BBA - General Subjects 1863 (2019) 1–12

Contents lists available at ScienceDirect

BBA - General Subjects

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

Myrtenal-induced V-ATPase inhibition - A toxicity mechanism behind tumor cell death and suppressed migration and invasion in melanoma

Brunna Xavier Martins[a], Raul Ferraz Arruda[a], Gildeíde Aparecida Costa[a], Hassan Jerdy[b], Sávio Bastos de Souza[a], Julianna Maria Santos[a,1], William Rodrigues de Freitas[c], Milton Masahiko Kanashiro[c], Eulógio Carlos Queiroz de Carvalho[b], Nadir Francisca Sant'Anna[a], Fernanda Antunes[b,d], Raul Martinez-Zaguilan[e], Sennoune Souad[e], Anna Lvovna Okorokova-Façanha[d,f], Arnoldo Rocha Façanha[a,d,*]

[a] *Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil*

[b] *Hospital Veterinário/Laboratório de Morfologia e Patologia Animal, Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil*

[c] *Laboratório de Biologia de Reconhecer, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil*

[d] *Unidade de Experimentação Animal (UEA), Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil*

[e] *Texas Tech University, Department of Physiology, Health Sciences Center, Lubbock, TX, USA*

[f] *Laboratório de Bioquímica e Fisiologia de Microrganismos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil*

ARTICLE INFO

Keywords:
Proton pumps inhibitors
Anti-melanoma drugs
Bioactive terpenes
pH signatures
Tumor acidification

ABSTRACT

Background: Metastatic tumor cells have acidic extracellular pH and differential electrochemical H^+ gradients generated across their cell membranes by V-type H^+-ATPases. This study shows that inhibition of the V-ATPases by the plant-derived monoterpene Myrtenal results in tumor cell death and decreased metastatic dissemination in mice.

Methods: The Myrtenal anticancer toxicity was evaluated *in vitro* using murine (B16F0 and B16F10) and human (SkMel-5) melanoma cell lines, and in *in vivo* mouse metastatic dissemination model. Proton flux and extracellular acidification were directly evaluated at the surface of living cells using a non-invasive selective ion electrode approach.

Results: The inhibition of V-ATPases by 100 μM Myrtenal disrupted the electrochemical H^+ gradient across the cell membranes, strongly induced cell death (4–5 fold), and decreased tumor cells migration and invasion *in vitro*. Myrtenal (15 mg/kg) also significantly reduced metastasis induced by B16F10 *in vivo*, further reinforcing that V-ATPase is a molecular target to halt the progression of cancers.

Conclusions: These data revealed the therapeutic potential of Myrtenal as inhibitor of melanoma progression proposing a mechanism of action by which once inhibited by this monoterpene the proton pumps fail to activate cancer-related differential electrochemical gradients and H^+ fluxes across the tumor cell membranes, disrupting pH signatures inherent in tumor progression, resulting in reprogrammed cell death and metastasis inhibition.

General significance: The work represents a new mechanistic strategy for contention of melanoma, the most aggressive and deadly form of cutaneous neoplasm, and highlights Myrtenal, other related monoterpenes and derivatives as promising proton pump inhibitors with high chemotherapeutic potential.

* Corresponding author: Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro. Campos dos Goytacazes-RJ, Brazil.
E-mail address: arnoldo@uenf.br (A.R. Façanha).
[1] Current address: Texas Tech University, Mechanical Engineering Department, Lubbock, TX, USA.

https://doi.org/10.1016/j.bbagen.2018.09.006
Received 11 February 2018; Received in revised form 9 September 2018; Accepted 10 September 2018
Available online 11 September 2018

1. Introduction

Over the years, many synthetic and natural plant compounds have been considered as potential anti-neoplastic drugs and have increasingly been used alone or in combination with conventional chemotherapeutic agents [1,2]. Myrtenal is an active principle of essential oils found in plants, such as cardamom, orange, lemon, spearmint, pepper and ginger, widely used as flavoring agent in food industry [3], for which no toxicity mechanism has been demonstrated up to now. Here, we provide a body of evidence that demonstrate a new bioactivity for this monoterpene, associated with a strong antimelanoma activity mechanistically related to the inhibition of V-ATPases, a molecular target that have been increasingly demonstrated to be overexpressed in tumor membranes of highly metastatic cancer cells [4,5].

Significant advances have been achieved in cancer therapy, leading to an improvement of overall patient survival and reduction of mortality rates in some, but not all types of cancer. Malignant melanoma, the most lethal skin cancer, evades most pro-apoptotic mechanisms and has shown an increase in death incidence over the last decades [6]. Progress in the treatment of this lethal disease has been made by using immunotherapy with Ipilimumab, a human monoclonal antibody that targets cytotoxic T-lymphocyte antigen 4 (CTLA-4), which was found to be effective in only 11% of the cases. Another chemotherapy drug, Vemurafenib, has shown to improve overall survival in 40–50%, but just for those patients with specific V600E mutation in the BRAF enzyme [7,8]. Therefore, new bioactive molecules with wider therapeutic spectrum are required for development of more effective and safer antimelanoma therapies.

In 1956, Otto Warburg reported that tumors generated an acidic extracellular microenvironment as result of increased glycolysis and other related changes in cancer bioenergetics [9]. However, only recently has this feature become a major focus to develop novel approaches for cancer therapy [10]. A series of seminal studies has uncovered that extracellular tumor acidification increases the malignant phenotype of tumor cells, by activating proteases with optima acidic extracellular pH that disrupts the extracellular matrix and contributes to tumor cell invasion and dissemination of highly metastatic tumors [11–13]. These works have proved that tumor acidification is associated with differential activations of V-type H^+-ATPases (V-ATPases), transmembrane ATP-dependent proton pumps composed by a multisubunit complex that energizes a myriad of secondary transport systems across cell membranes, pumping H^+ ions from the cytoplasm into the lumen of organelles or to the extracellular matrix [14]. Chronic disturbances of cellular and extracellular ionic homeostasis, and alterations in energy metabolism in carcinogenic processes are main factors in tumor progression, invasion and metastasis [15]. Alterations in pH are also involved in DNA damage and control of programmed cell death [16,17]. Tumor cells express differential electrochemical gradient of ions in their cell membranes, including the nuclear membranes, driven by V-ATPases [18], which has been linked to the apoptosis avoidance [16] and metastasis [15]. Recently, we have also demonstrated that disturbance of membrane microdomains promotes V-ATPase inhibition and disrupts pH signatures required for melanoma cell migration and invasion [19].

In this work, a putative anti-metastatic potential of the monoterpene Myrtenal is investigated through a toxicity/therapeutic mechanism of action by which inhibition of V-ATPase will induce programmed cell death and inhibit migration and invasiveness *in vitro* in human and mice tumor cell lines, inhibiting metastatic dissemination *in vivo* in a mouse melanoma model.

2. Materials and methods

2.1. Chemicals

(1*R*)-(-)-Myrtenal and concanamycin A purchased from Sigma Aldrich® were prepared as a stock solution in dimethyl sulfoxide (DMSO).

2.2. Cell lines and cell culture

B16F0 and B16F10 murine melanoma cell lines were used as models of low and high proliferative and metastatic potential, respectively, and compared with the highly metastatic human melanoma cell line SkMel-5. We also used a murine macrophage cell line (J774 A_1) that is known to express V-ATPase at the plasma membrane as non-cancer control cells [20] (Supplemental Fig. S2). Melanoma cell lines B16F0, B16F10 and SkMel-5 were cultured in DMEM medium (Sigma Aldrich®) supplemented with 10% FBS (Gibco™). The cells were incubated at 37 °C in a humidified chamber containing 5% CO_2. As expected, the highest metastatic cells, B16F10 and SkMel-5, exhibited a faster cell growth rate than the less metastatic B16F0 or the non-tumor J774 A_1 cell line (Supplemental Fig. S1A). The cell lines were obtained from the cell bank of the Federal University of Rio de Janeiro (UFRJ, Brazil).

2.3. Cell viability assay

The viability of the cells incubated with Myrtenal was evaluated by an MTT (3-(4,5 dimethyltiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, as described earlier [21]. Cells were plated in 96-well plates at 8.3×10^4 cells/mL, and then were treated with different concentrations of Myrtenal (10, 20, 50, 100, and 200 μM) or concanamycin A. The formazan crystals were dissolved in acidic isopropanol and their absorbance was determined at 570 nm using a microplate reader (Thermo Labsystems Multiskan, model 352). Treatment of these cells with increasing Myrtenal concentrations (10–200 μM) for 24 h resulted in a concentration-dependent decrease in cell viability, and above 50 μM this effect was stronger in the higher metastatic melanoma cell lines B16F10 and SkMel-5 (Supplemental Fig. S1B).

2.4. Evaluation of cell death

Apoptotic and necrotic cells were quantified by double staining with acridine orange and ethidium bromide essentially as previously described [22], a method in which viable/early apoptotic cells stain green with bright green dots in the nuclei, while late apoptotic and necrotic cells exhibit increasing orange fluorescence depending on the loss of membrane integrity due to co-staining with ethidium bromide. Cells were plated in 12-well plate at a density of 1×10^5 cells/mL, and 18–20 h after adhesion the cells were treated with Myrtenal (50 μM) or concanamycin A (5 nM) and incubated at 37 °C and 5% CO_2 for 12 h. Cells were stained with 10 μg/mL acridine orange and 10 μg/mL ethidium bromide (AO/EB) and immediately examined using fluorescence microscopy (Axioplan-Carl Zeiss). In order to prevent a possible photoactivated cytotoxicity [23], straight after AO/EB incubation in the dark, the cells were quickly washed with PBS and all procedure upon white light takes no > 5–7 min and just few seconds of blue light (450–490 nm bandpass filter) exposure, no longer than is necessary to achieve the images. The resulting images were subsequently analyzed by the Zen 2.3 (Blue Edition) software.

2.5. Flow cytometry

Cells were incubated with Myrtenal (100 μM) or concanamycin A (5 nM) for 24 h. Following double staining with FITC-Annexin V and propidium iodide (PI), cells were analyzed by flow cytometry (BD FACSCalibur™) using FlowJo V10 software. The flow cytometry allowed a more detailed quantitative analysis, which distinguished early and late apoptotic cells from the necrotic cells.