept constant at 0.25%. Control cultures received DMSO alone.

MTT bioassay

MTT assay was performed as described previously (Mossman and Churg, 1998). Briefly, the cells were set up 6×10^3 cells per well of a 96-well, flat-bottomed 200 μl microplate. Cells were incubated at 37 °C in a humidified 5% CO_2/95% air mixture and treated with sphaerophorin and pannarin at different concentrations (6–50 μM) for 72 h. Four hours before the end of the treatment time, 20 μl of 0.5% 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) in phosphate buffer saline (PBS) was added to each microwell. Cells were washed once before adding MTT. After 4 h of incubation at 37 °C, the supernatant was removed and replaced with 100 μl of DMSO. The optical density of each well sample was measured with a microplate