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The key role of mitochondria in human melanoma cells: a target of delta-tocotrienol anti-cancer activity and a stemness marker

Michela Raimondi

R11979

PhD Tutor: Prof. Patrizia Limonta

PhD School Coordinator: Prof. Chiarella Sforza

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ABSTRACT

Malignant melanoma is one of the most aggressive and treatment resistant cancers. Treatment options include surgery, chemotherapy, immunotherapy and targeted therapies. Unfortunately, the large heterogeneity of melanomas and the presence of stem cells within the tumor mass are the main causes of treatment resistance. Natural compounds have attracted great interest in cancer research for their capability to induce paraptosis, a non-canonical cell death characterized by endoplasmic reticulum (ER) dilatation, mitochondrial swelling, reactive oxygen species (ROS) production and Ca^{++} overload. Accumulating evidence supported the anticancer effect of tocotrienols (TTs); aim of this study was to investigate whether δ-TT might induce paraptosis in A375 and BLM human melanoma cells. We demonstrated that δ-TT induces paraptosis in melanoma cells, causing ER dilatation, mitochondria swelling and the activation of MAPKs. This effect was accompanied by an impairment of mitochondrial function, resulting in a reduction in OXPHOS levels, oxygen consumption, mitochondrial activity and energy depletion. This dysfunction leads to mitochondrial ROS production and increase in Ca^{++} levels. We then focused our attention on A375 stem cells. Cancer stem cells (CSCs) are a small tumor subpopulation responsible for chemotherapy resistance and they have been reported to be coupled with metabolic alterations. Melanoma SCs deriving from the A375 cell line are characterized by the expression of the ABCG2 transporter and δ-TT treatment counteracts A375 SCs growth. In this work we observed that melanoma SCs are characterized by higher mitochondrial mass, increased OXPHOS, increased PGC1α expression levels and increased mitochondrial fusion markers. We then analyzed whether δ-TT might affect stem cell mitochondria and we observed that δ-TT significantly impairs mitochondrial protein levels. Finally, preliminary data demonstrate that δ-TT affects mitochondria also in vemurafenib-resistant A375 cells. Taken together, all these data demonstrate that 1) δ-TT induces paraptotic cell death in melanoma cells, involving mitochondrial dysfunction, ROS production and Ca^{++} overload; 2) mitochondrial mass could be considered a novel marker to identify melanoma SCs; 3) δ-TT targets melanoma SCs affecting mitochondrial functions 4) δ-TT impairs mitochondria in vemurafenib-resistant A375 cells.

Index

INTRODUCTION

1. Melanoma

1.1 Epidemiology and risk factors

Cutaneous melanoma is the fifth most common cancer and one of the most aggressive and treatment-resistant human cancers. It represents the least common but the most aggressive and lethal form of skin cancers and it remains a potentially fatal malignancy. It accounts for only about 1% of skin cancer cases, but most of skin cancer deaths [1].

Its incidence has risen rapidly worldwide in the last decades and the incidence rates vary in relation to ethnicity, geographical location (Fig. 1) and even within populations across gender and age [2].

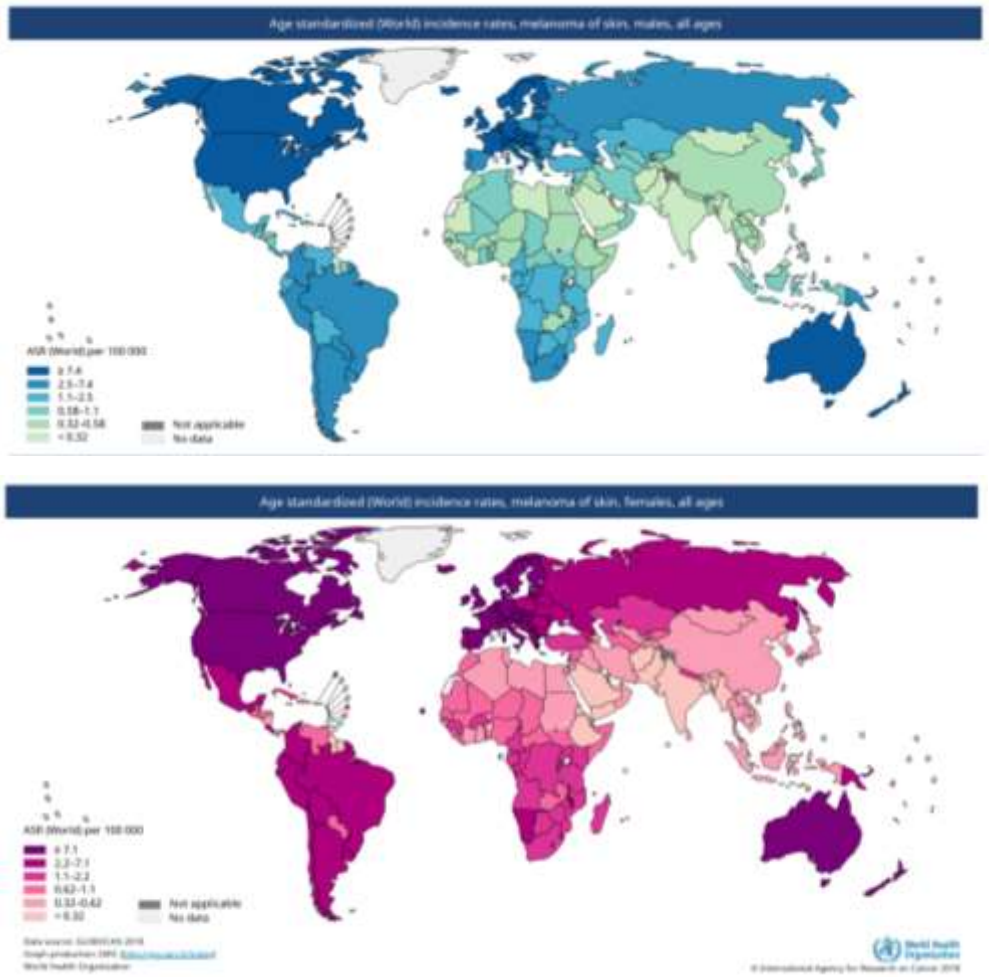


Fig. 1 Map of melanoma incidence rate.

Source: Cancer Facts & Figures, 2020.

According to ethnicity, melanoma demonstrates greater variation in incidence rate than most cancers, indicating that race represents one of the major risk factors.

Since white populations have an approximately 10-fold greater risk than black, Asian or Hispanic populations, phenotype is considered one of the major risk factors [3]. In fact, the risk of developing the disease is higher in fair skin and associated characteristics, such as blond or red hair, blue eyes, freckles [4].

This variation is partly attributed to reduced photo-protection due to different melanin synthesis: in darker-pigmented skins, melanin barrier decreases both ultraviolet (UV) A and B radiation [5]. Exposure to UV radiation (from sunlight or the use of indoor tanning) represents the most important risk factor for all types of skin cancer because of its genotoxic effect. Epidemiologic studies have shown the correlation of intense intermittent UV radiation (UVR) exposure and severe sunburns during childhood with the higher risk of developing melanoma [6]. Natural sunlight exists as a variety of wavelengths that are filtered by the atmosphere, therefore, differences in geographical location -that influence atmospheric absorption, latitude, altitude and cloud cover- can influence UVR exposure [7]. The carcinogenic UV effects are mostly due to the effect of UVB-photons on nucleic acids. However, UVR has multiple effects on the skin contributing to carcinogenesis, such as induction of reactive oxygen species (ROS) and alterations in cutaneous immune functions [8]. It is largely known that the risk of melanoma due to sun exposure is directly associated with the UV-B spectrum, while UVA radiation is involved in superficial sunburns. UVA radiation is a longer wavelength known to penetrate more deeply into the dermis then UVB and is the primary light used by tanning beds [9]. Multiple studies now clearly correlate the use of sunbeds with melanoma development, thus, UV light from tanning bed has been classified as a human carcinogen [10, 11]. Since sunlight is one of the principal causes of melanoma, skin sites normally exposed to the sun reported the highest incidence (face, neck, ears), followed by intermittently exposed sites (back, shoulders, and limbs). On the contrary, melanomas are rare on sites usually covered by clothes. However, it has been observed that certain types of melanomas arise in areas that are well protected from UVR (palms,

soles and areas under the nails), and others in sites that have no UV exposure at all (mucosal membranes) [12].

In addition to UV exposure, major risk factors playing a role in the development of melanoma include a personal or family history of melanoma, the number, the size and the type of congenital and acquired nevi. Approximately 25% of melanoma cases arise from a pre-existing nevus, while most cases arise de novo [13].

It has been estimated that a patient who has already had a melanoma has the 3-7% risk of tumor recurrence. Moreover, the presence of melanoma cases in family history increases the risk to develop a primary melanoma [14, 15].

Incidence rates vary in relation to age and peaks are observed at the sixth decade of life with differences between females and males. Overall, men are more susceptible to melanoma then women, but the risk is different for the two sexes in relation to age: females are more susceptible to melanoma during adolescence and younger ages, instead, after the age of 40, the risk reverses and is higher in men [16-21].

1.2 Pathologic description and melanoma subtypes

Malignant melanoma arises from melanocytes, neural crest-derived cells within the skin, eye and other tissues. Melanocytic neoplasms originate from melanocytic naevi, benign lesions that transform in malignant melanomas. Melanocytic evolution to malignant melanoma goes through different phases that ranges from mild, moderate, or severe dysplasia to atypical cell proliferation, to melanoma in situ (MIS), to early invasive melanoma.

MIS is a premalignant lesion characterized by a proliferation of melanocytes with increased dimension of the nuclei and an irregular growth pattern entirely within the epidermis. Despite the significant risk of progression to invasive melanoma, the metastatic potential is low, therefore when MIS is completely resected the survival rate is nearly 100% [22]. Once melanoma cells leave the epithelium and invade the subjacent mesenchymal tissue, such as the dermis or submucosa, melanoma has become invasive. Melanoma cells grow radially in the epidermis and superficial dermal layer (Radial Growth Phase, RGP). When the proliferation begins in the vertical direction, the lesions become palpable (Vertical Growth Phase, VGP) (Fig 2).

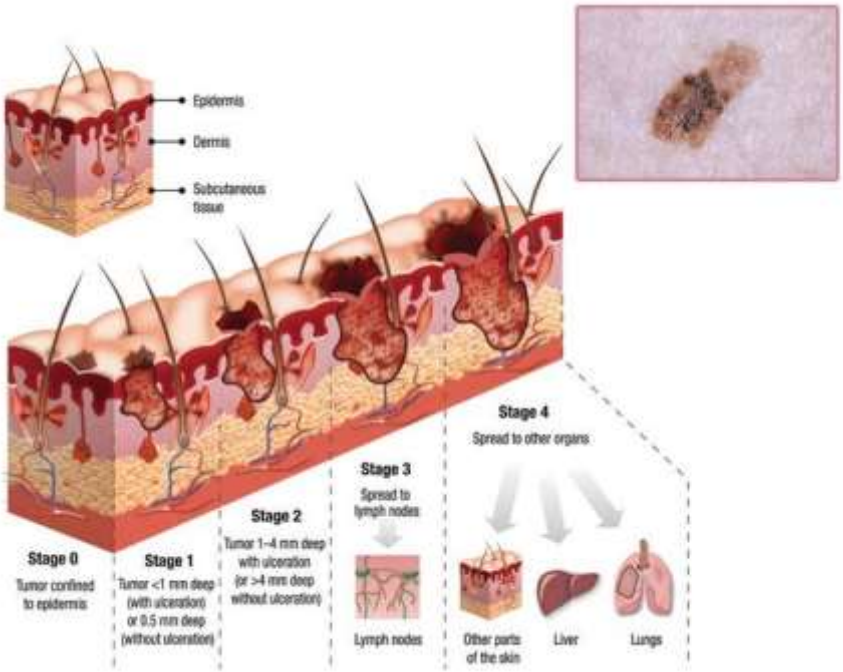


Fig. 2 Schematic description of melanoma progression. Stage 0: melanoma *in situ*. Stages 1-2: in situ, invasive melanoma. Stages 3-4: metastatic melanoma.

Source: Marzagalli,. 2016

The level of invasion, that can allow cancer cells to have access to blood or lymphatic vessels, is associated with the risk to develop metastasis and a poor prognosis [23].

From a clinical and histological point of view, based on growth pattern and location, it's possible to classify melanoma into different subtypes [24].

Superficial Spreading Melanoma (SSM) is the most common subtype that accounts for 60-70% of all cases. It is associated with intermittent exposure to the sun, and it arises on the back of the legs of women and on the back of men. SSM begins as a flat, well-circumscribed polycyclic patch with variegating shades of brown, gray and black growing in the radial dimension, but over time it would develop a vertical growth. SSM may arise de novo or in association with a nevus and it is characterized by different colors (tan, brown, black, grey, pink, violaceous and rarely blue or white). Transformed melanocytes are enlarged and often aggregated, forming a palpable protusion above the epidermis.

Nodular Melanoma (NMM) accounts for 5% of all melanomas. It is more common in men than women and occurs mainly in the trunk and limbs. NMM is correlated with intermittent sun exposure and the risk increases after the age of 50 and 60. NMM is characterized by only VGP, correlated with high metastatic potential. It appears as a brown, black or blue-black, raised popular lesion, with a smooth surface with high risk of ulceration, associated with a poor prognosis.

Lentigo Maligna Melanoma (LMM) represents 4% to 15% of cutaneous melanomas. It correlates with increasing age and, in contrast to NMM and SSM, with long-term exposure to the sun. It occurs on the face, on the neck and the head in patients with sun-damaged skins. It is localized on the basal layers of the epidermis and it presents as a flat, dark black or brown pigmented lesion, with irregular outlines, that slowly evolves before invading into the papillary dermis. Overall, the prognosis is better than other subtypes, but this slowly development can delay the diagnosis.

Acral Lentiginous Melanoma (ALM) is the most uncommon type of melanoma, accounting for 5% of all cases in white people, but it represents the most common one among Asian, Hispanic and African patients. It arises on glabrous skin such as the palms, the soles and the subungual areas. Typically it affects old people, with high prevalence in women. The localization in the nails bed can delay the diagnosis, because lesions are often mistaken for subungual hematomas.

LMM and ALM borders are not defined and melanocytes display a lentiginous growth pattern, making it difficult to determine the entire area in which the melanoma is localized.

Desmoplastic Melanoma (DM) is rare and only accounts for 1.7-4% of all melanomas. It is most common in males and the prevalence is between the age of 60 and 70 year, with a history of extensive sun exposure. It is commonly localized on the head and neck area but it can also occur in mucosal areas; it may presents as an amelanotic or flash-colored nodule. Its growth is highly invasive and has great propensity for local recurrence.

In some cases melanomas can arise in extracutaneous areas, such as the mucosal epithelium, or in the uvea. In general, the prognosis is not determined by the subtype,

but by an early detection: some melanoma subtypes are diagnosed in advanced stages and the late diagnosis indirectly affects prognosis [23, 25, 26].

1.3 Diagnosis

The most important factor that has a significant effect on patient survival is an early detection of melanoma. The stage of the tumor at the time of the diagnosis affects prognosis: for patients with melanoma in situ (MIS) life expectancy is the same as the general population [27].

The depth and the size of the neoplasm increases during progression, therefore it represents a prognostic sign. The cutaneous localization and the physical visibility of this type of cancer give the advantage of an early detection though non-invasive approaches. To recognize melanoma in its early stage, the "ABCD" rule was developed in 1985, and then it became "ABCDE". The ABCDE acronym stands for Asymmetry, Border irregularity, Color variegation, Diameter >6 mm and Dark, Evolving, which is especially important for the diagnosis of nodular melanomas (Fig. 3) [28].

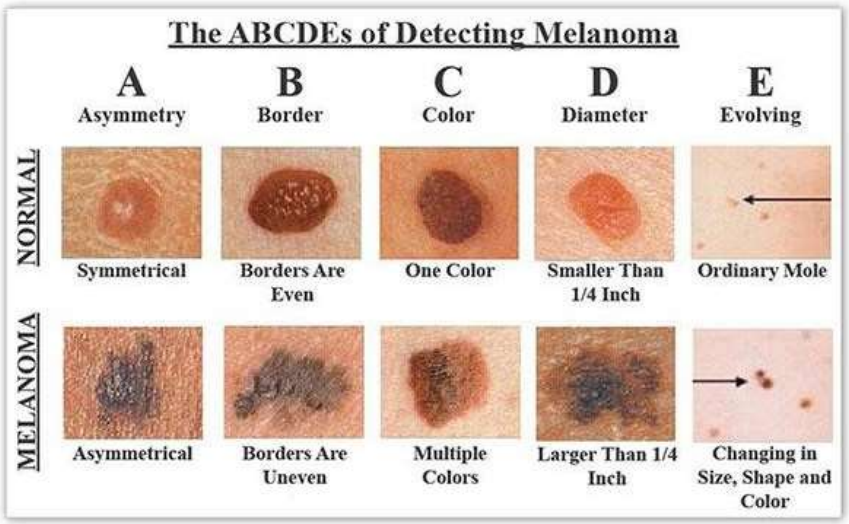


Fig. 3 ABCDE rule for clinical diagnosis of cutaneous melanoma.

Source: The Skin Cancer Foundation

This system allows to diagnose melanoma thanks to the observation of the features of the lesion and the examination of its history. This means the analysis of its evolution or

changes in its physical aspect, such as in shape, size and color. Melanoma usually appears as an irregularly pigmented skin lesion with an evolution of growth over time, that it does not involve only size and shape, but it might also include itching, bleeding or other subjective changes, such as the "ugly duckling" sign, individual lesions with different features from a person's typical nevus phenotype. In some cases primary cutaneous melanomas can be amelanotic and non-pigmented, and the diagnosis might be difficult [18].

The use of non-invasive diagnostic technique for the observation of the skin and a correct diagnosis is essential. Dermatoscopes or epiluminescent microscopes are devices that allow the visualization of morphological structures that are not visible to the naked eye and increase the accuracy of the diagnosis even when the lesion does not appear suspected at the first sight [29].

1.4 Staging and Prognosis

Pathologic staging is still the most important prognostic indicator in melanoma and is systematically defined by the American Joint Committee on Cancer (AJCC) Melanoma Staging Committee. This system is continuously evolving with regular updates to understand the biology and the progression of this cancer [30].

In the last edition of this system (8th edition, 2018), patients diagnosed before 1998 have been excluded, to omit patients who were managed without a standardized investigation and treatment protocol [31].

The National Comprehensive Cancer Network (NCCN) clarify that the most important factors to be included in the pathology report of melanoma are: (1) Breslow thickness (to the nearest 0.1 mm), (2) presence or absence of ulceration, (3) mitotic rate, (4) margin status (deep and peripheral), (5) presence or absence of microsatellites, (6) pure desmoplasia, (7) Clark level (for nonulcerated lesion, <1 mm, mitotic rate not determined) [32].

The synoptic reporting of the pathologic specimen recommended by the College of American Pathologists guidelines includes histologic type, tumor thickness, ulceration, macroscopic satellite nodules, microsatellite lesions, margins, mitotic rate,

lymphovascular invasion, neurotropism, tumor-infiltrating lymphocytes (TILs), and tumor regression [33].

Tumor Depth or Thickness

One of the most important prognostic factors is the depth or the thickness of the lesion, generally used to predict SLN positivity and recurrence free-survival [34, 35]. Breslow thickness was first described in 1970 by Alexander Breslow and represents the measure, in millimeters, of the vertical depth of invasion of melanoma [36]. Based on this classification, melanoma is considered “thin” (<1 mm), intermediate (1–4 mm), or thick (>4 mm). This stratification system is correlated with the prognosis and is used to determine the T stage.

Another classification system was previously proposed by Wallace Clark in 1969 and considers the anatomical layers of the skin invaded by the tumor (Clark’s level). In this system level I tumors are in situ melanoma and are confined in the epidermis, level II tumors extend to the papillary dermis, level III melanomas invade the papillary dermis, level IV arrive to the reticular dermis and level V tumors fill the subcutaneous fat [37]. Breslow system has become the most used system over the years, because of its easier classification and more accurate reliability.

Lymph Node Status and Sentinel Lymph Node Biopsy

Sentinel Lymph Node (SLN) is the first lymph node in which tumor cells metastasize and SLN biopsy represents a standard method for the evaluation of tumor stage. The status of SLN, the number of positive nodal status, the nodal tumor burden and extranodal extension are important prognostic factors for patient survival [38, 39].

Protocols recommend SLN biopsy for intermediate-thickness lesions and for thin and thick melanomas with specific clinical features [32].

Histopathologic analysis of the SLN is performed by hematoxylin and eosin staining, in association with the immunohistochemical staining for at least two immunohistochemical markers (such as S-100 and HMB-45 markers), in order to increase accuracy [40].

The highest association of SLN-positivity has been observed in ALM subtype [41].

Different criteria for SLN classification have been proposed. The Rotterdam criteria is based on the dimension of the tumor deposit (<0.1 mm, 0.1–1 mm, and >1 mm), that is associated with tumor positivity rate in non-sentinel nodes, while the Dewar criteria is based on the localization of tumor cells within the LN: subcapsular, parenchymal, combined, multifocal, and extensive. Subcapsular location is associated with best prognosis [42, 43].

In-transit Lesions

Tumor cells that have not reached the draining nodal basin and get trapped in dermal lymphatics are identified as “in-transit lesions” and give rise to small pigmented or non-pigmented nodules, observed in 5% to 10% of patients [44].

In-transit lesions are commonly found in patients with thick melanomas and positive SLNs and are associated with other malignant features, such as increased thickness and ulceration. They usually predict a poor prognosis with reduced disease-free survival (DFS) and overall survival (OS) [45].

Ulceration

Ulceration consists of loss of continuity of the epithelium overlying the lesion, and it is regularly accompanied by inflammation. It is a predictor of poor prognosis: ulceration has been shown to be associated with a more aggressive phenotype, with higher metastatization potential and with higher nodal positivity rate. A better response to interferon therapy has been observed in patients with ulceration [34, 46, 47].

Mitotic Rate

Mitotic rate is defined as mitoses/mm2, in the area with maximum mitosis within the lesion. It was included as a prognostic factor in the 7th AJCC staging, for its inverse correlation with survival. It has recently been excluded from the 8th edition, however it represents an independent predictor of SLN positivity and patient survival [48-50].

Lymphovascular Invasion

Lymphovascular invasion (LVI) is the condition in which tumor cells are found in the dermal lymphatics and blood vessels in the area surrounding the primary tumor. It is associated with SLN positivity and poor prognosis [51].

The detection of LVI is possible with staining techniques and the identification of lymphatic endothelial markers, such as D2-40 [52, 53].

Neurotropism or Perineural Invasion

Neurotropism or perineural invasion is identified as the invasion of perineural spaces by melanoma cells and it is associated with increased frequency of local relapse [54].

Tumor Margins

The most important thing during the surgical excision of melanoma is to obtain clear margins of the pathologic specimen. The presence of tumor cells in peripheral and deep margins must be assessed and the measure of the distance between the tumor mass and the margin measured. If deep margins are positive for tumor cells, the tumor depth is more than the reported one [32].

Tumor-infiltrating Lymphocytes

The presence of immune cells in the tumor bulk has been reported by Clark and colleagues [37]. Tumor-infiltrating lymphocytes (TIL) are lymphocytes infiltrated in the tumor nests and represent markers of good response to immunotherapy [55-57].

Clinical stage grouping

Clinical stage groups in the 8th edition are based on the Tumor-Node-Metastasis (TNM) system classification. Stage I and stage II include in situ melanoma. Tumor thickness and ulceration status are evaluated to define T subgroups. Stage III melanomas include patients with metastases localized in regional lymph node or satellite and/or in-transit metastasis. The number of metastatic nodes is a predictor of patient survival and, in association with the distinction between micro- and macro-metastases is used to identify subcategories. Stage IV melanomas indicate tumors with distant metastases.

Prognosis is poor and patient survival is very low. Patients classification is based on the site of the metastasis [31].

In the following tables TNM system and melanoma stages are described (Table 1,2) [31].

CHANGE	DETAILS OF CHANGE/HIGHLIGHT
Definition of primary tumor (T)	All principal T-category tumor thickness ranges are maintained, but T1 is now subcategorized by tumor thickness strata at 0.8-mm threshold
	Tumor mitotic rate is removed as a staging criterion for T1 tumors: T1a melanomas are now defined as nonulcerated and <0.8 mm in thickness; T1b is now defined as melanomas 0.8–1.0 mm in thickness regardless of ulceration status OR ulcerated melanomas <0.8 mm in thickness
	T0 definition has been clarified: T0 should be used to designate when there is no evidence of a primary tumor or that the site of the primary tumor is unknown (eg, in a patient who presents with an axillary metastasis with no known primary tumor); staging may be based on the clinical suspicion of the primary tumor with the tumor categorized as T0 (Tis, not T0, designates melanoma in situ)
	Tumor thickness measurements are now recorded to the nearest 0.1 mm, not the nearest 0.01 mm, because of impracticality and imprecision of measurements, particularly for tumors >1 mm thick; tumors ≤1 mm may be measured to the nearest 0.01 mm when practical but should be reported rounded to the nearest 0.1 mm (eg, melanomas measured to be anywhere in the range from 0.75 mm to 0.84 mm are reported as 0.8 mm in thickness [and hence T1b])
	Tis (melanoma in situ), T0 (no evidence of or unknown primary tumor), and TX (tumor thickness cannot be determined) may now be used as the T-category designation for stage groupings
Definition of regional lymph node (N)	The number of metastasis-containing regional lymph nodes is retained
	Previously empirically defined "microscopic" and "macroscopic" descriptors are redefined as "clinically occult" (ie, clinical stage I–II with nodal metastasis determined at sentinel node biopsy) and "clinically apparent" regional node disease (clinical stage III), respectively
	Sentinel node tumor burden is considered a regional disease prognostic factor that should be collected for all patents with positive sentinel nodes but is not used to determine N-category groupings
	Non-nodal regional disease, including microsatellites, satellites, and in-transit cutaneous and/or subcutaneous metastases, is more formally stratified by N category according to the number of tumor- involved lymph nodes (the presence of microsatellites, satellites, or in-transit metastases is now categorized as N1c, N2c, or N3c based on the number of tumor-involved, regional lymph nodes, if any)
	"Gross" extranodal extension no longer used as an N staging criterion (but the presence of "matted nodes" is retained)
Definition of distant metastasis (M)	M1 is now defined by both anatomic site of distant metastatic disease and serum lactate dehydrogenase (LDH) value for all anatomic site subcategories
	Descriptions of distant anatomic sites of disease are clarified in M subcategories
	Descriptors are now added to M1 subcategory designation that provides LDH values (designated as "0" for "not elevated" and "1" for "elevated") for all sites of distant disease; eg, skin/soft tissue/nodal metastases with elevated LDH are now M1a(1), not M1c
	A new M1d designation is added to include distant metastasis to the central nervous system (CNS), with or without any other distant sites of disease; M1c no longer includes CNS metastasis
	Elevated LDH level no longer defines M1c
AJCC prognostic stage groups	No overall change in T subcategories, but definitions of stages IA and IB are refined
	N category is now composed of 4 substages rather than 3, and stage III subgroupings are based on multivariable models, including T-category (tumor thickness and ulceration) and N-category (number of lymph nodes, satellites/in-transits/microsatellites) elements that demonstrate a significant impact of primary tumor factors in assigning N substage
	Clarified that stage IV is not further substaged (ie, M1c is stage IV, not stage IVC)

Table 1: Major Changes Introduced and Highlights of the Eighth Edition of the AJCC Melanoma Staging System.

Source: AJCC cancer staging manual 8th edition

WHEN T IS...	AND N IS...	AND M IS...	THEN THE CLINICAL STAGE GROUP IS...
Tis	N0	M0	0
T1a	N0	M0	IA
T1b	N0	M0	IB
T2a	N0	M0	IB
T2b	N0	M0	IIA
T3a	N0	M0	IIA
T3b	N0	M0	IIB
T4a	N0	M0	IIB
T4b	N0	M0	IIC
Any T, Tis	≥N1	M0	III
Any T	Any N	M1	IV

Table 2: AJCC Clinical Prognostic Stage Groups (cTNM)

Source: AJCC cancer staging manual 8th edition

1.5 Mutations implicated in melanoma development and progression

Malignant melanoma develops from the transformation of melanocytes, cells within the skin (but also eyes, mucosal epithelia and meninges) responsible for melanin production, the pigment that confers skin pigmentation and provides photo-protection. Melanin consist of two pigments: brown/black eumelanin and red pheomelanin. The key enzyme in melanin production is tyrosinase, responsible for tyrosine conversion in dihydroxyphenylalanine (DOPA). The signaling activity of the melanocortin receptor MC1R determines then the production of the two pigments. Since the intermediates are toxic for cells, melanin synthesis occurs in melanosomes, and when synthesis is completed, melanin granules are transferred to keratinocytes to protect skin from UV radiation [58, 59]. α- melanocyte-stimulating hormone (α-MSH) stimulates melanin synthesis through the binding to MCR1, activating intracellular cAMP-signaling and resulting in the activation of microphtalmia-associated transcription factor (MITF), the transcription factor involved in melanin synthesis and melanocytes differentiation [27].

It has become clear that clinical and histopathological features of melanomas are driven by specific genetic mutations. Genes that drive melanocytes development have been found mutated in melanomas, supporting that development, migration and

differentiation of melanocytes can be involved in the susceptibility of malignant transformation. The main player involved in melanoma development is the MAPK pathway: mutations in NRAS and BRAF have been found in more than 90% of melanoma cases and the constitutive activation of this signaling pathway in implicated in cell proliferation and survival [60]. The most common pathways found mutated in melanoma are showed in Table 3.

Pathway	Gene	Mutation	Subtype*	Progression phase*	Role
MAPK	*BRAF*	V600E	Non-CSD	Naevi	Initiation
	BRAF	V600K, K601E and G469A, among other clustered non-V600E alterations	CSD	Intermediate and MIS lesions	Initiation
	NRAS	Q61R and Q61K, among other less common alterations affecting codon 61 or 12	CSD	Intermediate and MIS lesions	Initiation
	NF1	Disabling mutations occurring throughout the gene and deletions	CSD	MIS	Initiation
Telomerase	*TERT*	Promoter mutations affecting hg19 coordinates 1,295,228 or 1,295,250, among less common, nearby mutations	CSD and non-CSD	Intermediate and MIS lesions	Progression
RB	*CDKN2A*	Deletions and disabling mutations occurring throughout the coding region	CSD and non-CSD	Invasive melanoma	Progression
Chromatin remodelling	*ARID1A*, *ARID1B* and/or *ARID2*	Disabling mutations occurring throughout the protein	CSD and non-CSD	Invasive melanoma	Progression
PI3K	*PTEN*	Disabling mutations occurring throughout the protein and deletions	Non-CSD	Thicker invasive melanomas	Advanced progression
p53	*TP53*	Disabling mutations occurring throughout the protein	CSD	Thicker invasive melanomas	Advanced progression

ARID, AT-rich interaction domain; CDKN2A, cyclin-dependent kinase inhibitor 2A; CSD, chronically sun damaged; MIS, melanoma in situ; NF1, neurofibromin 1; TERT, telomerase reverse transcriptase. *Subtype refers to the melanoma subtype(s) predominantly associated with the mutation. ‡Progression phase refers to the earliest progression phase at which the mutation typically occurs.

Table 3: Most common mutations in melanoma

Source: Shain et al, 2016

BRAF mutations

It has been shown that approximately 40-50% of melanoma patients have an activating mutation in BRAF and approximately 70-88% of these mutations involve a single substitution of glutamic acid for valine at amino acid 600 (V600E). Other mutations in BRAF comprise V600K, V600R, and V600M, accounting for 11–20%, 2–5%, and 1–4% of all BRAF mutated melanomas respectively. Less common BRAF mutations (5% of all melanomas) include non-V600 alterations at codons 466, 469, 597, and 601, and BRAF fusions [61-64].

BRAF mutation results in the enhanced activation of kinase function that leads to the activation via phosphorylation of MEK and ERK, independently of the upstream activation of RAS. The activation of MEK and ERK results in the promotion of cell growth

through the stimulation of G1/S transition of the cell cycle, the inhibition of proliferation inhibitors such as p27 and the inhibition of apoptosis [65, 66].

Different studies have shown the association between BRAF mutation and intermittent sun exposure instead of chronical sun exposure or unexposed skins [67]. BRAF mutation is also most commonly found in superficial spreading melanoma patients and occurs mainly in younger ages [61].

It has been observed that 70-80% of acquired melanocytic nevi have BRAF mutation, but the majority of them will not progress to tumor, suggesting that the mutation itself is not sufficient to drive malignant transformation of the nevi and other genetic mutations are necessary [68, 69].

Melanoma development requires the mutation of other signaling pathways. BRAF activation together with PTEN inactivation or CDKN2A and Lkb1 loss results in melanoma development in mouse models [70-73].

Phenotypic features of BRAF-mutant melanomas are the presence of nest of melanocytes, organized in little cluster instead of single cells, and the pigmentation due to the maintained melanin production [12].

RAS mutations

The first gene identified to be involved in melanoma development was RAS [74], that encodes for a GTPase located at the plasma membrane. The RAS family includes three proto-oncogenes: NRAS, KRAS and HRAS, involved in cell proliferation and apoptosis. Mutations at this level cause the activation of MAPK and PI3K downstream pathways, leading to cell cycle progression, growth and survival. The most frequently mutated protein of this family is NRAS (>20% of melanomas), generally in codon 61 and in less cases in codons 12 and 13 [64, 75]. NRAS mutation is associated with aggressive progression and poor prognosis and it has been observed that in most cases it correlates with non-exposed sites [76].

KIT mutations

KIT is a proto-oncogene coding for a transmembrane receptor tyrosine kinase located on the cell membrane involved in melanocytes development, proliferation,

differentiation, migration and survival. Its downstream signaling pathway activates MAPK, PI3K/Akt and JAK/STAT. KIT mutations occur in 1-3% of all melanoma cases, mainly in exon 11 or 13 [77], and are mainly reported in acral or mucosal melanomas (15%) and in chronically sun-damaged (CSD) skins [78, 79]. It has been reported that in KIT mutated melanomas BRAF mutations are not common. Acral and mucosal melanomas and CSD-melanomas show different characteristics from BRAF-mutant melanomas: the incidence is higher in old people with a lentiginous growth pattern. In normal conditions, KIT pathway is essential for melanocytes migration from the neural crest to the skin, and its reactivation is responsible for an increased lateral mobility of the transformed melanocytes, resulting in a poorly circumscribed lesion that reflects its augmented cell mobility [80].

NF1 mutations

NF1 (neurofibromatosis type 1) gene encodes for a GTPase protein that downregulates RAS activity, thus inhibiting MAPK signaling. Mutations of this gene consist of nonsense mutations, insertions or deletions, resulting in the activation of MAPK [81]. NF1-mutated melanomas represent the third most common subset of this type of cancer and account for 14% of The Cancer Genome Atlas (TCGA) melanoma samples [64].

CDKN2A mutations

CDKN2A is the primary gene identified to be involved in familial cases of melanoma. The product of this gene are suppressor proteins, p16INK4A and p14ARF, that take part in cell cycle regulation. p16INK4A inhibits cyclin-dependent kinase 4 and 6 (CDK4 and CDK6) functions, blocking the phosphorylation of the retinoblastoma protein (pRb) and resulting in cell cycle arrest in G1/S phase. p14ARF inhibits the degradation of P53, stimulating apoptosis activation. CDK2A mutations or epigenetic inactivation improve cell growth and survival. Only 1.2% of primary melanomas harbor these alterations, but it has been reported in 20-57% of families with at least three cases of melanoma [82].

TP53

TP53 is an onco-suppressor gene and it has been found mutated in about 15% of TCGA melanomas, most frequently in late-stage tumors and commonly in BRAF, NRAS and NF1 mutated melanomas [64].

PTEN

PTEN is a tumor suppressor protein that negatively regulates PI3K pathway, involved in cell growth and survival. PTEN mutations or deletions are found in <10% of melanoma cases, resulting in the activation of PI3K /AKT signaling [83]. PI3K itself is not mutated in melanomas, but PI3K/Akt pathway is activated in 53% of primary and 67% of metastatic melanomas (by both PTEN mutations and NRAS mutations), stimulating cell proliferation and tumor progression through the upregulation of cyclin D3 and the downregulation of p21 and p27 [66].

RAC1

RAC1 mutations have been reported in approximately 10% of sun-exposed melanomas and have been associated with BRAF or NRAS mutations. RAC1 mutations result in the activation of signaling pathways involved in proliferation and migration [84]. Some studies have reported that RAC1 mutations mediate resistance to BRAF and MEK inhibitors and may influence immunotherapy response, upregulating PD-L1 [85, 86].

TERT

TERT mutations account for 69% of all melanomas and 86% of cutaneous melanomas. TERT gene encodes for the telomerase reverse transcriptase and mutations generally occur in the promoter, resulting in an increased gene expression [87].

GNAQ/GNA11

Mutations in G-proteins have been described in approximately 90% of uveal melanomas and in some cases in non-uveal melanomas, but are rare in non-melanocytic tumors [88, 89]. GNAQ and GNA11 genes encode for Gq and G11 proteins respectively. Mutations of these proteins activate MAPK and PI3K/AKT pathways [90]. In particular, mutations of Gq protein result in its constitutive activation and augmented expression

of its downstream signaling, such as ERK [91]. GNAQ mutations have been found in blue nevi with a lower frequency in malignant tumors, while GNA11 mutations have an opposite trend: they are common in uveal melanoma metastasis and lower cases have been reported in blue nevi, suggesting that GNA11 may have a higher oncogenic potential than GNAQ [12].

Other mutations

MYC amplifications have been found in 8% of melanomas and are associated with a non-pigmented lesion and a poor prognosis [92]. Moreover, other evidences showed that MYC amplifications result in immune exclusion a T cell dysfunction in the tumor microenvironment [93].

In 10% of melanoma cases have been reported mutations in the WNT/CTNNB1 pathway, especially in APC and CTNNB1, which are responsible for few clinical implications but CTNNB1 has been involved in immune cell exclusion [94].

MITF amplifications occurs in 10% of primary and 20-40% of metastatic melanomas [95]. In normal conditions, microphtalmia-associated factor is involved in melanocyte differentiation, growth and development. Its activation can both stimulate the expression of cancer promoting and cancer suppressor genes, and the final effect depends on the balance of them [66].

Low frequency mutations with unclear clinical implications have been found in ARID2, PPP6C, MAP2K1, IDH1, RB1 [61, 96].

1.6 Therapeutic options

1.6.1 Surgical resection

The primary treatment for in situ melanoma is the surgical removal of the neoplasia and the surrounding healthy borders. In patients with a tumor characterized by ulceration or a thickness greater than 0.8 mm the surgery is associated with sentinel lymph node biopsy [97]. Sentinel lymph node and the other lymph nodes in the area are removed if melanoma cells are detected. For metastatic melanoma, surgery is not resolutive and need to be associated with other therapies [98].

1.6.2 Chemotherapy

Surgical resection for metastatic melanoma is not curative, thus patients need drug treatments. Chemotherapy has been the only treatment option available for patients until the development of more recent target therapy and immunotherapy [99].

The first clinical trial for metastatic melanoma was conducted in 1968, testing 1-phenylalanine mustard, that resulted toxic. The first chemotherapeutic drug approved by FDA for the treatment of melanoma was dacarbazine, an alkylating agent, in 1975 [97, 100, 101]. Studies reported that dacarbazine offers a median survival from 5 to 11 months and only 27% of 1 year survival rate [97, 100, 102, 103]. Despite the poor overall survival, dacarbazine is included in standard treatments alone or in combination with other drugs.

A dacarbazine analog, available for oral intake, is temozolamide. Due to the good oral bioavailability and the penetration in the central nervous system, is used to treat and prevent melanoma brain metastasis [104, 105]. However, clinical studies comparing the response of dacarbazine and temozolomide reported no significant differences between the two drugs, also in the treatment and the prevention of central nervous system metastasis [99, 102, 106].

1.6.3 Targeted therapy

Maliganant melanoma consists of several heterogeneous diseases carrying different genetic mutations involved in tumor development and progression. The identification of the signaling pathways implicated is crucial for the treatment choice.

BRAF inhibitors

The most common mutation in melanoma is in the *BRAF* oncogene, which is responsible for the constitutive activation of RAS–RAF–MEK–ERK-signaling pathway [107].

The first targeted drug approved by the FDA in 2011 for BRAF-mutated melanomas is vemurafenib, selective for BRAFV600E mutations. Vemurafenib has demonstrated anti-proliferative activity in BRAF-mutated cell line, with no effects on BRAF wild type cells. Phase I clinical trials showed significant clinical benefits [107-109]. Phase II study reported an increase in overall response rate and a complete response of 5%. Side

effects reported were photosensitivity, arthralgia, fatigue, alopecia, skin reactions, nausea, vomiting and diarrhea [110]. Increased overall survival with respect to dacarbazine treatment has been observed in phase III trial [111]. Despite these results, the response is limited due to the development of resistance [112].

Another monoclonal antibody against BRAF-mutated melanomas is dabrafenib, approved by the FDA in 2013. Dabrafenib showed activity against both BRAFV600Eand BRAFV600K mutations and a significant clinical activity against brain metastasis [112, 113]. Progression free survival and response rate to dabrafenib resulted to be similar to vemurafenib in controlled phase III trial conducted on patients with BRAFV600E mutation [114]. Limited response is due to the development of resistance and severe side effects [115].

MEK inhibitors

Other inhibitors of the MAPK pathway target MEK. Trametinib was approved by FDA in 2013 for melanoma patients harboring BRAFV600E or BRAFV600K mutations [112]. The mechanisms of action of trametinib consist in the induction of the cell cycle arrest through the inhibition of pERK1/2. In a phase III trial the efficacy of trametinib was compared with dacarbazine and paclitaxel and the progression free survival was reported to be 4.8 months in patients treated with the MEK inhibitor and 1.5 months in the group treated with chemotherapy [116]. Another study highlighted the increase in the overall survival: it showed that in patients treated with trametinib the 6 months overall survival was 81%, while in patients treated with chemotherapy the overall survival was 67% [117]. In patients previously treated with BRAF inhibitors, trametinib showed a reduced activity [118].

KIT, PI3K/Akt and VEGF pathway

KIT-mutated melanoma represents a small percentage and frequently occurs from acral, mucosal, and chronically sun-exposed skins. c-KIT mutations have been reported to be associated with the development of drug resistance to other therapeutic strategies in melanoma patients. This mutation has been suggested as an emerging target and c-KIT inhibitors have been studied in clinical trials. Imatinib, sunitinib, dasatinib, and nilotinib

have been investigated and results showed a good response to therapy [119]. These results have led to the approval of KIT-targeted therapy for metastatic and unresectable melanoma, in association with immunotherapy [120].

The inhibition of the the PI3K/Akt/mTOR pathway has shown anti-cancer activity in melanoma cell lines and different compounds are under investigation in clinical trials. Rapamycin, a mTORC1 inhibitor, have shown anti proliferative activity in melanoma cell lines [121, 122]. Everolimus and temsirolimus, two rapamycin analogs, showed inhibition of tumor growth in preclinical studies. Results were not confirmed in phase II clinical study for everolimus, but it exhibited antiangiogenic activity [123]. Temsirolimus, in association with temozolomide, reduced tumor progression in vemurafenib-resistent melanoma cells [124].

High VEGF production is correlated with poor prognosis in metastatic melanoma patients. Moreover, VEGF is associated with immune suppression, neoangiogenesis and progression. Bevacizumab is a VEGF inhibitor that showed results in different tumors, but clinical trial in melanoma patients did not confirmed an improvement of the disease [125].

1.6.4 Immunotherapy

Melanoma is known to be an immunogenic tumor. Melanomas are able to downregulate tumor associated antigens and melanoma differentiation antigens, resulting in the inhibition of the recognition by the immune system [55, 126]. Moreover, melanomas have been reported to produce immune modulatory molecules to inhibit the immune system, such as immune suppressive cytokines, transformation growth factor beta (TGF-β) and prostaglandin E2 (PGE2) [55, 127-129].

As knowledge of cancer cells escape the immune system and immune cells react against cancer, researches have been conducted to redirect the immune system in order to stimulate an anti-cancer response [98].

The first immune therapy approved for melanoma was interleukein-2 (IL2) in 1992, which stimulates melanoma T-cells but the percentage of response was low and the toxicity high [100, 101, 126, 130]. In the 1990's several anti-cancer vaccines have been

developed to sensitize the immune system against tumor antigens, however no vaccines have passed clinical trials [131-134].

Immune checkpoint receptors on immune cells regulate the immune response. Programed cell death protein 1 (PD-1) on T-cells membrane is activated by PD-1 ligand (PD-L1/2) on somatic cells surface. The activation of PD-1 inhibits the immune response, triggering apoptosis in T-cells. PD-L1/2 has been found overexpressed in cancers, including melanoma, and allows cancer cells to "switch off" the immune system and escape immune clearance [98, 135].

Another immune checkpoint receptor is cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which has a similar function as PD-1. This receptor is expressed on T-reg cells and it binds to B7-1/2 receptor on APCs, repressing the immune response [101, 136]. On the other hand, CD28 on T-cells binds to B7-1/2 activating the immune system response [136-138].

Immune checkpoint inhibitors (ICI) are antibodies against PD-1, PDL1/2 and CTLA-4 that inhibits the binding to their ligand, resulting in the stimulation of the immune system response [55, 101, 136-138]. Thus, the use of immune checkpoint inhibitors restores the anti-tumor activity of the immune cells. In melanoma treatment, the first ICI was approved in 2011 [55, 130, 139]. Ipilimumab, an anti-CTLA-4 antibody, nivolumab and pembrolizumab, two anti-PD1 antibodies, are the three ICIs that have been approved for melanoma treatment [138].

Ipilimumab is an ICI against CTLA-4 and showed a significant improvement of survival rates at 3 years in metastatic melanoma patients. In 20% of melanoma cases it showed survival of up to 10 years. The most common adverse effects reported observed were rashes, colitis, hepatitis and endocrinopathies [140].

Nivolumab and pembrolizumab were approved in 2014. In metastatic melanoma patients, 37-38% of them showed a response to pembrolizumab treatment and overall survival of 74% at 12 months has been observed [141]. About 40% of patients exhibited a response to nivolumab and 12 month overall survival was 73%, compared with 43% of patients treated with dacarbazine [142]. Fatigue, pruritus and diarrhea were the sides effects observed in ICI treatment [140].

2. Cancer stem cells

Cancer stem cells (CSCs) are a rare subpopulation of the tumor bulk characterized by the capacity of self renewal and the tumor-generating potential [143]. They are able to give rise to the tumor mass when transplated in vivo in mice thanks to their high tumorigenicity [144-148]. They represents the main drivers of malignant growth, resistance to therapy and metastases [149, 150].

Different methods have been developed to isolate CSCs form cancer cell lines in vitro or in patient-derived tumors. The most used method in cancer research is the CSCs-enrichment of the cell culture through stem-cell-specific medium. This particular cell culture system allows CSCs selection and spherical colonies formation which grow in suspension [147, 151]. Moreover, by fluorescence-activated cell sorting (FACS) it is possible to isolate a pure population based on the expression of CSC-specific markers. [147, 152-154].

Different specific cell makers to detect CSCs have been identified (i.e., CD44+/CD24−/low for breast cancer, CD44+/CD24+/ESA+/CD133+ for pancreatic cancer, CD133+ for glioma), but the existence of a specific marker for melanoma SCs is still under study [155]. CD271, CD133, ATP-binding cassette transporters (ABCs), metabolic features (ALDH activity) have been proposed for melanoma, but they have been found on different cell subclones [156-159].

2.1 Surface markers

CD271, also known as p57, is one of the most used cell surface makers in the study of melanoma SCs but its reliability is still under debate. Its expression could be associated with cell undifferentiation, since it is usually expressed in the neural crest during embryonic development, but it has been reported that it can be expressed in 2.5% to 40% of melanoma cells [156, 160-163]. Other studies suggest that CD271 could be involved in melanoma aggressive behavior, progression and prognosis, rather than stemness [164, 165].

The other most common used marker for CSCs in melanoma research is CD133 (Prominin-1), identified in murine neuroepithelial stem cells and related to primitive

human cell differentiation. It seems to be involved in tumor progression and aggressiveness [166], but its role in tumor SCs is still controversial [159, 167, 168].

2.2 Markers associated with drug-resistance

Drug resistance in tumor cells is mediated by different factors. One of these is the expression of ATP-binding cassette (ABC), usually expressed in CSCs [169].

ABCB5 was found to be correlated with higher tumorigenic potential, tumor progression and the expression of other SCs markers, such as CD133 [170-173]. Similar results have been reported for ABCG2, another ABC transporter, found to be associated with stemness in different tumors [174, 175]. Moreover, ABCG2+ cells are characterized by higher autofluorescence compared with ABCG2- cells, probably due to the influx of riboflavin, thus autofluorescence itself has been proposed as a SCs marker in tumors [163, 176-178].

2.3 Intracellular markers

Enzymes such as aldehyde dehydrogenases (ALDH), transcription factors such as Sox2 and Klf4 have been suggested as CSCs markers. ALDH1 has been reported to be overactivated in different tumor SCs, including melanoma [179, 180]. Sox2 and Klf4 are considered as pluripotency markers and they have been associated with a SCs phenotype, since they induce cell dedifferentiation, proliferation and inhibition of apoptosis. Moreover, they have been reported to induce tumor initiation and treatment resistance [181-184].

2.4 EMT markers and tumor progression

Tumor progression is often associated with metastases formation, the process in which cancer cells escape from the primary tissue and colonize distant sites [184]. Before spreading, tumor cells undergo a phenotypic switch that involves the modulation of the expression levels of cell-matrix or cell-cell adhesion molecules (i.e., integrins, cadherins, EpCAM, cytokeratins) [185].

In the last years, several studies strongly supported the involvement of CSCs in the growth and dissemination of tumors, including melanoma [186-189]. Indeed, different

CSCs surface markers have been found to be expressed both on CTCs and at the metastatic sites [190]. CD271, CD133, ABCB5, ABCG2 and ALDH have been shown to be associated with augmented tumorigenic properties, increased metastases incidence and resistance to therapy [165, 186, 191-195]. Moreover, large evidence reported that the knockdown of stemness marker expression suppressed melanoma metastatic ability in vitro and in vivo and increased the anti-proliferative activity of standard drugs [172, 196].

Epithelial to mesenchymal transition (EMT) is a cellular process that play a role in tumor progression. This phenotypic switch allows cancer cells to lose adhesion properties and acquire invasive and migratory abilities [197, 198]. EMT is also responsible for the de-differentiation of tumor cells into cells with SCs features, such as SCs superficial markers and self-renewal capacity [154, 199-202]. In particular, the transcription factors Slug, Snail, Zeb, Twist, Sox, MITF (Melanogenesis-associated Transcription Factor) and ESRP1 (Epithelial Splicing Regulatory Protein1) were described to be involved in the acquisition of SCs features by melanoma cells. In melanoma cells, Twist1 and Zeb1 (but not Twist2 and Zeb2) promote the switch to a staminal phenotype, in particular Zeb1 is associated with stem cells markers CD133 and CD44 in murine melanoma cells (B16F10) [202-205] and it suppresses the epithelial marker E-cadherin in both murine and human melanoma cells [206, 207]. Moreover, the suppression of EMT-TFs reduces the metastatic potential of melanoma stem cells.

Another factor found to be involved in EMT and stemness in different tumors is IGF-1 (insulin-like growth factor-1). IGF-1 silencing in murine B16F10 melanoma cells decreases Zeb1, mesenchymal markers and stem cells markers (CD133, Sox2, Oct3/4), and IGF-1 suppression results also in a reduction of melanospheres formation and ALDH1 activity [208]. Furthermore, in melanoma cells, IGFBP5 (Insulin-like Growth Factor Binding Protein 5) downregulates the expression levels of stem cell markers (CD133, Sox2, Oct4) [209].

Moreover, melanoma progression is associated with the 'mesenchymal-to-ameboid' transition (MAT), a process related to the expression of EphA2 (ephrin type-A receptor

2) and RacN17 (a dominant-negative form of Rac1), in which melanoma cells acquire stemness marker and an increased metastatic potential [210].

These evidences support that CSCs play a crucial role in melanoma growth, dissemination and metastases. Moreover, stem-like cells are generated during EMT through the loss of cell differentiation [143].

2.5 CSCs and metabolic reprogramming

Metabolic reprogramming is a process occurring in most types of cancer during neoplastic transformation [211-213]. Cancer cells, to support their fast and uncontrolled growth, need high amount of energy and nutrients, thus they display augmented glycolysis and mitochondrial pathways. The increased glycolytic activity in cancer cells, also with oxygen availability, was first described by Otto Warburg in 1956 and is now known as the "Warburg Effect" [214]. Now it is largely confirmed that cancer cells also display increased mitochondrial activity with augmented oxidative phosphorylation (OXPHOS), upregulated expression of electron transport chain (ETC) complexes and elevated oxygen consumption [215, 216]. Another metabolic pathway increased in cancer cells is the fatty acid synthesis and the glutaminolysis, which leads to the conversion of glutamine into glutamate that enters into the TCA cycle (tricarboxylic acid cycle) [217, 218]. Metabolic reprogramming has been found also in melanoma, with a different adaptation based on the specific genetic mutation and different characteristics that reflect the high heterogeneity of this type of cancer [218-222].

Since CSCs are responsible for tumor resistance and recurrence, they need a high amount of energy and also CSCs undergo a metabolic rewiring to support their functions [216, 223, 224]. Data from literature support that CSCs metabolism could rely on different metabolic pathways depending on the tumor type and on the mutations pattern. Breast and hepatocellular carcinoma are characterized by a SCs subpopulation that is dependent on glycolysis: it has been reported high levels of glucose transporter GLUT1, high expression of glycolytic enzymes and decreased levels of gluconeogenic enzymes. On the other hand, other studies showed that CSCs from ovarian, lung, pancreatic and prostate cancer rely on an increased mitochondrial metabolism to

supply their energy demand [149, 225-229]. In these tumors, CSCs display increased mitochondrial mass, OXPHOS activity and PGC1-α expression levels [230, 231]. For these reasons mitochondrial mass has been proposed as a new metabolic biomarker for CSCs identification in different tumors and a new pharmaceutical target for their eradication, since it has been associated with chemo-resistance (Fig. 4) [149, 150, 232-236].

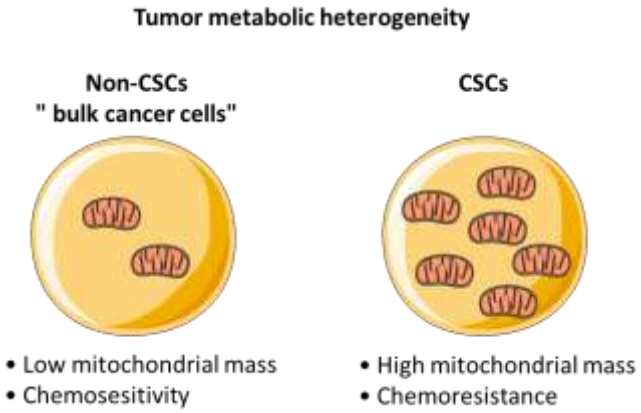


Fig. 4: The relationship between mitochondrial mass, "stemness" and chemo-resistance in cancer cells.

Other studies reported a rewiring of lipid metabolism in CSCs. Increased level of intracellular lipids, fatty acid synthase (FASN) activity, cholesterol synthesis have been shown to be associated with stemness features in different tumors and CSCs were shown to depend on fatty acid oxidation [149, 237-241]. To increase intracellular lipid levels, CSCs exploit CD36 transporter on their membranes, to allow lipid influx from the microenvironment [242]. CD36 was found to be associated with increased intracellular fatty acids, augmented cell proliferation and metastatic potential and it is considered a poor prognosis factor [243]. Fatty acids and cholesterol esters are normally stored in "lipid droplets" (LD), organelles developing from Golgi or ER membranes [239]. In colorectal, breast and ovarian cancer it has been reported an association between LD and stemness features. Moreover, LD content in lipid biopsies (i.e., blood) is now accepted as a biological marker to evaluate tumor aggressiveness [244].

Another metabolic pathway exploited by CSCs is glutamine (Glu) metabolism. Glu enters cells through ASCT2 (SLC1A5) transporter and it is metabolized to provide aminoacids, nucleotides and lipids [238, 245]. Glu is converted in α-ketoglutarate that fuels the TCA cycle for ATP production. An hyper activation of the Glu-aspartate-oxalacetate pathway has been reported also in CSCs: an augmented expression of ASCT2, Glu synthetase and glutaminase 1 has been found in CSCs and ASCT2 knockdown has been reported to suppress colon cancer SCs. Moreover, it has been observed that CSCs resistant to standard drugs are coupled with ad altered glutamine metabolism and its inhibition restored their sensitivity to the anti-cancer treatment [237, 238, 246-249].

Also melanoma SCs metabolism has been studied to better understand their features and behavior. Melanoma cells escaping the anticancer activity of the BRAF inhibitor vemurafenib are still able to form melanospheres when cultured in the specific tumor cell medium, thus supporting that resistant cells possess stemness features [163, 177, 178, 250]. It has been reported that melanoma cells resistant to the anticancer activity of BRAF and MEK inhibitors upregulate the expression of the mitochondrial biogenesis activator PGC1α and this results in increased OXPHOS activity (Fig. 5) [251-253].

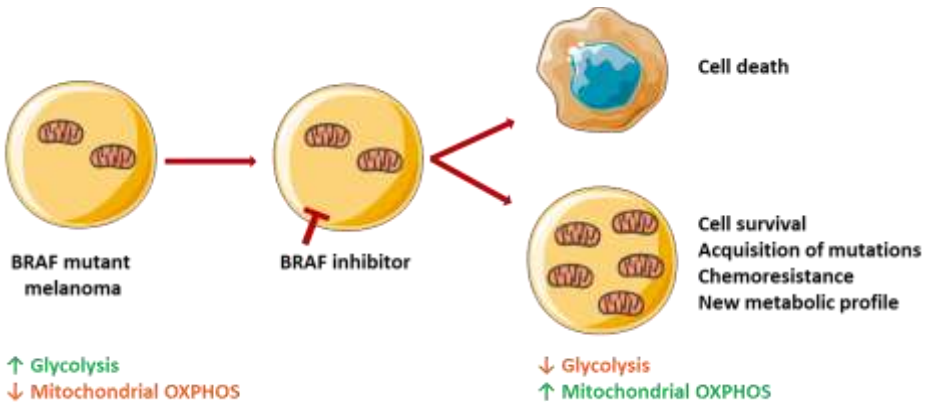


Fig. 5: metabolic reprogramming in BRAF-mutated melanoma cells treated with BRAF-inhibitors.

Moreover, BRAF inhibition has been associated with changes in mitochondrial dynamics in melanoma cells: BRAF-treated cells have been found to be characterized by increased mitochondrial fusion and decreased mitochondrial fission (Fig. 6) [143, 253].

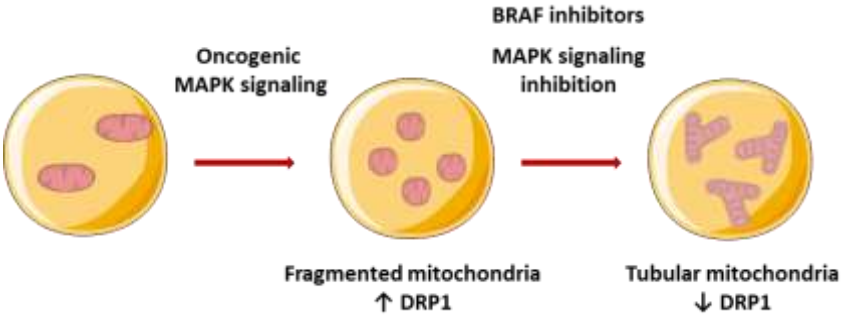


Fig. 6: MAPK-mediated mitochondrial dynamics remodeling in melanoma cells.

It has been reported that cells resistant to BRAF and MEK inhibitors, together with increased mitochondrial biogenesis and fusion, have an augmented ER-mitochondria Ca^{++} axis: the treatment triggers ER stress pathways and results in Ca^{++} release from ER and its influx into mitochondria. In mitochondria, the increase in Ca^{++} levels stimulates OXPHOS mechanism and the enzymes involved, leading to an improved mitochondrial respiration [253, 254].

Given these data, it has been developed a 'mitochondrial based oncology platform' to study and individuate CSCs in different tumors [231]. Metabolomics analysis revealed that ABCB5 positive melanoma cells display increased glycolysis, increased membrane phospholipid metabolism and glutaminolysis [255]. Other studies revealed that mitochondrial phosphoenolpyruvate carboxykinase 2 (PKC2) expression is decreased in melanoma SCs. This results in the release of citrate from mitochondria that fuels glycolysis and fatty acids synthesis [256]. Moreover, PKC1 was found to be upregulated in melanoma SCs and this was associated with glucose consumption and the glycolytic pathway [257]. Other evidences reported that β3- adrenoreceptor mediates a reduction in mitochondrial activity with increased glycolytic pathway [258].

Also lipid metabolism undergoes alteration during cancer development. It has been demonstrated that melanoma cells displaying high lipid content have high metastatic potential and the expression of CD36 receptor was found to be associated with self-renewal and metastatic behavior [243, 259]. High lipid content was described in CD133+ melanoma cells, that are characterized by LD accumulation and high expression of SREBP-1 (sterol regulatory element- binding protein-1) and PPAR-γ (peroxisome

proliferator-activated receptor-γ) [259]. Monounsaturated fatty acids are synthetized by SCD1 (stearoyl-CoA desaturase 1), that has been found to be upregulated in CSCs in different tumor types. Melanoma cells resistant to MAPK inhibitors show increased SCD1 levels accompanied by augmented Hippo pathway, that is known to be associated with CSCs features. The inhibition of SDC1 activity revealed a reduction in resistant stem cells [260].

Given these data, the metabolic reprogramming of melanoma stem cells is still controversial and remains under debate. Moreover, metabolic alterations of CSCs might represent a novel target for anti-cancer drugs in different type of tumors, including melanoma [143, 236].

2.6 Melanoma SCs and intracellular signaling pathways

CSCs play a role in tumor development, progression, EMT, treatment resistance and other malignant behaviors [154, 186, 249]; different are the molecular pathways involved in these mechanisms and their identification might help find novel molecular targets. Several signaling pathways have been found to be involved in melanoma SCs, such as TGFβ, Notch, Wnt/β-catenin, Hedghog (HH) [186, 261-264].

TGFβ (transforming growth factor beta) plays a key role in cell growth, differentiation, EMT and stemness [265-267]. TGFβ produced by mesenchymal SCs stimulates B16 mouse melanoma cells to metastasize, to increase migration and invasion, to undergo EMT [268, 269]. Moreover, Nodal, a member of the TGFβ family, is involved in melanoma chemoresistance and vasculogenic mimicry [270, 271]. Another growth factor involved in melanoma progression is IGF-1 and its inhibition prevent tumor metastases and EMT process and stemness features and markers (Sox2, Oct3/4, CD133, ALDH activity, sphere formation) [209]. The Notch receptors family is composed of different cell surface receptors that are highly activated in cancer cells and are associated with CSCs markers and features [263, 264, 272, 273]. Notch1 was found to be upregulated in melanoma cells, leading to metastatic progression, stemness, CD133 expression, invasion and metastases [186, 274]. Wnt signaling pathway has been demonstrated to support stemness, controlling CSC expansion, EMT, self-renewal, resistance to therapies, mobility, polarity and stem cells markers [261]. Another

pathway activated in melanoma SCs is the Hedgehog (HH) signaling, that has been reported to be activated in melanoma SCs, contributing to EMT, melanosphere formation and ALDH expression [275, 276].

2.7 Melanoma SCs, chemoresistance and novel targets

Different are the mechanisms involved in resistance to therapy and tumor recurrence: drug efflux, metabolism reprogramming, DNA repair capacity, uncontrolled growth, genetic mutation amplification, quiescence, immunosuppression, cell plasticity, cell death escape and tumor heterogeneity (Fig. 7) [277-280].

CSCs, compared with others tumor subpopulations, possess typical traits and abilities that allow them to survive and escape the antitumor activity of standard treatments [187, 281-284].

The expression of 'ATP-binding cassette (ABC) transporters', such as ABCB5, ABCG1, ABCG2, ABCG5, on CSCs membranes permits to extrude chemotherapeutics out of cells [285-287]. Elevated activity of ALDH has been observed in cells resistant to taxanes, cyclophosphamide, doxorubicin and its activity allows the transformation of chemotherapeutic drugs into inactive compounds [283, 288, 289]. The overexpression of Bcl-2 protein and the over-activation of the Notch/Hedgehog signaling are some of the mechanisms involved in the resistance to therapy [290, 291]. The capacity to repair DNA confers to CSCs resistance to radiotherapy: it has been observed that CD133+ glioblastoma cells can escape cell death thanks to efficiently repair of the DNA when damaged by radiations [292]. Moreover, CSCs activate anti-oxidant scavengers against treatment-induced ROS [293]. Given these observations, it is clear that CSCs are involved in the development of resistance.

It has been demonstrated that BRAF-mutated A375 melanoma cells treated with vemurafenib were still able to form melanospheres [163] and BRAF and MEK resistance has been found to be associated with increased Sox2 its downstream target CD24 [181]. Another study showed that vemurafenib and binimetinib resistant melanoma cells have higher expression of YAP/TAZ stem cell markers [260] and dabrafenib resistant cells have higher expression of CD20 stem cell marker [294].

Similar results were observed in melanoma cells resistant to chemotherapy. Paclitaxel has been found to increase ABCB5 expression in mouse B16F10 melanoma cells, dacarbazine treatment resulted in CD133+ subpopulation upregulation and Doxorubicine and methotrexate resistance has been associated with integrin β3 [295-297].

Based on these evidences, a strategy to overcome drug resistance might be the targeting of CSCs features and markers, in order to eradicate this aggressive subpopulation. An example is the target of CD20, a well known marker of melanoma SCs: it has been reported that CD20 targeting inhibits tumor growth in vivo and it decreases inflammatory markers [298, 299]. Innovative formulations have been developed to target this resistant subpopulation, such as liposomes and nanoparticles. Liposomes containing vincristine and directed against CD20+ cells were reported to reduce tumor growth in mice [300] and salinomycin- loaded lipid polymer nanoparticles significantly reduced CD20+ cells proliferation, metastatic potential and melanospheres formation [301]. Similar results were achieved with antibodies against CD133 [302]. The AC133-saponin is a complex composed of the drug saponin and a monoclonal antibody against CD133 that specifically inhibits CD133-positive cells proliferation [294]. Morin, a natural compound from Moraceae family, was reported to reduce cell growth, sphere formation ability and the expression of CSCs markers CD133, CD20 and CD44 in melanoma cells [303]. The co-treatment with antibodies targeting CD47 and CD271 was shown to inhibit the development of metastasis in melanoma patient-derived cells xenografted in nude mice [193].

In recent years the reliability of surface markers to identify CSCs has been discussed. Recently autofluorescence and the ABCG2 transporter have been proposed as novel CSCs markers [176]. In A375 melanoma cells, ABCG2 has been associated with stemness features and the natural compound δ-tocotrienol was found to decrease its expression, melanosphere formation and spheres disaggregation [163]. Another stemness marker is ALDH and it has been shown that it is responsible for the activation of nifuroxazide in ALDH positive cells, resulting in the inhibition of their stemness features [304]. A signaling pathway involved in stemness is Wnt/ β- catenin and its targeting through

pimozide has an anticancer effect [305]. Another compound affecting Wnt pathway is 35b, that counteracts melanospheres formation and ALDH activity [306].

As discussed above, mitochondrial reprogramming is an hallmark of CSCs. It has been shown that antibiotics (tetracyclins, erythromycins, glycylcyclines) targeting cells mitochondria are able to eradicate CSCs and these results were confirmed by clinical trials [233]. The inhibition of mitochondrial functionality through mitochondrial complex V inhibitors such as oligomycin A counteracts breast cancer spheres formation in vitro [232].

Thus, the identification of specifics markers to individuate CSCs is necessary to target and eradicate this aggressive subpopulation and overcome drug resistance and tumor relapse [143, 149, 150].

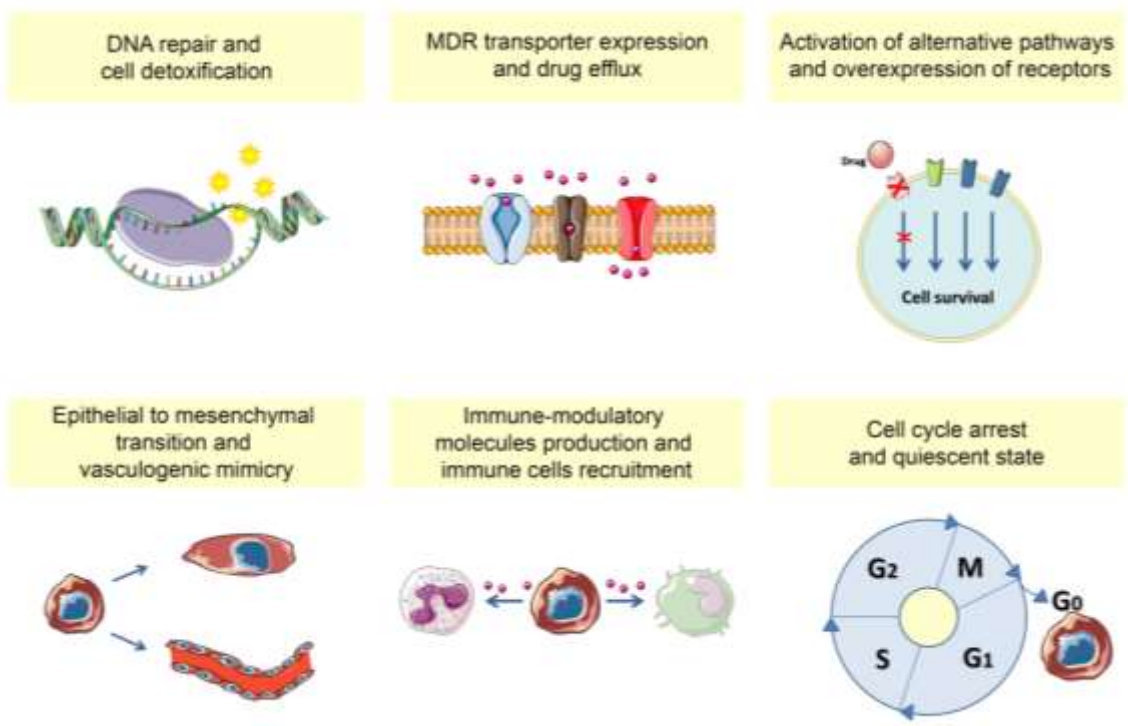


Fig. 7: Mechanisms of aggressiveness and resistance in cancer stem cells.

3. Natural compounds, cancer and cell death mechanisms

3.1 Nutraceuticals and anti-cancer activity

The term “nutraceutical” refers to “food, or parts of a food, that provide medical or health benefits, including the prevention and treatment of disease” and was first coined in 1989 by Dr. Stephen De Felice. It includes natural foods and vegetables rich in phytochemicals (such as polyphenols, flavonoids and carotenoids) with medical properties [307]. Different natural compounds have been found to exert health benefit properties, including anti-cancer activity, targeting signalling pathways involved in tumor development and progression. Some natural compounds, such as taxanes, vinca alkaloids and podophyllotoxin analogues, are currently used in chemotherapy, others have been tested in vitro, pre-clinical and clinical studies [308]. A lot of phytochemicals have demonstrated anti-cancer activity in melanoma: polyphenols (flavonoids, curcumin and resveratrol), organosulfur compounds (sulforaphane), terpenoids (artemisinin, oridonin and ursolic acid), saponins (ginsenosides), tocotrienols (γ- and δ-isoforms), alkaloids (berberine, harmine and capsaicin) and hydroxycinnamic acids (caffeic acid and its phenethyl ester) [309].

The study of natural compounds and their anti-cancer effects is aimed not only at finding new therapeutic strategies, but also at clarifying the molecular mechanisms. Numerous natural compounds were found to trigger canonical (apoptosis) and non-canonical (paraptosis) cell death mechanisms, involving several pathways such as endoplasmic reticulum (ER) stress, autophagy, mitochondrial dysfunction, ions homeostasis dysregulation [309].

3.2 Apoptosis

Apoptosis is the most common known type of cell death. In this process cells commit a suicide after a certain stimuli using energy. It was first described in 1972 and it is involved physiological processes and pathological conditions [310]. The main morphological features observed in apoptosis have been described by light and electron

microscopy [311]. In the first phases of apoptosis, cell size decreases and cytoplasm appears dense, with organelles packed and chromatin condensation (shrinkage and pyknosis). Cell shape appears round or oval and with aggregates of chromatin fragments under the nuclear membrane. The next step is the rupture of nuclear membrane, with release of its content (karyorrhexis). Cell membrane undergoes a process named “blebbing” and cell is fragmented into “apoptotic bodies”, vesicles surrounded by plasma membrane that are phagocytised by macrophages. This process involve single cells or only small clusters and occurs without triggering an inflammatory response. Apoptosis can be classified in intrinsic (mitochondrial) or extrinsic (death receptors), depending on the pathways involved [312, 313].

The extrinsic pathway is activated by surfaces receptors of the tumor necrosis factor receptor superfamily (TNFRS), that, when activated by extracellular ligands, transmit death stimuli and stimulate intracellular pathways. A death-inducing signalling complex (DISC) is recruited, pro-caspase-8 is activated and pro-caspase-3, after cleavage, executes the apoptotic process [314, 315].

The intrinsic pathway is known as “mitochondrial” and it is regulated by Bcl-2 family enzymes [316]. This family is composed of two groups of proteins: the anti-apoptotic proteins inhibit cell death, while the pro-apoptotic proteins stimulate the release of cytochrome C from mitochondria that recruits apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9, forming the complex “apoptosome”, which stimulate caspase-9/3 signaling cascade [317-319].

Apoptotic caspases have been divided in initiator caspases (caspase-8 and -9) and executioner caspases (caspase-3, -6, and -7). Proteolytic activity of caspases results in the cleavage of proteins, regulator of apoptosis, DNA repair proteins and cell cycle proteins [320, 321].

Another feature of apoptosis is the expression of cell surface markers known as “eat me” signals, such as phosphatidylserine, annexin I, calreticulin. These markers are recognised by phagocytes and lead to the elimination of cells and apoptotic bodies without the generation of an inflammation response [322].

3.3 Apoptosis in cancer

One of the mechanisms involved in drug resistance and tumor progression is the evasion of cell death. Several mechanisms are exploited by cancer cells to survive to anti-cancer treatments: a dysregulation of pro-apoptotic/anti-apoptotic proteins balance has been observed in the Bcl-2 protein family, in the guardian of the genome p53 and in the inhibitor of apoptosis proteins (IAPs), but also a downregulation of different cell death receptors has been reported in various tumors [323]. Given these evidences, triggering alternative cell death mechanisms could be a strategy to overcome apoptosis resistance and eliminate resistant cancer cells [324].

3.4 Paraptosis: a non-canonical cell death

Paraptosis is a non-canonical programmed cell death described for the first time by Sperandio et al. in 2000 [325]. The main typical feature observed in paraptosis is cytoplasmic vacuolization, deriving from mitochondrial swelling and/or ER dilatation (Fig. 8). Since it requires energy and protein synthesis, this type of programmed cell death can be blocked by the protein synthesis inhibitor cycloheximide [326].

It has been shown that during paraptosis caspases are not activated and, as a consequence, caspases inhibitors do not prevent this type of cell death; moreover, cell is not fragmented in apoptotic bodies. Paraptosis is characterized by the activation of mitogen-activated protein kinase (MAPK) family members, such as c-Jun N-terminal protein kinase 1 (JNK1), p38 and extracellular signal-regulated kinase (ERK1/2) and it is inhibited by the multifunctional adapter protein AIP-1/Alix [327].

This non-canonical cell death is often accompanied by reactive oxygen species (ROS) production, ER stress and the dysregulation of Ca^{++} homeostasis (Fig. 8). ER is composed of membranous tubules and sacs starting from the nuclear membrane and it is involved in protein synthesis, folding and post-translational modifications. The condition in which unfolded proteins are accumulated in ER is defined ER stress and it is responsible for the activation of pro-death signals. Together with mitochondria, ER is involved in the control of intracellular Ca^{++} and its homeostasis depends on several channels. Ca^{++} levels regulate the activity of ER chaperone proteins responsible for protein correct folding, such as BiP, calnexin, calreticulin and PDI [328, 329].

It has been reported that several natural compounds cause paraptosis in cancer cells triggering these mechanisms. Thapsigargin, a blockade of SERCA channel derived from the T. garganica, and tunicamycin, an inhibitor of N-acetylglucosamine transferase, extracted from S. lysosuperificus, have been tested in preclinical studies [330, 331].

However, features described in paraptosis are not always visible and the expression "paraptosis-like cell death" has been coined to describe this situation, in which cell death is characterized by paraptosis-features without one or more of these characteristics [332, 333].

Paraptotic cell death has been described during neural development, during neurodegeneration and in different neurological diseases [334]. In addition, paraptosis has been documented to be implicated in pathologies affecting retina after glucocorticoid treatment, reperfusion injury and glaucoma [335-338].

Several mediators of paraptosis have been identified: epidermal growth factor (EGF) [339, 340] insulin-like growth factor 1 receptor (IGFR-1) [327], TAJ/TROY, an orphan TNF receptor family member [341], the neuropeptide substance P [342] and adenine nucleotide translocase 1 (ANT1) [343].

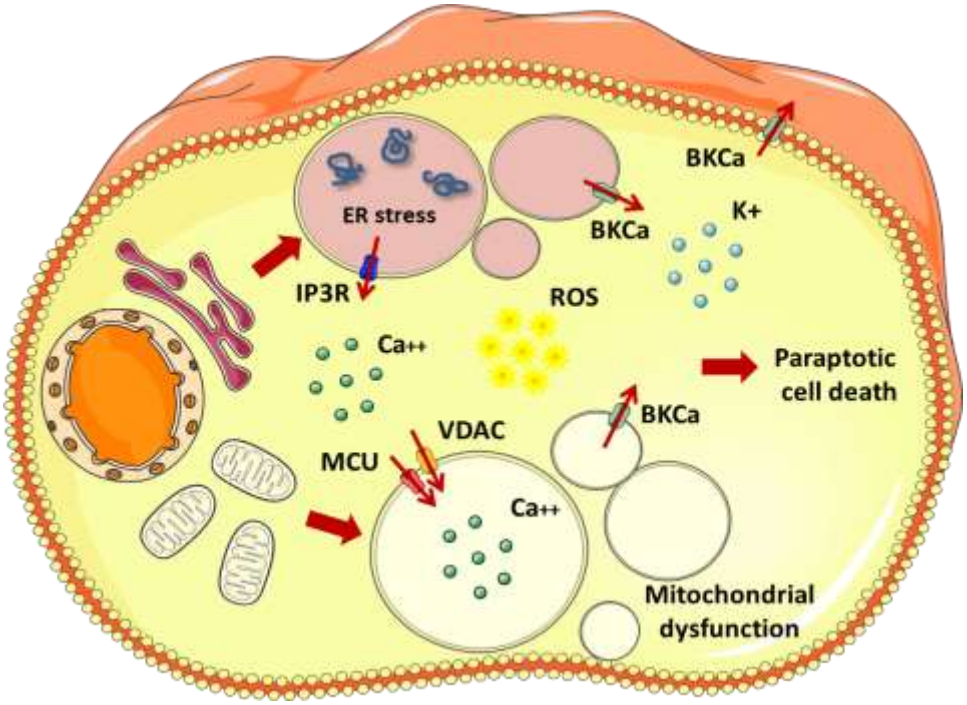


Fig. 8: Mechanisms involved in paraptotic cell death.

3.5 Mitochondrial dysfunction, ROS production and calcium homeostasis dysregulation

Mitochondrial dysfunction

Mitochondria play a key role in different cell deaths, such as apoptosis and paraptosis and different pro-death signalling such as ER stress and autophagy. Mitochondria are highly dynamic organelles with close interaction with ER and they are involved in the control of metabolism, Ca^{++} homeostasis and ROS production. Mitochondria are responsible for ATP generation via oxidative phosphorylation (OXPHOS) mediated by electron transport chain (ETC), in addition to metabolites oxidation by tricarboxylic acid (TCA) cycle and fatty acids oxidation by β-oxidation. Therefore, mitochondrial dysfunction leads to redox homeostasis alteration, ions dysregulation and metabolic impairment. Mitochondria impairment is responsible for ROS production [344-347] which can lead to cell death, both canonical (apoptosis) and non-canonical (paraptosis, necroptosis) [348-351].

Oxidative stress

Reactive oxygen species originate from molecular oxygen after redox reactions involving electron transfer. These reactions give rise to superoxide anion radical $O2^{\bullet-}$ and then hydrogen peroxide (H_2O_2). These reactions can be spontaneous or can be the results of the enzyme superoxide dismutases (SOD). $O2^{\bullet-}$ and H_2O_2 can react with iron in the cell and generate hydroxyl radicals that react and damage proteins, RNA, DNA and lipids, spreading the free radical chain. In the presence of nitric oxide or fatty acids, ROS react and produce peroxynitrite or peroxyl radicals. Superoxide can cross organelle membranes through ion channels and ROS produced into the mitochondrion can be released into the cytoplasm via voltage-dependent anion channels located on the outer mitochondrial membrane [352]. High ROS levels can be produced after stress stimuli, both endogenous and exogenous. The role of mitochondrial ROS is not completely understood: ROS have been considered only a result of a mitochondrial dysfunction, however they can be involved in different signalling pathways, including cell proliferation, migration and death [353].

In physiological conditions, about 1% of the electrons moved through the ETC generates superoxide [354]. Complex I (site IQ) and complex III (site IIIQo) of the ETC are the main responsible for superoxide generation in mitochondria and ROS produced can be released in the cytosol [355]. Mitochondrial ROS production depends on the metabolic state of the cell: the availability of substrates of the respiratory chain, the mitochondrial membrane potential, the matrix pH and the oxygen concentration directly affect ROS generation in mitochondria [356].

Calcium homeostasis and overload

Intracellular Ca^{++} is involved in different signalling pathways and it represents a second messenger, regulating cell metabolism, gene expression, contraction and cell death/survival [357]. Calcium channels are transmembrane proteins (either voltage-dependent, or receptor-operated) which allow ions influx into the cells without energy consumption, while calcium pumps (ATP-ases) permit calcium flow in a ATP-dependent mechanism.

Ca^{++} concentrations are different depending on cell compartments: Ca^{++} levels are higher in the endo/sarcoplasmic reticulum (ER/SR) and the Golgi apparatus (ranging from 300 to 1000 μM) [358, 359], while levels are lower in in the cytoplasm, mitochondrial matrix and peroxisomal lumen (between 100 and 500 nM), where it represents the modulator of signalling pathways [360].

Ca^{++} influx into ER is mediated by the sarco/endoplasmic reticulum calcium pump (SERCA) channel [361-363], while its release is mediated by inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3R) [364] or ryanodine receptors (RyR) [365, 366]. In non-excitable cells, the IP_3R located on the ER membrane, after the activation mediated by inositol phosphate 3 (IP_3), opens and allows Ca^{++} release from ER to the cytosol, resulting in a significant increase in cytoplasmic Ca^{++} levels. The increase in cytoplasmic Ca^{++} levels promotes the activation of pathways involved in protein synthesis, transcriptional regulation, metabolic control, cytoskeletal remodelling and secretion [367].

The Na^{+}/Ca^{++} exchanger and Ca^{++} ATPase on cell membrane have been found involved in Ca^{++} removal from the cell [368].

Membrane voltage-dependent anion channel 1 (VDAC1), on the outer mitochondrial membrane, is responsible for Ca^{++} influx into mitochondria, modulating its access into the mitochondrial intermembrane space [369]. The outer mitochondrial membrane is highly permeable to Ca^{++}, while the inner mitochondrial membrane represents a more selective barrier for Ca^{++} influx into the matrix [370] and mitochondrial calcium uniporter (MCU) has been found to be the Ca^{++} selective channel on the inner mitochondrial membrane [371-373].

Mitochondrial Ca^{++} depends on MMP: the voltage generated by OXPHOS complexes across the inner mitochondrial membrane drives Ca^{++} uptake, that consequently affects mitochondrial functionality, energy production and cell death [353, 374-376]. Indeed, energy metabolism is controlled by mitochondrial Ca^{++} levels, which affects NADH production, modulating enzymes involved in TCA and fatty acid oxidation [353].

Mitochondrial Ca^{++} levels are fundamental for the homeostasis of cell physiology. After stress stimuli, the increase in intracellular and mitochondrial Ca^{++} over physiological levels leads to the opening of the voltage- and Ca^{++} - dependent high-conductance channel (also known as the mitochondrial permeability transition pore: mPTP), located in the inner mitochondrial membrane, causing the activation of apoptotic cell death [377, 378]. In cancer cells, the dysregulation of Ca^{++} homeostasis mediated by several compounds has been associated with ER stress, ER dilatation, mitochondrial swelling and paraptosis [332].

Calcium-ROS interplay and MAMs

A close bidirectional interconnection between Ca^{++} levels and ROS production has been reported. Ca^{++} levels have been found to modulate ROS production and redox state regulates Ca^{++} levels [353, 379, 380].

In physiological conditions Ca^{++} controls ROS generation from complex I and III, however, it is involved in ROS overproduction after the pharmacological inhibition of these complexes [381]. Ca^{++} stimulates Krebs cycle and OXPHOS, promoting ATP synthesis. An augmentation of the metabolic rate is accompanied by an increase in oxygen consumption and as a consequence ROS production [381]. The increase in ATP synthesis leads to mitochondrial membrane potential reduction and ROS production

augmentation, however, an extensive Ca^{++} influx into mitochondria results in ROS generation independently of the metabolic activity [382].

The opening of the voltage and Ca^{++} -dependent mitochondrial permeability transition pore (mPTP) leads to a higher permeability of the inner mitochondrial membrane [383], accompanied by a depolarization of the MMP, a reduction in oxygen consumption and an increase in ROS production and Ca^{++} flow. These events cause the swelling of mitochondria and the opening of the OMM with the release of intermembrane proteins, triggering the activation of cell death mechanisms. mPTP opening depends on both Ca^{++} and ROS levels, suggesting that these two mechanisms can generate an amplification loop that affects mPTP activation [353, 379].

Several cytotoxic molecules have been reported to trigger cell death through mPTP opening, causing Ca^{++} overload [384, 385]. Since cancer cells are able to reduce mPTP sensitivity to Ca^{++} levels improving their resistance to treatments, this channel could be a target to improve therapy response [385].

Ca^{++} overload and mitochondrial ROS have been reported to be involved in different pathological conditions, such as ischemia [386], neurodegenerative diseases [387], osteoarthritis and sepsis [388, 389]. Furthermore, mitochondrial alteration and dysfunction have been found implicated in cancer growth and development and mitochondria have been proposed as a promising target for cancer treatment [390, 391].

Another mechanism that has been found associated with cell death, ROS and calcium overload is ER stress [353, 379]. ER is involved in protein assembly and folding, thus it contains chaperones, folding enzymes, ATP and calcium. To ensure disulphide bond formation it is characterized by an oxidizing environment. ER function impairment is known as “ER stress” and it activates a cascade of signaling pathways, aimed at restoring cell homeostasis or activating pro-death mechanisms [392, 393].

ER stress is characterized by a rapid increase in ROS in ER lumen and Ca^{++} release to the cytosol, through IP_3R or RyR [393]. ER is responsible for about 25% of intracellular ROS, required for the generation of an oxidative environment necessary for the correct protein folding and disulphide bonds formation. These physiological ROS are generated

by protein disulfide isomerase (PDI) and endoplasmic reticulum oxidoreductin-1 (ERO1α) [394]. ERO1α upregulation during ER stress leads to an extensive oxidation over normal conditions: this change in the redox state affects the interaction between IP_3R and endoplasmic reticulum resident protein 44 (ERp44), causing Ca^{++} release from ER and triggering pro-death mechanisms [395].

Mitochondria and ER interacts with each other thanks to physical contacts named mitochondria-associated ER membranes (MAMs), involved in the exchange of lipids, metabolites and calcium [396]. In these sites different channels regulates Ca^{++} passage. VDAC on the OMM has been reported to be physically associated with the IP_3R on ER membrane through the glucose-regulated protein 75 (GPR-75) molecular chaperone. These interactions allow Ca^{++} influx and accumulation in mitochondria [397].

It has been reported that VDAC channel is involved in the transport of superoxide anions and, since the IP_3R is sensitive to redox changes, a mutual interaction between mitochondrial ROS and Ca^{++} has been reported. The impairment of mitochondrial functionality has been shown to affect redox homeostasis, with increase in ROS production both in mitochondria and, due to the presence of MAMs, in ER [398, 399].

Stress events causing Ca^{++} overload are responsible for Ca^{++} release from ER and its subsequent influx into mitochondria. The calcium flow causes changes in mitochondrial pH, thereby increasing ROS production and altering MMP (Fig. 9). This alteration triggers the opening of the mPTP, which allows the release of cytochrome c and the activation of calcium-dependent proteins [400]. These events cause a self-amplifying loop, in which Ca^{++} dysregulation cause an increase in ROS production and ROS increase Ca^{++} flow [401]. Hence, ER stress triggers mitochondrial Ca^{++} overload, ROS generation and ATP reduction, resulting in the activation of pathways involved in cell death [402].

The dysfunction of Ca^{++}/ROS homeostasis at the ER-mitochondria interface has been found implicated in several disorders. In neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's diseases and Alzheimer's disease, it has been reported a disruption of the Ca^{++}-ROS balance in neurons involved [403, 404]. Ca^{++} overload accompanied by mitochondrial ROS has been described also in ischemic

diseases, myocardial infarction, diabetes and other metabolic diseases and inflammatory diseases [405, 406].

ER-mitochondrial Ca^{++} and ROS have been found implicated also in tumor progression and survival [407]. On the other hand, this axis involving ER stress/calcium/ROS has been described involved in cell death induced by several chemotherapeutics [408, 409].

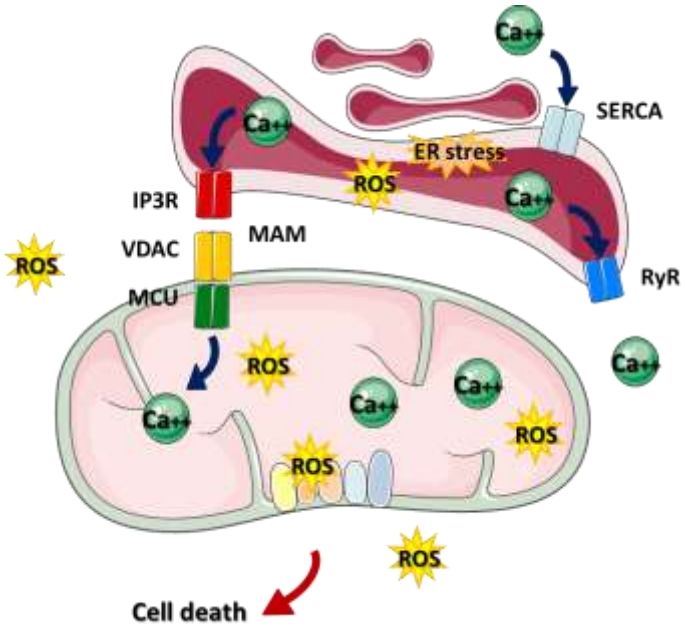


Fig. 9: ROS and calcium crosstalk.

3.6 Paraptosis and natural compounds

Several natural compounds have been reported to induce cell death in tumor cells through the activation of paraptosis, showing promising effects: celastrol, chalcomoracin, cis-nerolidol, cyclosporine A, curcumin, DL-selenocystine,gambogic acid, ginsenosides, hesperidin, honokiol, manumycin A, morusin, ophobiolin A, plumbagin,procyanidins, taxol, tocotrienols, tunicamycin, yessotoxin, 1-desulfoyessotoxin, 6-shogaol, 8-p-hdroxybenzoyl tovarol and 15-deoxy-Δ12,14-prostaglandin J2 [326, 410].

3.7 Tocotrienols

Vitamin E was first discovered by Herbert Evans and Katharine Bishop in 1922 [411]. Vitamin E family is composed of 8 hydrophobic compounds classified in two groups: α-, β-, γ- and δ-tocopherols (TPs) and α-, β-, γ- and δ-tocotrienols (TTs). These compounds are constituted by a chromanol ring that is linked to an isoprenoid side chain, which is saturated in TPs and unsaturated in TTs. Each compound of TPs and TTs differs from the others in the substituents on the chromanol ring: the α and β TPs and TTs are trimethylated, the γ isomers are dimethylated and the δ isomers are monomethylated (Fig. 10) [178].

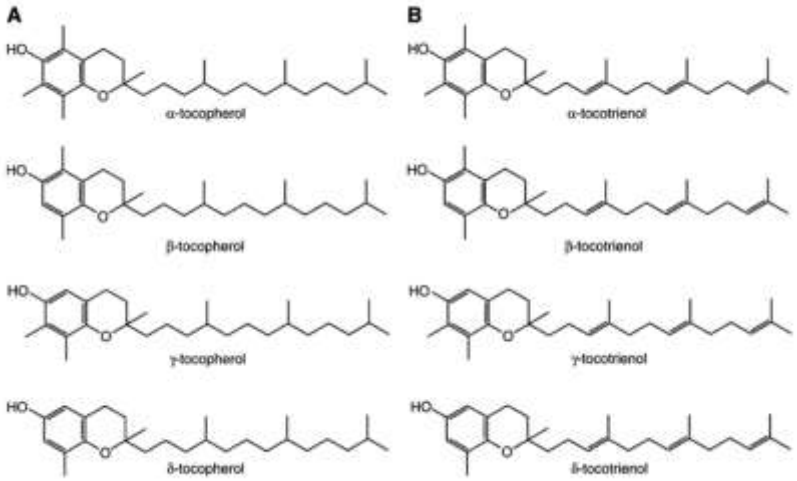


Fig. 10: Chemical structure of TPs and TTs.

Source: Shahidi et al, 2016

TPs and TTs are absorbed in the small intestine through the action of the bile salts and then transported through the blood into liver lipoproteins, thanks to α-TP transport protein-mediated sequestration. It has been shown that TPs have higher affinity than TTs for α-TP transport protein, and this results in lower TTs intestinal absorption and increased catabolism in the liver [412, 413]. Despite these evidences, it has been reported high activity in vivo after oral administration, and no side effects or toxicity have been observed [414, 415]. Moreover, in healthy human subjects and patients with breast cancer TTs demonstrated high bioavailability and safety [416-418].

TTs are mainly found in cereals, grains, seeds, nuts and vegetal oils. The richest sources are represented by annatto (*Bixa orellana L.*) seeds (150 mg δ-TT/100 g dry seeds with no TPs) [419-422].

In the last decade TTs have been largely studied for their health benefits properties. They have been shown to exert cardio- and neuro- protective activity and have been demonstrated preventive and ameliorating properties in hyperlipidemia, inflammation, diabetes and osteoporosis [423-426]. Moreover, it has been largely shown a potent anti-cancer, anti-metastatic and anti-angiogenetic activity in a wide range of tumors (Fig. 11) [326].

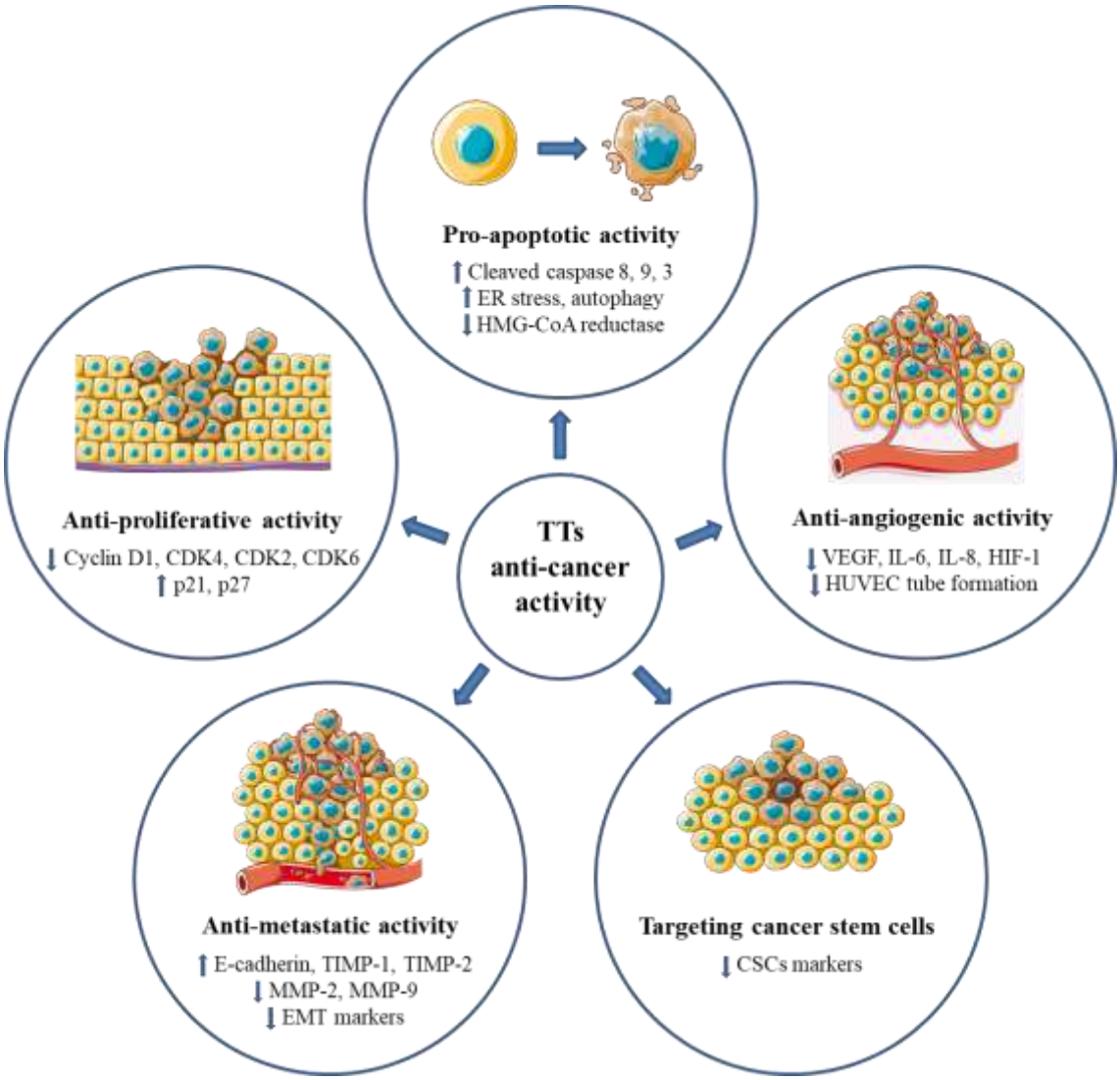


Fig. 11: Anti-cancer activity of TTs.

Source: Fontana et al, 2019

Tocotrienols in melanoma and other skin cancers

In A375 and BLM human melanoma cell lines it has been shown that δ-TT induces apoptosis triggering ER-stress pathway. In particular, Marzagalli et al. have shown that δ-TT exerts a dose-dependent-cytotoxic effect in A375 and BLM melanoma cell lines, with no effect on melanocytes. They demonstrated that δ-TT activates intrinsic apoptosis, increasing Bax/Bcl-2 proteins ratio, releasing cytochrome *c* from mitochondria and inducing pro-caspase-3 and PARP cleavage. The anti-proliferative (as evidenced by the inhibition of colony formation) and proapoptotic effects were accompanied by the activation of ER-stress signaling pathway: after δ-TT treatment, BiP, PERK, IRE1α, p-eIF2α, ATF4 and CHOP were found to be upregulated. These results were confirmed in preclinical models of A375 melanoma cell xenografts in nude mice: it was found that δ-TT significantly decreased tumor volume, tumor mass and tumor progression [250]. Moreover, in A375 cell line it has been identified a subpopulation of ABCG2-expressing cells characterized by higher autofluorescence, stem-cells markers and high resistance to vemurafenib treatment. Unlike BRAF-inhibitor vemurafenib, δ-TT has been demonstrated to be able to target ABCG2+ cells in A375 cell line, counteracting melanosphere formation ability and inducing sphere disaggregation. These data clearly indicate that this natural compound is able to target the most aggressive sub-population in A375 cells [163]. γ-TT has been found to induce apoposis, suppress invasion, downregulate mesenchymal markers levels and restore E-cadherin and γ-catenin expression in malignant melanoma cells [427]. In B16 mouse melanoma cells, TTs were reported enhance sensitivity to baicalein and lovastatin, resulting in tumor growth inhibition [428, 429]. In A431 and SCC-4 human keratinocyte cancer cells, a nano-emulsified TTs delivery system has been tested demonstrating anti-proliferative activity [430]. To overcome bioavailability problems, transferrin-bearing multilamellar vesicles entrapping α-TT have been developed, to exploit transferrin receptors to deliver drugs to the tumor bulk. This innovative delivery system was found to suppress tumor growth in A431 human epidermoid carcinoma cancer cells and B16-F10 murine melanoma cells lines. No side effects has been reported in mice bearing A431 and B16-F10 tumors after intravenous administration of these vesicles [431].

Tocotrienols in prostate cancer

In prostate cancer (PCa) cell lines TTs were reported to exert anti proliferative, pro-apoptotic and paraptotic activity, accompanied by a sever dysfunction of mitochondrial metabolism and function, ROS production and Ca^{++} overload [432-435]. In PC3 and DU145 castration-resistant PCa cells γ-TT was found to downregulate CD133 and CD44 CSCs expression, thus counteracting anchorage-independent growth and spheroidogenic ability [436]. Moreover, TTs potentiate the anti-cancer activity of lovastatin and geranylgeraniol, through the downregulation of HMG-CoA reductase [437, 438].

Tocotrienols in breast cancer

In breast cancer cells γ-TT was reported to inhibit proliferation and induce cell death, triggering both intrinsic and extrinsic apoptosis [439-441]. It has been found that δ-TT inhibits cholesterol synthesis through the downregulation of HMG-CoA reductase activity [442] and the accumulation of TTs in lipid draft microdomains has been reported to dysregulate and inactivate HER-2 receptors and their downstream signaling pathways [443]. Moreover, TTs have been demonstrated to exert anti-metastatic and anti-angiogenic properties and they were shown to eliminate breast CSCs [444-447]. A synergic effect has been reported combining TTs with erlotinib, gefitinib, statins, celecoxib and different natural compounds [177]. A clinical trial showed that TTs given in combination with tamoxifen reduced the mortality risk, but results were not statistically significant, probably for the small sample size of the experiment [448].

Tocotrienols in cervical cancer

In HeLa cervical cancer cells TTs were reported to inhibit proliferation through the downregulation of cyclin D3, p16 and CDK6 and the upregulation of Interleukin-6 (IL-6) [449]. γ- and δ-TT -induced apoptosis in cervical cancer cells was found to be associated with ER stress, Ca^{++} overload and dysregulation of lipid metabolism [450]. Finally, γ-TT was found to target CSC subpopulation growth [451].

Tocotrienols in colon cancer

In RKO human colon cancer cells, a TRF combination was found to induce intrinsic apoptosis [452] and in HCT-116 cells γ-TT was reported to affect lipid metabolism [453]. In SW620 and HCT-8 human colon carcinoma cells TTs induce paraptosis [454, 455]. A synergistic antitumor activity of γ-TT has been observed with capecitabine, atorvastatin, celecoxib, 6-gingerol [456-458].

In DLD-1 human colorectal adenocarcinoma cells δ-TT was found to inhibit VEGF, IL-8 and COX-2 synthesis and results were confirmed also in vivo [459-461].

Tocotrienols in gastric cancer

It has been found that γ-TT induces apoptosis in human gastric cancer cells through the suppression of the MAPK signaling [462]. It has been shown to exerts anti-metastatic and anti-angiogenic activity through the downregulation of MMP-2 and MMP-9, the upregulation of tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 [463] and the counteraction of the hypoxia-mediated HIF-1α and VEGF synthesis [376]. Moreover, γ-TT was reported to improve the anticancer activity of capecitabine in vitro as well as in nude mice [464].

Tocotrienols in lung cancer

In lung cancer δ-TT inhibits cell proliferation and tumor growth through the upregulation of miR-34a, which leads to decreased expression of Notch-1, Hes-1, cyclin D1, survivin and Bcl-2 [465]. In addition, δ-TT was found to potentiate cisplatin and lovastatin activity [429, 466].

Tocotrienols in pancreatic cancer

In pancreatic cancer cells it has been shown that δ-TT induces apoptosis and cell cycle arrest through p27Kip1 [467]. Moreover it inhibits HMG-CoA reductase [467], HER2 [468] and EGR-1/Bax pathway [469]. It downregulates EMT biomarkers (such as N-cadherin, vimentin and MMP9) reducing the invasive potential and it downregulates Oct4 and Sox2, thus reducing pancreatic ductal adenocarcinoma (PDAC) stem-like cells [470]. Tocotrienols derivative or combinations have been tested in vitro, showing an improvement of the anti-cancer activity [471, 472].

AIM

Malignant melanoma represents the most aggressive form of skin cancer. An early diagnosis of a melanoma in situ is usually associated with a good prognosis, because it allows the surgical removal of the entire lesion. If melanoma is not diagnosed in time, it could invade surrounding tissues, becoming invasive and giving rise to distant metastases. Different therapeutic options are available to improve patient survival, depending on the tumor subtype, the stage and the mutation pattern. Therapy includes chemotherapy, radiotherapy, targeted therapy and immunotherapy. Unfortunately, the high level of melanomas heterogeneity can result in low response, drug resistance and tumor recurrence.

In cancer therapy several are the main causes of drug resistance. First, the resistance to apoptosis. In fact, cancer cells are able to escape programmed cell death, de-regulating apoptotic pathways and leading to drug resistance. For this reason, a strategy could be exploiting the induction of non-canonical cell deaths, such as paraptosis, a programmed cell death with different features from apoptosis, characterized by extensive cytoplasmic vacuolization related to ER dilatation and mitochondrial swelling, ROS production and calcium overload.

Another cause of drug resistance is the presence of CSCs. CSCs are a rare subpopulation of the tumor bulk, characterized by the ability to generate all heterogeneous tumor cell lineages, to give rise to the tumor mass and to recapitulate continuous tumor growth. They play a crucial role in treatment resistance and they contribute to tumor relapse. CSCs have been shown to be coupled with metabolic alterations and several studies have proposed new metabolic markers for their identification.

For these reasons it is necessary to find novel compounds exploiting alternative cell death mechanisms and it is necessary to identify specific markers in order to characterize and target different cell subpopulations. Thus, this project focused on two tasks: 1) the study of the anti-cancer activity of δ-TT in melanoma cells 2) the identification of a new metabolic marker and target for melanoma SCs.

The first task of this project was to assess the role paraptotic cell death in the δ-TT-mediated cytotoxicity in A375 and BLM cells. We previously demonstrated that δ-TT exerts a pro-apoptotic effect on A375 and BLM human melanoma cells triggering ER

stress, while sparing normal melanocytes; the aim of this work was to analyze the induction of paraptosis and the molecular mechanisms associated with its activity. We first analysed morphological changes induced by δ-TT to clarify cytoplasmic vacuolization formation and the activation of MAPK signaling. We then evaluated mitochondrial functional and structural impairment. Since mitochondria are also involved in the regulation of redox and ions homeostasis, we focused on the clarification of the mechanisms underlying the pro-death pathways activated by δ-TT, with special regard to ROS production and calcium homeostasis dysregulation. Finally we investigated the relationship between ROS and Ca^{++} signaling.

In the second part we focused on melanoma SCs, in particular on SCs mitochondria: we investigated their role as a marker of stemness and as target for the anticancer activity of δ-TT. In melanoma SCs we analysed mitochondrial protein expression and proteins involved in mitochondrial dynamics, in order to characterize melanosphere model. Then we evaluated δ-TT activity on melanoma SCs mitochondria, to verify the target of this compounds in CSCs. Finally, preliminary experiments have been conducted on melanoma cells resistant to vemurafenib, in order to confirm mitochondria as a possible target to overcome drug resistance.

MATERIALS AND METHODS

Chemicals

δ-TT was purified from a commercial extract of Annatto seeds (*Bixa orellana*) (kindly provided by American River Nutrition Inc., Hadley, MA, USA), as previously described [473].

For western blot analysis the following primary antibodies were utilized: JNK (9252), p-JNK (4668), P38 (8690), p-P38 (4511), ERK(4695), p-ERK (4370), AMPK (5832), p-AMPK (5832), OPA1 (80471), MFN2 (9482), Drp1 (14647), GAPDH (5174) (all from Cell Signaling Technology Inc., Danvers, MA, USA), β-tubulin (8328) (from Sigma-Aldrich, Milano, Italy), Total OXPHOS (ab110413), PGC1-α (ab110411) (from Abcam, Cambridge, UK). Most of the antibodies were utilized at the concentration 1:1000; tubulin at 1:2000 and PGC1-α at 1:500. Horseradish-peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents were from Cyanagen (Bologna, Italy).

For flow cytometry ABCG2 antibody (NB110-93511AF405) from Biotechne, USA was used.

The translation inhibitor cycloheximide, NAC (N-acetyl-L-cysteine - the ROS scavenger), 2APB (2-Aminoethyl diphenylborinate – the blocker of IP3R), DIDS (disodium 4,4′-diisothiocyanostilbene-2,2′-disulfonate - the blocker of VDAC), and analytical grade solvents were from Sigma-Aldrich.

Cell cultures

The human A375 melanoma cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). A375 mutation status: BRAFV600E mutated NRAS wild-type.

The human BLM melanoma cell line was provided by Dr. G.N. van Muijen (Department of Pathology, Radbound University Nijmegen Medical Center, Nijmegen, The Netherlands). This cell line is a subline of BRO melanoma cells isolated from lung metastases after subcutaneous inoculation of nude mice with BRO cells; mutation status: BRAF wild-type, NRAS mutated.

A375 and BLM cells were cultured in DMEM medium supplemented with FBS (7.5% and 10% respectively), glutamine and antibiotics. Cells were cultured in humidified

atmosphere of 5% CO_2/95% air at 37°C. Original stocks of cells were stored frozen in liquid nitrogen; after resuscitation, cells were kept in culture for no more than 10-12 weeks. Cells were detached through trypsin-EDTA solution and passaged once/week. Cell lines were recently authenticated by Short Tandem Repeat (STR) analysis as described in ANSI Standard (ASN-0002) by ATCC Standards Development Organization (SDO).

To obtain A375 melanospheres, A375 cell line was grown in Euromed-N medium, supplemented with 10 ng/ml EGF, 10 ng/ml FGF2 and 1% N2 in normal flasks. Floating tumor spheres with stem features were formed within 5-7 days. To enrich melanoma cell culture of initiating-tumor cells, floating spheres were harvested and passaged every ten days, or once reached 100 to 150 µm diameter size, otherwise, cells in the center of the sphere will undergo apoptosis. The supernatant in the flask was recovered, and centrifuged for 5 minutes at low speed (100xg), because spheres are quite fragile. Moreover, since their volume is higher than single cells, centrifugation at low speed allows to separate voluminous melanospheres from single cell-suspension. Spheres were then resuspended in 3 ml Euromed-N, mechanically dissociated, and splitted in new 25-cm2 flasks. 1 ml Euromed-N was added every 48 hours to the culture, to provide fresh medium to the cells. Conditioned medium derived from culturing spheres was centrifuged to eliminate single cells and collected to be used for subsequent assays, mixed with 70% fresh medium.

Vemurafenib-resistant A375 cells were obtained culturing A375 cell line in presence of increasing dose (from 1 µM to 100 µM) of Vemurafenib for 3 months. Vemurafenib resistance was assessed via MTT assay and after the achievement of vemurafenib resistance cells were maintained in culture medium with 1 µM of vemurafenib to prevent the loss of resistance.

Morphological analysis

Cells were seeded at 3 or 1.5×10^5 cells/dish (A375 and BLM respectively) in 6-cm dishes, respectively, and treated with δ-TT or δ-TT and NAC/DIDS/2APB. Cytoplasmic vacuolization was analyzed by light microscopy from different fields under a Zeiss

Axiovert 200 microscope with a 20 or 32 × 0.4 objective lens linked to a Coolsnap Es CCD camera (Roper Scientific-Crisel Instruments).

For TEM analysis, cell pellets were fixed overnight in a solution containing 2% of paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Samples were post-fixed in 1% osmium tetroxide in cacodylate buffer at 0°C for 90 minutes, washed, dehydrated and embedded in Epon-Araldite resin. Ultrathin sections were cut by a Leica Supernova ultramicrotome (Reichert Ultracut E) and stained with lead citrate. TEM was performed with a Zeiss EM10 electron microscope.

Western blot assay

Cells were seeded at 8 or 4×10^5 cells/dish (A375 and BLM respectively) in 10-cm dishes. After each treatment, cells were lysed in RIPA buffer; protein preparations (15-30 μg) were resolved on SDS-PAGE and transferred to nitrocellulose membrane. Membranes were incubated with the specific primary antibodies. Detection was done using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Westar Etac Ultra 2.0, XLS075,0100; Cyanagen Srl). β-Tubulin or GAPDH were utilized as loading controls.

Oxygen consumption analysis

The analysis of OCR (oxygen consumption rate) on whole cells after δ-TT treatment was performed using a Clark oxygen electrode (DW1 electrode chamber, Hansatech Instruments Ltd, Norfolk, UK), as previously described [435]. A375 and BLM cells were rinsed in pre-warmed (37 °C) PBS and suspended in coupled respiration buffer (2% free-fatty acid BSA, 25 mM D-glucose, 1 mM Na-pyruvate, 40 μg/ml digitonin) or electron flow buffer (2% free-fatty acid BSA, 2 mM malate, 10 mM Na-pyruvate, 40 μg/ml digitonin, 4 μM carbonyl cyanide m-chlorophenyl hydrazine, CCCP). Then, samples were transferred to the electrode chamber for the measurement of OCR. After the measurement of basal respiration, uncoupled and maximal respiration were analyzed by adding oligomycin (10 μM) and CCCP (10 μM), respectively.

Flow cytometry analysis

After δ-TT treatment, flow cytometry analyses were performed to analyze: mitochondrial activity and mass (using the fluorescent MitoTracker Orange CMTMRos dye and MitoTracker Green FM; ThermoFisher Scientific); mitochondrial ROS levels (using the fluorescent probe MItoSOX Red; Invitrogen Life Technologies) and cytosolic and mitochondrial Ca^{2+} levels (using the fluorescent probes Fluo-3 AM and Rhod-2 AM, respectively; Invitrogen Life Technologies, Monza, Italy). The flow cytometry analyses were performed with a Novocyte3000 instrument (ACEA Biosciences, San Diego, CA). Data were analyzed with Novoexpress software.

ATP measurement

The effects of δ-TT treatment on ATP production in A375 and BLM cells were investigated using a specific ATP colorimetric/fluorimetric assay kit (GeneTex, Alton Pkwy Irvine, CA, USA). Luminescence was analyzed to quantify ATP production.

MTT viability assay

Cells were seeded at a density of 3×10^4 cells/well in 24-well plates for 24 hours and then exposed to the specific compounds. The medium was then changed with MTT solution (0.5 mg/mL) in DMEM without phenol red and FBS; cells were incubated at 37 °C for 20-45 min and violet precipitate was dissolved with isopropanol. Absorbance at 550 nm was measured through an EnSpire Multimode Plate reader (PerkinElmer, Milano, Italy).

Statistical analysis

Statistical analysis was performed with a statistic package (GraphPad Prism5, GraphPad Software San Diego, CA, USA). Data are represented as the mean ± SEM of three-four independent experiments. Differences between groups were assessed by t-test or one-way analysis of variance (ANOVA) followed by Dunnet's or Bonferroni's test. A P value < 0.05 was considered statistically significant.

RESULTS

δ-TT induces cytoplasmic vacuolization

Data reported above suggested that cytotoxic activity of natural compounds is frequently accompanied by morphological alterations.

In order to evaluate morphological changes associated with the cytotoxic effect of δ-TT, melanoma cells treated with our compound have been observed via optical microscopy. A375 and BLM cell lines were treated with 15 µg/mL of substance and cell morphology was analysed. We found that δ-TT induces an extensive cytoplasmic vacuolization in both A375 and BLM cell after 12 hours of treatment (Fig. 12a).

In order to better elucidate the nature of these vacuoles, we performed TEM analysis to understand which organelles and cell structures were damaged by the treatment.

TEM images showed that untreated cells exhibit a normal appearance with intact mitochondria and normal ER, with a regular profile of cisternae. On the other hand, melanoma cells treated with δ-TT 15µg/mL showed enlarged and damaged mitochondria with loss of/altered cristae and ER cisternae dilation (Fig. 12b).

These data suggested that in human melanoma cells δ-TT induces extensive morphological changes related to mitochondrial swelling and ER dilation that may be responsible for cell organelles dysfunction and cell death.

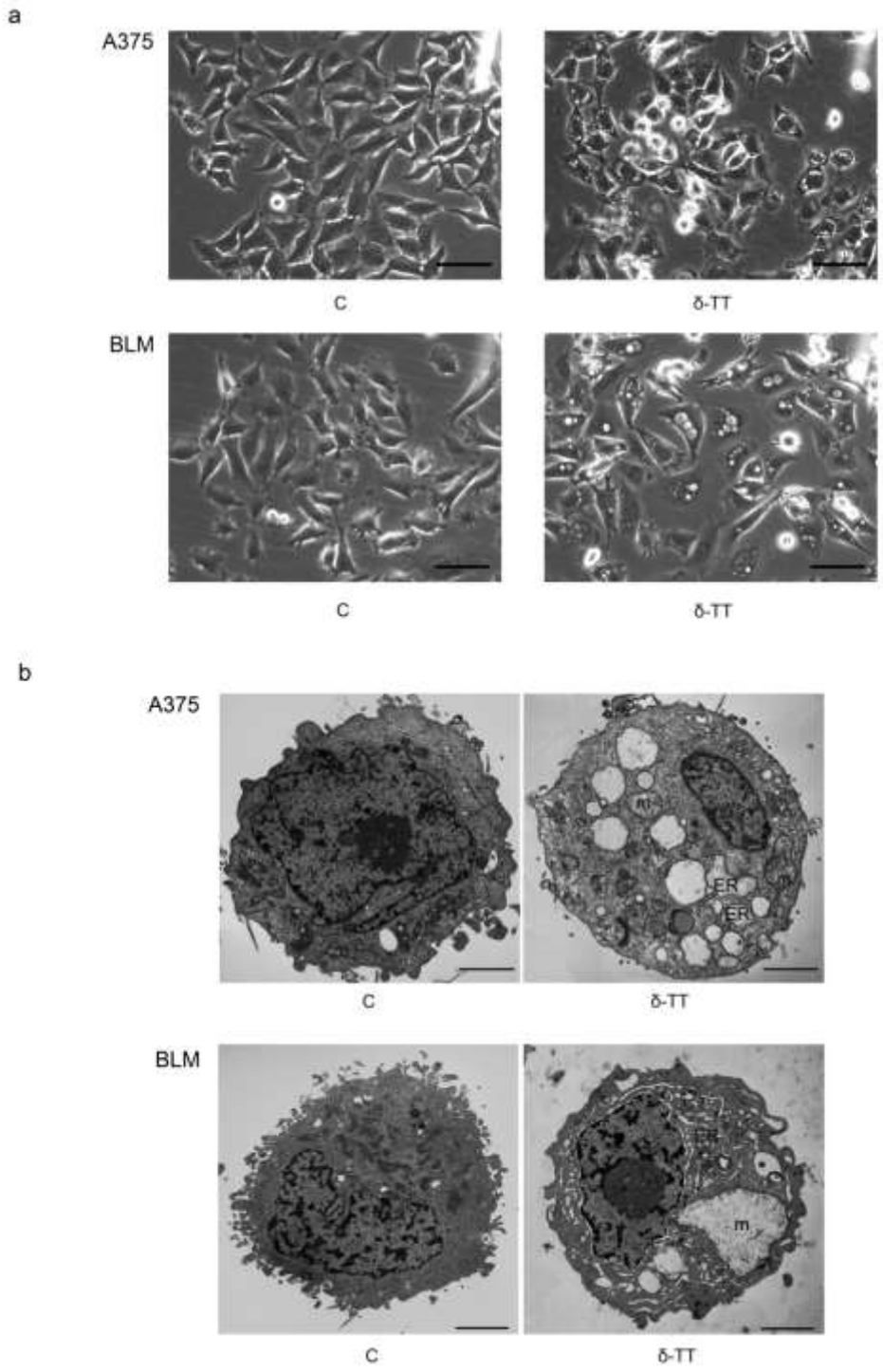


Fig. 12 δ-TT induces cytoplasmic vacuolization in melanoma cells

(a) A375 and BLM cells were treated with δ-TT (15 μg/mL, 12 h) and then analyzed by light microscopy, showing extensive cytoplasmic vacuolization. Scale bars are 20 μm. (b) A375 and BLM cells treated with δ-TT (15 μg/mL, 12 h) and then analyzed by electron microscopy

exhibited swollen mitochondria with rare cristae (m), dilated endoplasmic reticulum (ER) cisternae and enlarged nuclear envelope. Scale bars are 2 µm.

δ-TT -induced vacuolization is associated with paraptotic cell death

In literature, cell vacuolization induced by natural compounds has been linked to a non-canonical cell death, paraptosis. Moreover, it has been reported that δ-TT may exert its anticancer activity by triggering alternative cell death mechanisms in addition to apoptosis. Paraptosis is characterized by morphological alteration related to cell vacuolization, due to mitochondrial swelling and ER dilatation. Moreover, paraptosis was reported to be a cell death dependent on protein synthesis, thus can be prevented by cycloheximide, a protein synthesis inhibitor. Recently, we have demonstrated that δ-TT induces a paraptotic cell death in prostate cancer cells, we then analysed whether this mechanism occurs also in melanoma cells.

To confirm the involvement of paraptotic cell death in the cytotoxic effect of δ-TT, A375 and BLM cells were pretreated with cycloheximide 20 µM 3 hours before the treatment with δ-TT 15µg/mL.

We observed that protein synthesis inhibition strikingly prevented the cytoplasmic vacuolization caused by δ-TT in both A375 and BLM cell lines, supporting the relationship between vacuoles formation and paraptosis (Fig. 13a).

Paraptosis is known to be characterized also by the activation of MAPK signaling, thus we analysed MAPK phosphorylation after 24 hours of δ-TT treatment in A375 and BLM cells.

We observed that δ-TT 15µg/mL increased the levels of pJNK, pP38 and pERK kinases starting from 12 hours of treatment in both A375 and BLM cell lines (Fig 13b).

These data support that paraptosis is involved in the cytoplasmic vacuolization induced by δ-TT and demonstrate that the cytotoxic effect of δ-TT involves the activation of pJNK, pP38 and pERK kinases in A375 and BLM cells.

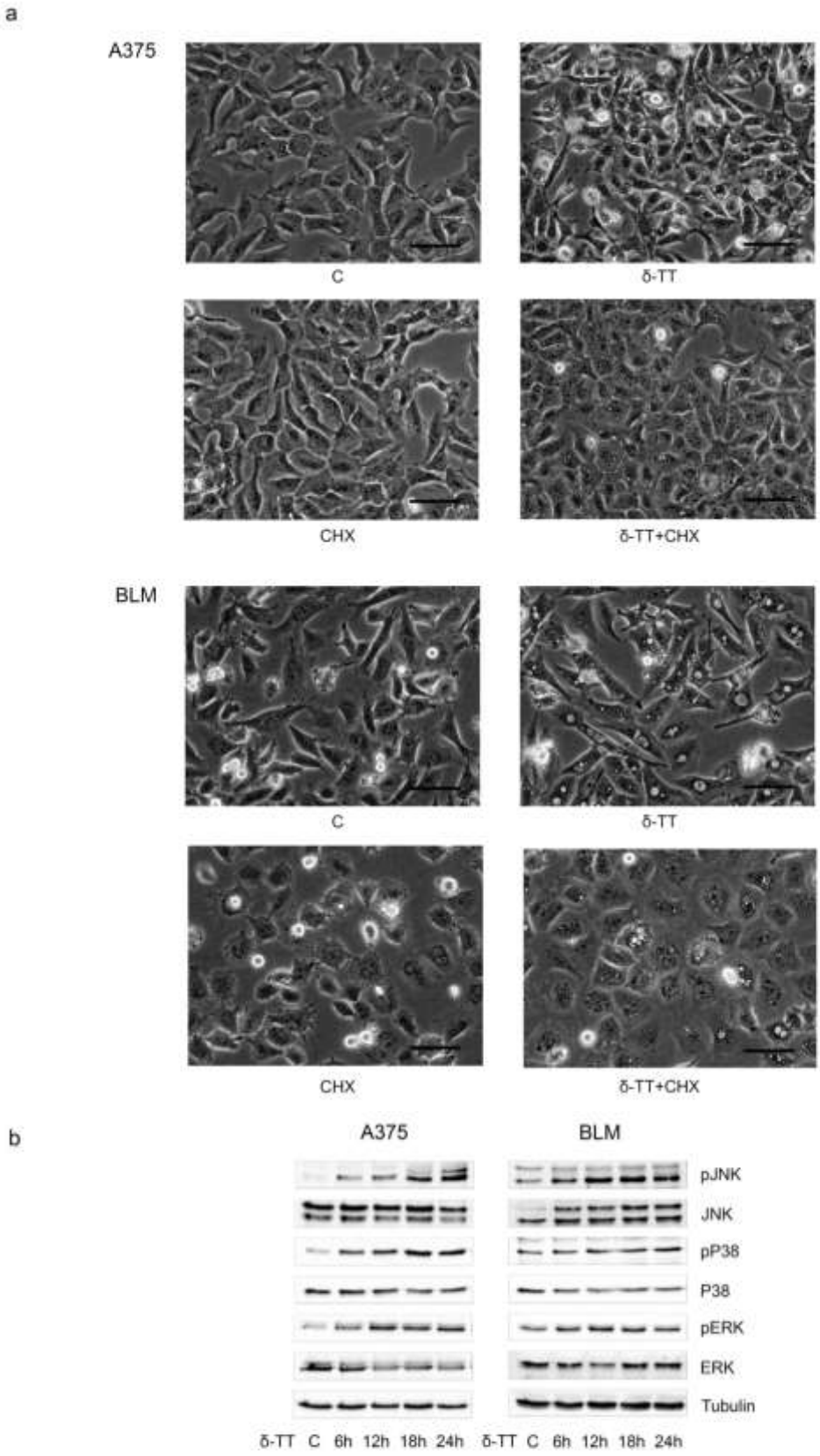


Fig. 13 δ-TT induces paraptosis in melanoma cells

(a) A375 and BLM cells were pretreated with cycloheximide (CHX) (20 μM, 3 h) and then with δ-TT (15 μg/mL, 12 h). Images show that CHX suppressed δ-TT-induced vacuolization in melanoma cells. Cell morphology was analyzed by light microscopy. Scale bars are 20 μm. (b) A375 and BLM cells were treated with δ-TT (15 μg/mL) for 6-24 h. Western blot analysis was performed to evaluate protein levels of p-JNK, p-p38 and p-ERK1/2. Tubulin was used as a loading control. One representative experiment of three performed is shown.

δ-TT induces a mitochondrial damage, affecting mitochondrial function

Given the fact that δ-TT induces an extensive mitochondrial swelling, we next focused our attention on the analysis of mitochondrial function, to clarify the role of mitochondria in the anti-cancer activity of our compound. Mitochondria are responsible for producing almost 90% of the energy needed to sustain cell proliferation and survival. Thus, we examined the effects of δ-TT on mitochondrial function, in particular on the protein levels of mitochondrial electron transport chain (ETC) complexes (that are responsible for oxidative phosphorylation (OXPHOS) process), on the oxygen consumption capacity and on the mitochondrial membrane potential.

In order to assess the presence of a molecular damage, we performed Western Blot analysis to examine OXPHOS proteins levels.

A375 and BLM cells were treated with δ-TT 15 μg/mL for 24 hours and proteins levels were checked at different time points. We could find out that δ-TT significantly downregulates the expression of OXPHOS complex I (Fig. 14a), demonstrating the presence of a molecular damage in ETC.

As a direct consequence, this molecular damage resulted in a reduction of oxygen consumption, measured after 12 hours of δ-TT treatment. In particular, by Clark type electrode we could observe that oxygen consumption was affected in basal, as well as in uncouples (oligomycin, 10 μM) and maximal (carbonyl cyanide m-chlorophenyl hydrazine, CCCP, 10 μM) respiration conditions (Fig. 14b).

Mitochondrial membrane potential (MMP) is determined by OXPHOS activity. Electron flow through ETC generates energy used to transport protons across the inner

mitochondrial membrane, resulting in a generation of a pH gradient and therefore an electrical potential across the membrane. Thus, a reduction in OXPHOS functionality could lead to a membrane depolarization. In order to assess the polarization status, we performed cytofluorimetric analysis after staining with MitoTracker Orange CMTMRos (10 µM, 30 min) fluorescent probe and we found that δ-TT 15 µg/mL significantly reduced MMP (Fig. 14c), resulting in a reduced mitochondrial activity.

These data demonstrate that δ-TT significantly impairs mitochondrial function, damaging OXPHOS proteins, impairing oxygen consumption capacity and resulting in an extensive mitochondrial membrane depolarization.

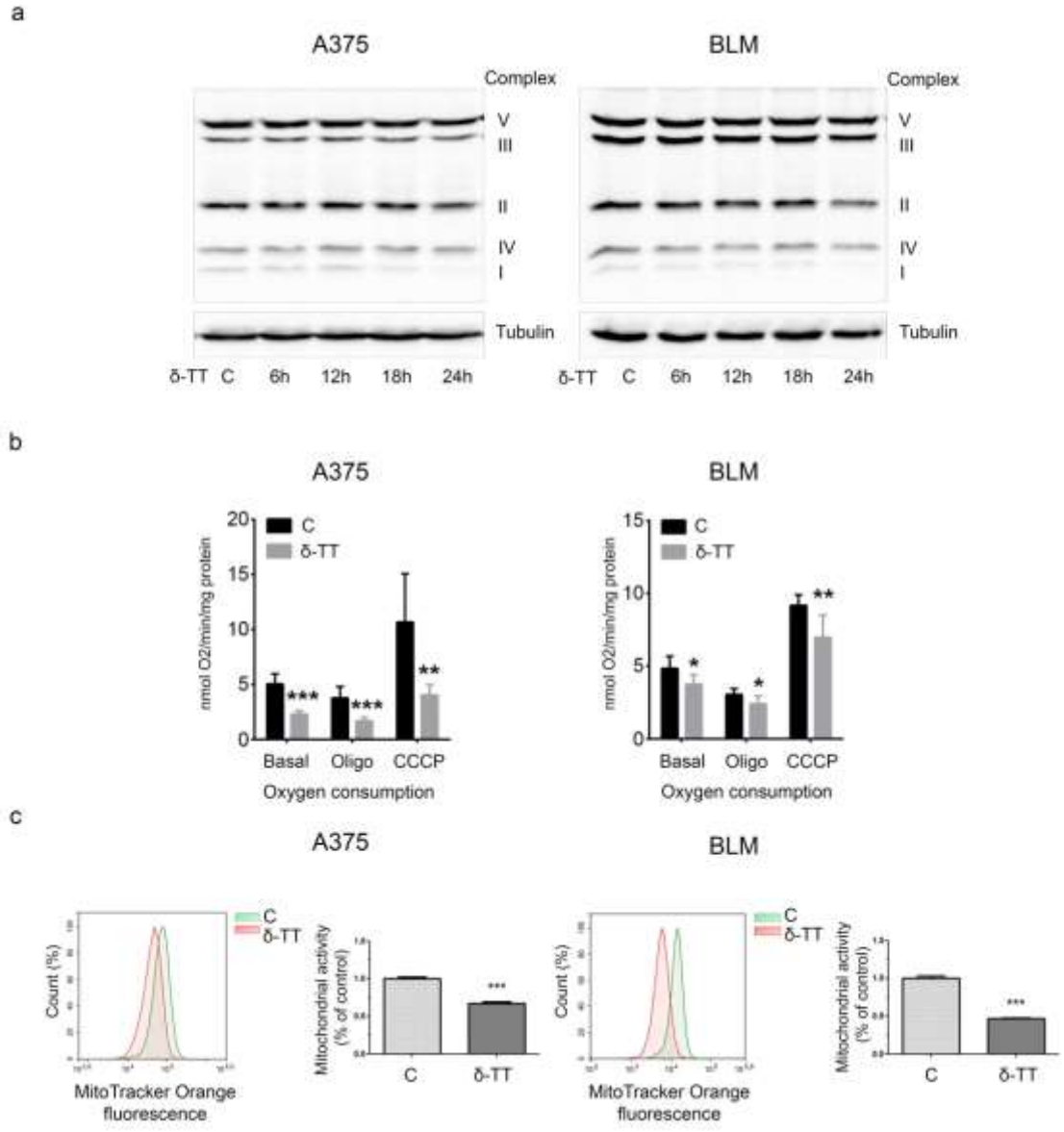


Fig. 14 δ-TT impairs mitochondrial function in melanoma cells

(a) A375 and BLM cells were treated with δ-TT (15 μg/mL) for 6-24 h. Western blot analysis was performed to evaluate OXPHOS proteins levels. Tubulin was used as a loading control. One representative experiment of three performed is shown. (b) A375 and BLM cells were treated with δ-TT (15 μg/mL, 12 h). Clark electrode was used to measure oxygen consumption in basal as well in uncoupled (oligomycin, oligo) and maximal (carbonyl cyanide m-chlorophenyl hydrazine, CCCP) respiration conditions. One representative experiment of three performed is shown. Data represent mean values ± SEM and were analyzed by t-test. ∗P < 0.05 vs C, controls (vehicle); ∗∗P < 0.01 vs C, controls (vehicle); ∗∗∗P < 0.001 vs C, controls (vehicle). (c) A375 and BLM cells were treated with δ-TT (15 μg/mL, 12 h) and then stained with MitoTracker Orange CMTM Ros (10 nM, 30 min) fluorescent probe. Mitochondrial activity was measured by flow cytometry. One representative experiment of three performed is shown. Data represent mean values ± SEM and were analyzed by t-test. ∗∗∗P < 0.001 vs C, controls (vehicle).

Mitochondrial damage leads to energy depletion

Mitochondria are the major source of ATP production within the cell. Protons accumulated between the inner and the outer mitochondrial membrane flow back across the membrane through ATP synthase, which exploits this energy to phosphorylate ADP into ATP.

Since an OXPHOS damage could lead to a variation of ATP generation, resulting in an alteration of energy homeostasis, we performed colorimetric assay in order to detect ATP production.

A375 and BLM cells were treated with δ-TT 15 μg/mL for 12 hours and we observed that ATP production was significantly lower in treated cells compared with control cells (Fig. 15a).

The main regulator of energy homeostasis is AMPK, known as the “energy stress sensor”. A decrease in ATP levels induces an activation (phosphorylation) of this kinase. We could observed that the reduction of energy production induced by δ-TT 15 μg/mL triggered the activation via phosphorylation of AMPK (18-24 hours) (Fig. 15b).

Taken together, these data demonstrate that the mitochondrial damage induced by δ-TT severely alters cell energy homeostasis.

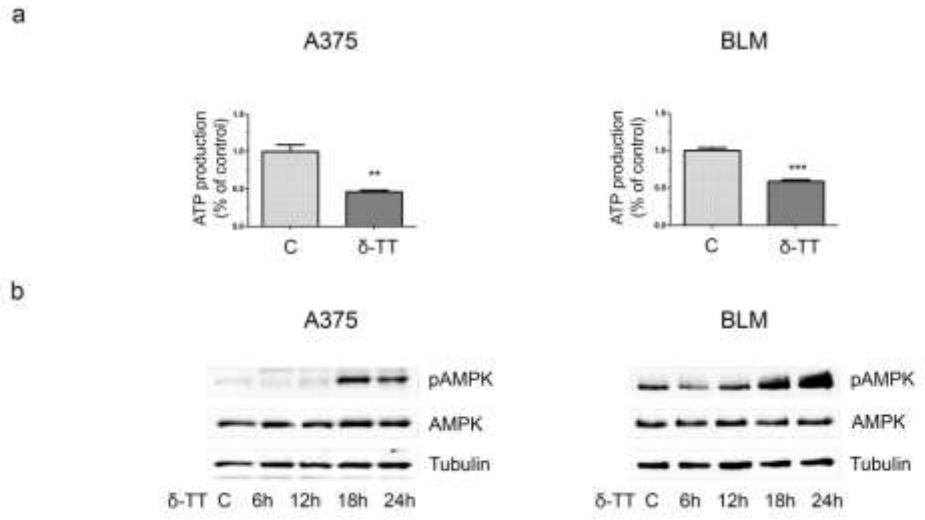


Fig. 15 δ-TT causes energy depletion in melanoma cells

(a) A375 and BLM cells were treated with δ-TT (15 μg/mL, 12 h). ATP levels were measured by colorimetric assay. One representative experiment of three performed is shown. Data represent mean values ± SEM and were analyzed by t-test. ∗∗P < 0.01 vs C, controls (vehicle); ∗∗∗P < 0.001 vs C, controls (vehicle). (b) A375 and BLM cells were treated (15 μg/mL) for 6-24 h. Western blot analysis was performed to evaluate p-AMPK protein level. Tubulin was used as a loading control. One representative experiment of three performed is shown.

δ-TT increases ROS production

It is largely demonstrated that high intracellular levels of ROS can oxidize and subsequently damage macromolecules (such as nucleic acids, proteins and lipids), organelles and cell membranes. The propagation of this damage could trigger cell death pathways such as apoptosis and paraptosis. Large evidence demonstrate that different natural compounds exert their anticancer activity through the induction of ROS production [326, 410, 474].

Given the fact that δ-TT induces an extensive mitochondrial dysfunction, we then monitored by flow-cytometry its effect on mitochondrial ROS generation, using the fluorescent probe MitoSOX Red (5 μM for 10 min). We observed that δ-TT 15 μg/mL treatment significantly increased ROS levels in mitochondria after 12 hours, in both A375 and BLM cells (Fig. 16a).

To verify whether ROS are involved in the cytotoxic effect of δ-TT, we pre-treated for 2 hours A375 and BLM with NAC (N-acetyl-L-cysteine, 4 mM), an inhibitor of ROS generation. MTT assay showed that the pre-treatment with NAC significantly (even if not completely) counteracted δ-TT-mediated cell death (Figure 16b).

These data show that the cytotoxic effect of δ-TT, responsible for mitochondrial swelling and OXPHOS impairment, is partially mediated also by intracellular ROS generation in A375 and BLM cells, and a free-radical scavenger could limit the anti-cancer effect of δ-TT.

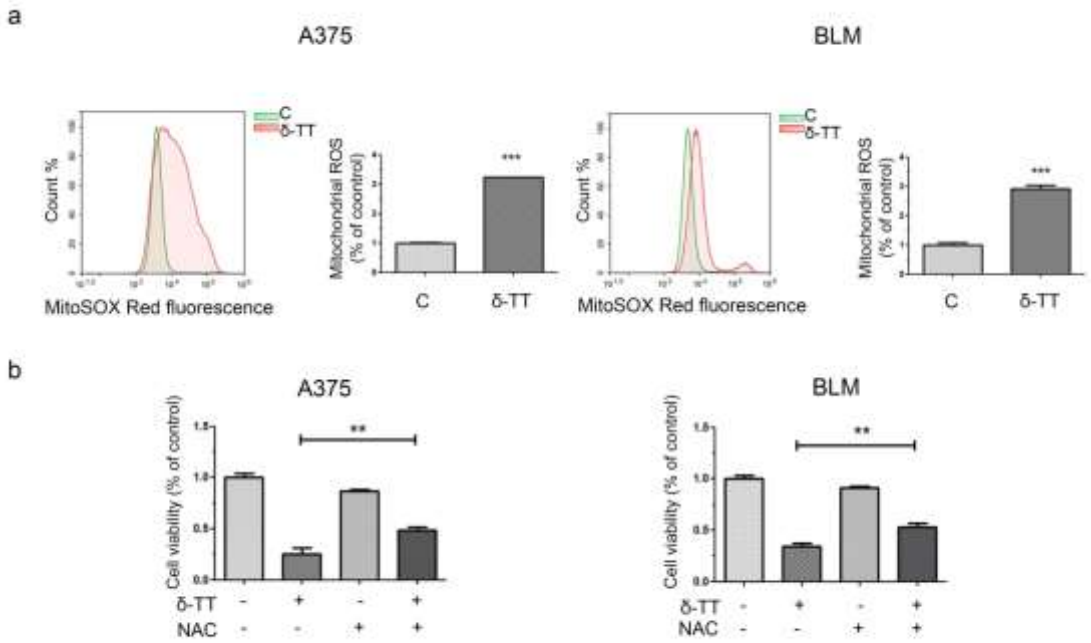


Fig. 16 δ-TT induces mitochondrial ROS overproduction in melanoma cells

(a) A375 and BLM cells were treated with δ-TT (15 µg/mL, 12 h) and then stained with MitoSOX Red (5 µM, 10 min) fluorescent probe. Mitochondrial ROS generation was measured by flow cytometry. One representative experiment of three performed is shown. Data represent mean values ± SEM and were analyzed by t-test. ***P < 0.001 vs C, controls (vehicle). (b) A375 and BLM cells were pretreated with the ROS scavenger NAC (N-acetyl-l-cysteine, 4 mM, 2 h) and then with δ-TT (15 µg/mL, 24 h). Cell viability was assessed by MTT assay. Each experiment was repeated three times. Data represent mean values ± SEM and were analyzed by one-way analysis of variance followed by Bonferroni's test. **P < 0.01 vs C, controls (vehicle).

ROS are involved in δ-TT - induced paraptosis

Several studies demonstrated that ROS production is associated with paraptotic features. Different natural compounds trigger paraptotic cell death mediated by ROS generation, which are strictly connected with cytoplasmic vacuolization and MAPK activation [326]. Based on these observations we analysed the relationship between ROS and paraptosis in the anti-cancer activity of δ-TT in melanoma cells.

As described before, we pretreated A375 and BLM cells with NAC (4 mM, 2 hours) and then with δ-TT. When next analysed vacuoles formations by optical microscopy and MAPK phosphorylation by Western Blot analysis.

We observed that NAC pre-treatment markedly reduced cytoplasmic vacuolization (Fig. 17a) and JNK, P38 and ERK phosphorylation (Fig. 17b) induced by δ-TT.

These results demonstrate that the redox imbalance caused by δ-TT is correlated with dysfunctional changes and morphological alteration. ROS generation is responsible for paraptotic vacuolization and MAPK activation in human melanoma cells.

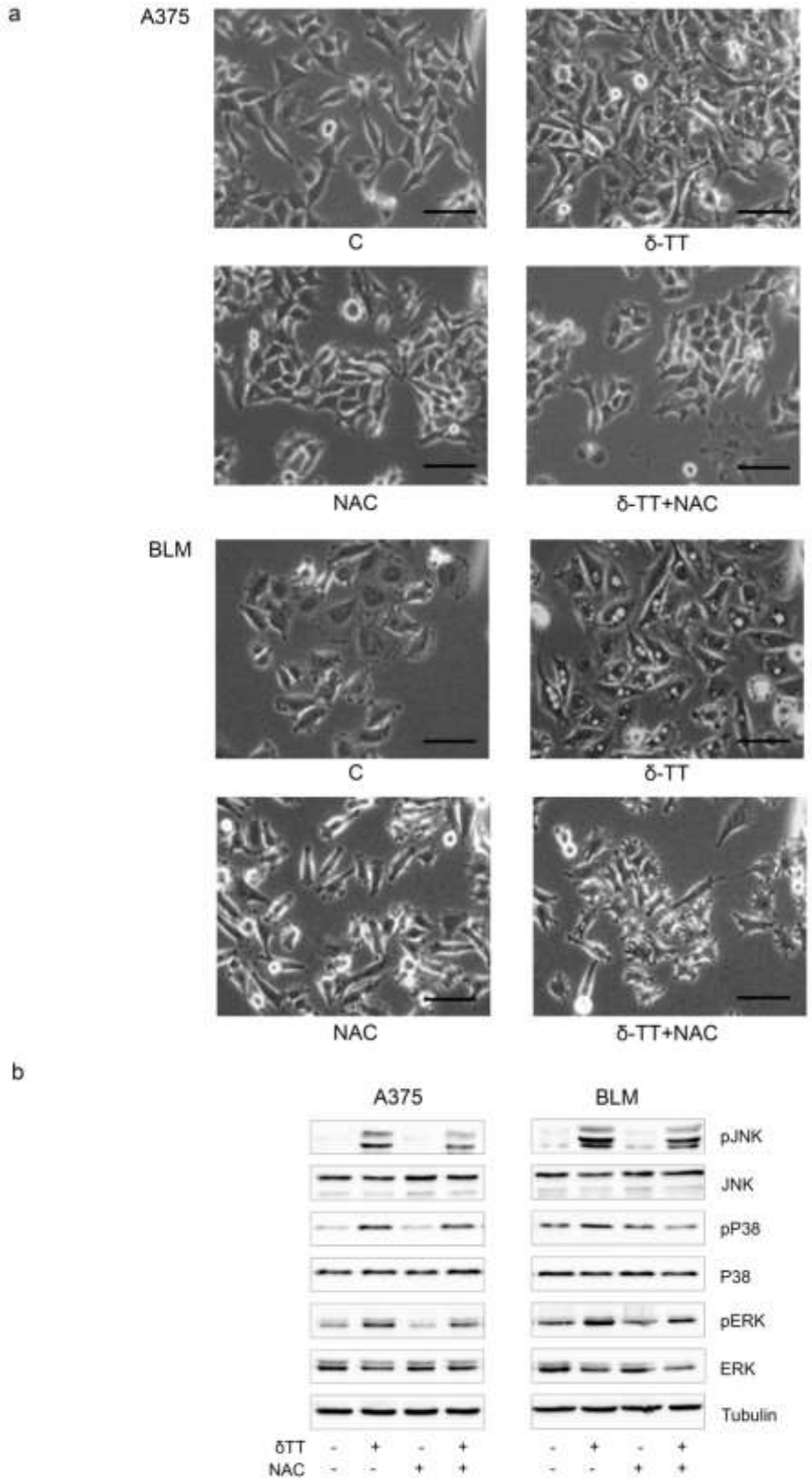


Fig. 17 δ-TT-induced ROS are involved in paraptotic vacuolization and MAPK activation

(a) A375 and BLM cells were pretreated with NAC (4 mM, 2 h) and then with δ-TT (15 μg/mL, 12 h). Cell morphology was analyzed by light microscopy. Scale bars are 20 μm. (b) A375 and BLM cells were pretreated with NAC (4 mM, 2 h) and then with δ-TT (15 μg/mL, 24 h). Western blot analysis was performed to evaluate the proteins levels of p-JNK, p-p38 and p-ERK1/2. Tubulin was used as a loading control. One representative experiment of three performed is shown.

δ-TT damage leads to cytoplasmic and mitochondrial Ca^{++} overload

As described above, loss of MMP, ROS production and Ca^{++} overload are the main features associated with apoptotic and paraptotic cell death. Different compounds, either natural or synthetic, were reported to induce apoptosis and paraptosis by dysregulating Ca^{++} homeostasis [367, 410, 475-478].

The two main intracellular storages of Ca^{++} are mitochondria and ER. Damages to these structures could alter ions fluxes and homeostasis, resulting in a dysregulation of ions homeostasis [379]. It has been reported that different anti-cancer compounds affecting mitochondrial function and triggering ER stress also cause Ca^{++} efflux from ER and subsequently its influx into mitochondria. This flow is mediated by ion channels on ER and mitochondrial membranes. In particular, Ca^{++} efflux from ER is mediated by inositol trisphosphate receptor (IP_3R), while voltage-dependent anion channel (VDAC) on the OMM is involved in its influx into mitochondria [379].

By flow cytometry analysis we investigated the effects of δ-TT on cytoplasmic (using Fluo-3 AM fluorescent staining, 5 μM, 30 min) and mitochondrial (using Rhod-2 AM fluorescent staining, 5 μM, 30 min) Ca^{++} levels and their role in the cytotoxic effect of this compound on melanoma cells.

Flow cytometry analysis revealed that the treatment with δ-TT 15 μg/mL for 12 hours significantly increased both cytoplasmic (Fig. 18a) and mitochondrial (Fig. 18b) calcium levels in A375 and BLM cells, suggesting a severe dysregulation of calcium homeostasis.

To confirm the involvement of Ca^{++} overload in the pro-death effect of δ-TT we pretreated A375 and BLM cells with 2APB (2-Aminoethyl diphenylborinate, 20 μM, 2 hours), the blocker of IP_3R channel, and DIDS (disodium 4,4′-diisothiocyanostilbene-2,2′-

disulfonate, 75 µM, 2 hours), the inhibitor of the VDAC channel. MTT assay showed that the inhibition of Ca^{++} efflux from ER and its influx into mitochondria significantly (even if not completely) counteracted δ-TT-mediated cell death (Fig. 18c, 18d).

Taken together these data confirm that δ-TT induces a significant dysregulation of calcium homeostasis, causing its release from ER and its influx into mitochondria. Moreover, this Ca^{++} movement is involved in the cytotoxic effect of δ-TT in human melanoma cells.

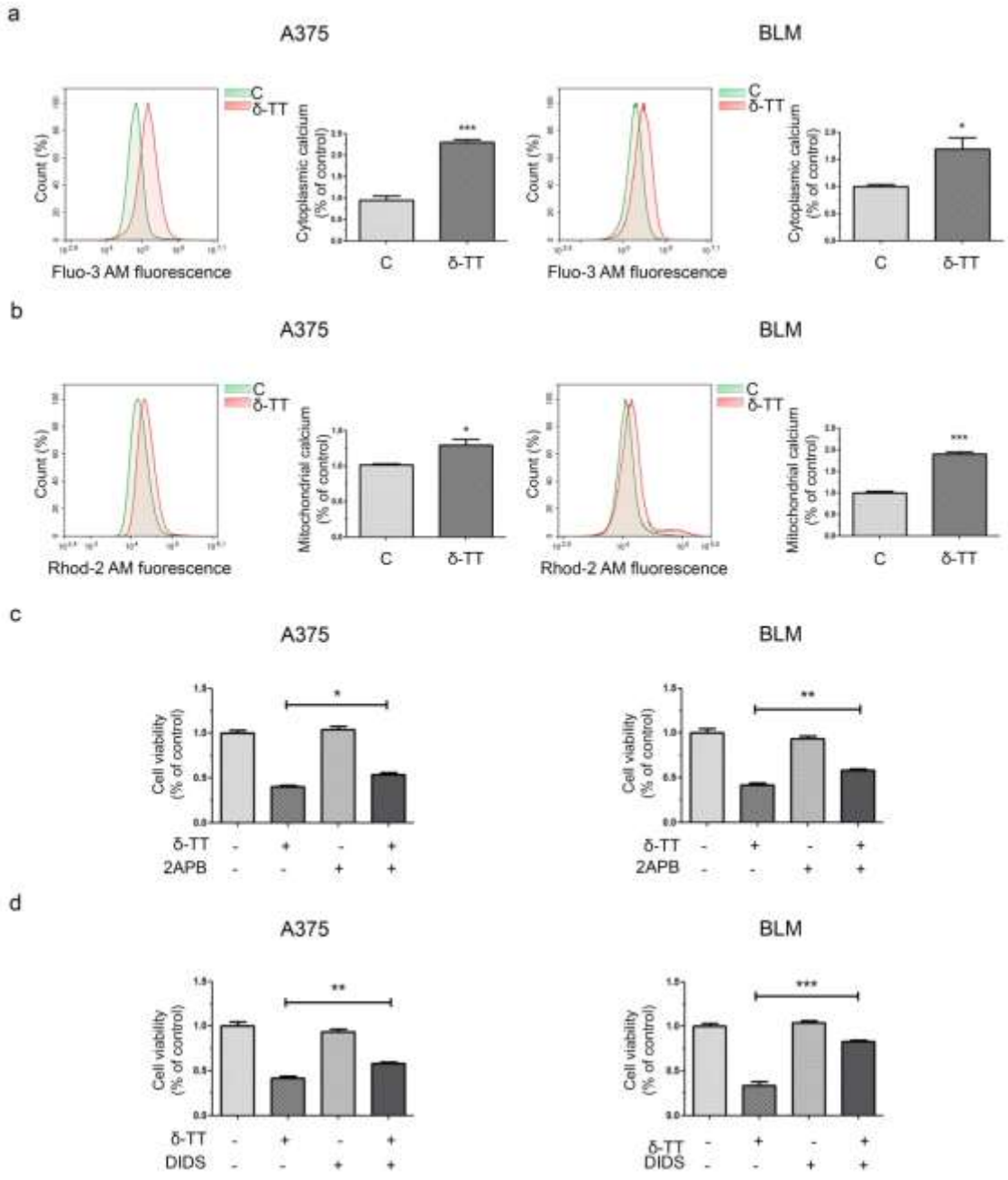


Fig. 18 δ-TT causes Ca^{++} homeostasis dysregulation in melanoma cells

(a) A375 and BLM cells were treated with δ-TT (15 μg/mL, 12 h) and then stained with Fluo-3 AM (5 μM, 30 min). Cytoplasmic Ca^{++} levels were measured by flow cytometry. One representative experiment of three performed is shown. Data represent mean values ± SEM and were analyzed by t-test. ∗P < 0.05 vs C, controls (vehicle); ∗∗∗P < 0.001 vs C, controls (vehicle). (b) A375 and BLM cells were treated with δ-TT (15 μg/mL, 12 h) and then stained with Rhod-2 AM (5 μM, 30 min). Mitochondrial Ca^{++} levels were measured by flow cytometric analysis. One representative experiment of three performed is shown. Data represent mean values ± SEM and were analyzed by t-test. ∗P < 0.05 vs C, controls (vehicle); ∗∗∗P < 0.001 vs C, controls (vehicle). (c) A375 and BLM cells were pretreated with the IP3R inhibitor 2APB (20 μM, 2 h) and then with δ-TT (15 μg/mL, 24 h). Cell viability was assessed by MTT assay. Each experiment was repeated three times. Data represent mean values ± SEM and were analyzed by one-way analysis of variance followed by Bonferroni's test. ∗P < 0.05 vs C, controls (vehicle); ∗∗P < 0.01 vs C, controls (vehicle). (d) A375 and BLM cells were pretreated with the VDAC blocker DIDS (75 μM, 2 h) and then with δ-TT (15 μg/mL, 24 h). Cell viability was assessed by MTT assay. Each experiment was repeated three times. Data represent mean values ± SEM and were analyzed by one-way analysis of variance followed by Bonferroni's test. ∗∗P < 0.01 vs C, controls (vehicle); ∗∗∗P < 0.001 vs C, controls (vehicle).

Ca^{++} homeostasis dysregulation is involved in paraptotic cell death

Large evidence have associated Ca^{++} overload with paraptotic features. Vacuoles and MAPK phospohorylation, indeed, were found to be correlated with calcium dysregulation homeostasis in the anti-cancer activity of different natural compounds. As cytoplasmic and mitochondrial Ca^{++} levels were significantly increased after δ-TT treatment, we further analysed their role in δ-TT-induced paraptosis.

To investigate the role of Ca^{++} overload in vacuoles formation and in MAPK activation, we pretreated A375 and BLM cells with 2APB (20 μM, 2 hours) and DIDS (75 μM, 2 hours), and then with δ-TT 15 μg/mL.

Via optical microscopy we observed cell morphology and we found that, inhibiting Ca^{++} release from ER and blocking calcium influx into mitochondria, δ-TT-induced

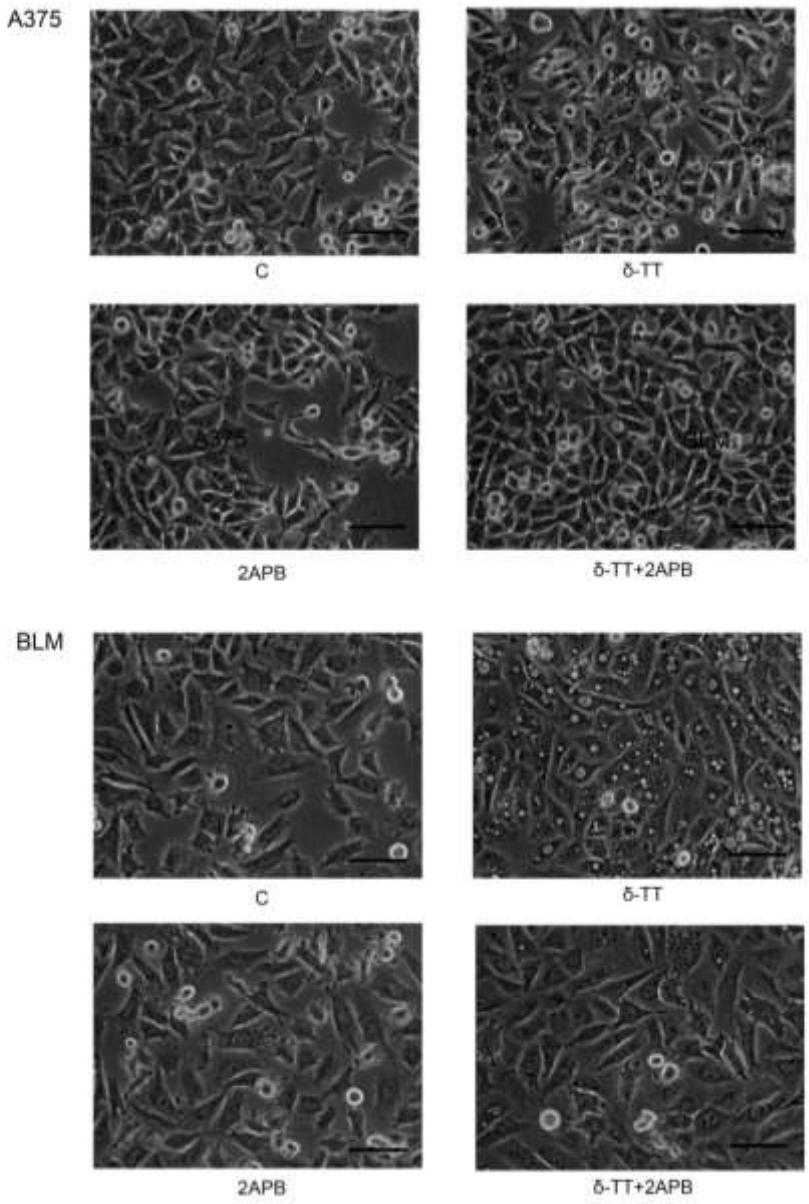
vacuolization was significantly reduced in both cell lines (Fig. 19a,b), suggesting the role of ER calcium in paraptotic vacuolization.

Moreover, by western blot we observed that the increase in cytoplasmic and mitochondrial calcium levels was involved in MAPK activation: indeed, 2APB and DIDS pretreatment reduced the phosphorylation of JNK, P38 and ERK in both melanoma cell lines (Fig. 19c,d), suggesting that this signaling pathway is partially activated by ions homeostasis dysregulation.

These data suggest that ER Ca^{++} is involved in mitochondrial swelling and subsequent paraptotic pathways observed in melanoma cells treated with δ-TT. Furthermore, TEM analysis revealed the presence of some sites tightly associated between ER and mitochondria in A375 and BLM cells after δ-TT treatment (Fig. 20). These communication sites are defined mitochondria-associated membranes (MAMs) and are involved in the fluxes of ions and molecules.

Taken together, these data suggest that calcium overload induced by δ-TT is involved not only in the reduction of cell viability and cell death, but also in the paraptotic features observed. Indeed, both cytoplasmic and mitochondrial calcium increase are responsible for vacuoles formation and MAPK activation in A375 and BLM cells treated with δ-TT, suggesting that ions homeostasis dysregulation is one of the mechanisms underlying the anti-cancer activity of this compound. Moreover, the high presence of MAMs supports the existence of direct Ca^{++} fluxes at the ER-mitochondria interface.

a



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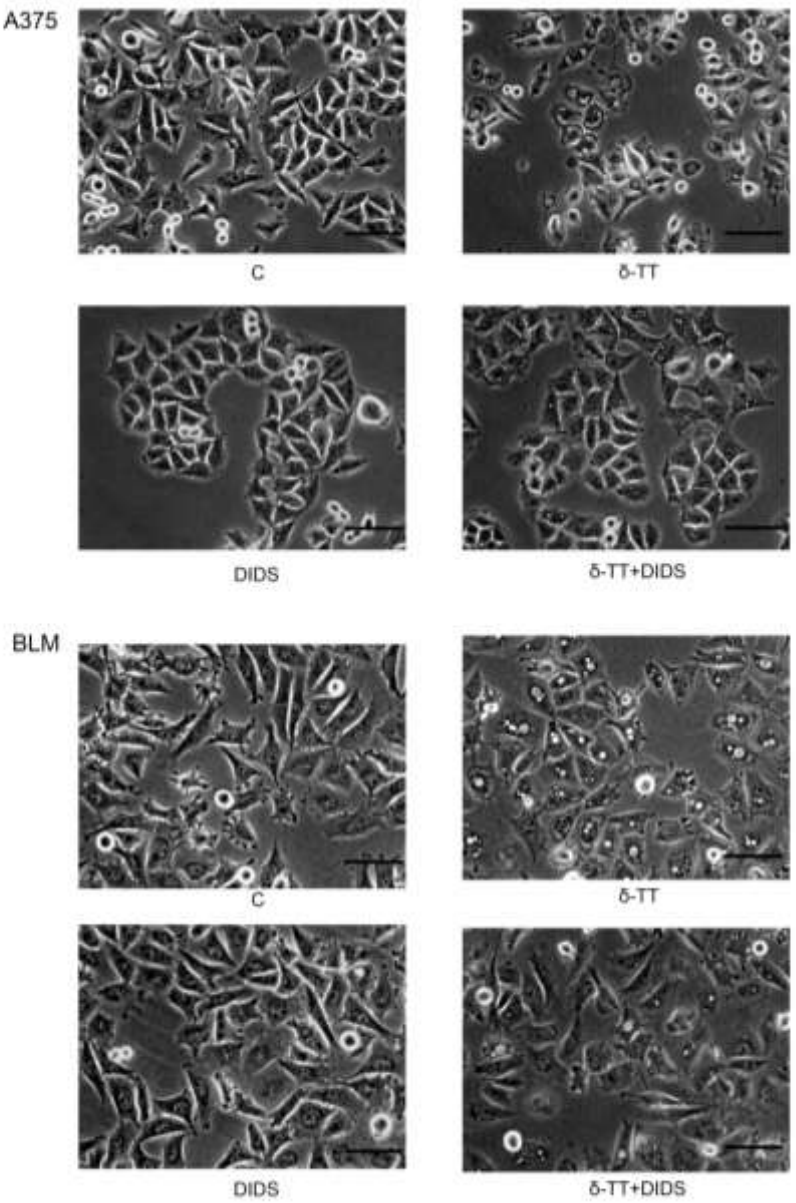

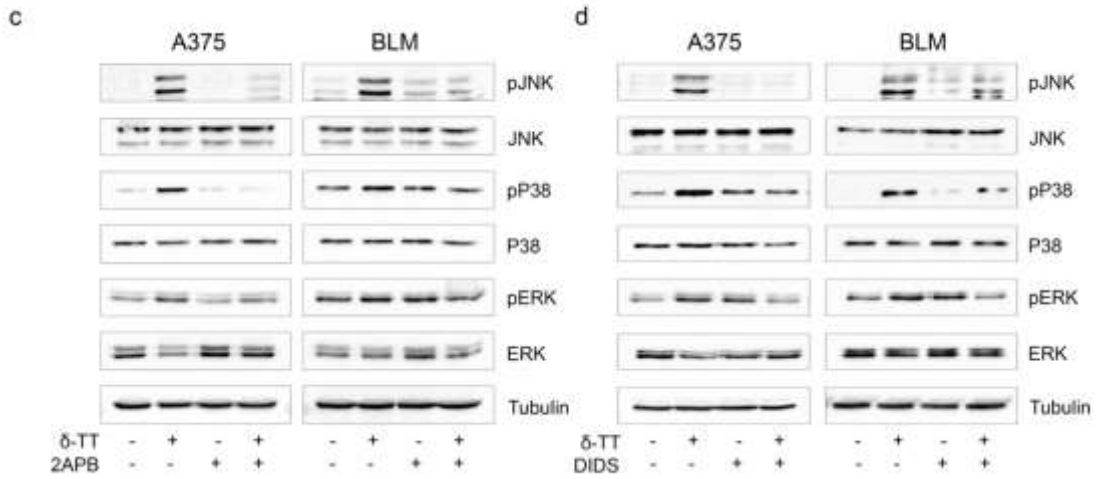

Fig. 19 δ-TT-induced Ca^{++} overload is involved in paraptotic vacuolization and MAPK activation

(a) A375 and BLM cells were pretreated with the IP3R inhibitor 2APB (20 μM, 2 h) and then with δ-TT (15 μg/mL, 12 h). Cell morphology was analyzed by light microscopy. Scale bars are 20 μm. (b) A375 and BLM cells were pretreated with the VDAC blocker DIDS (75 μM, 2 h) and then with δ-TT (15 μg/mL, 12 h). Cell morphology was analyzed by light microscopy. Scale bars are 20 μm. (c) A375 and BLM cells were pretreated with 2APB (20 μM, 2 h) and then with δ-TT (15 μg/mL, 24 h). Western blot analysis was performed to evaluate the proteins levels of p-JNK, p-p38 and p-ERK1/2. Tubulin was used as a loading control. One representative experiment of three performed is shown. (d) A375 and BLM cells were pretreated with DIDS (75 μM, 2 h) and then with δ-TT (15 μg/mL, 24 h). Western blot analysis was performed to evaluate the proteins levels of p-JNK, p-p38 and p-ERK1/2. Tubulin was used as a loading control. One representative experiment of three performed is shown.

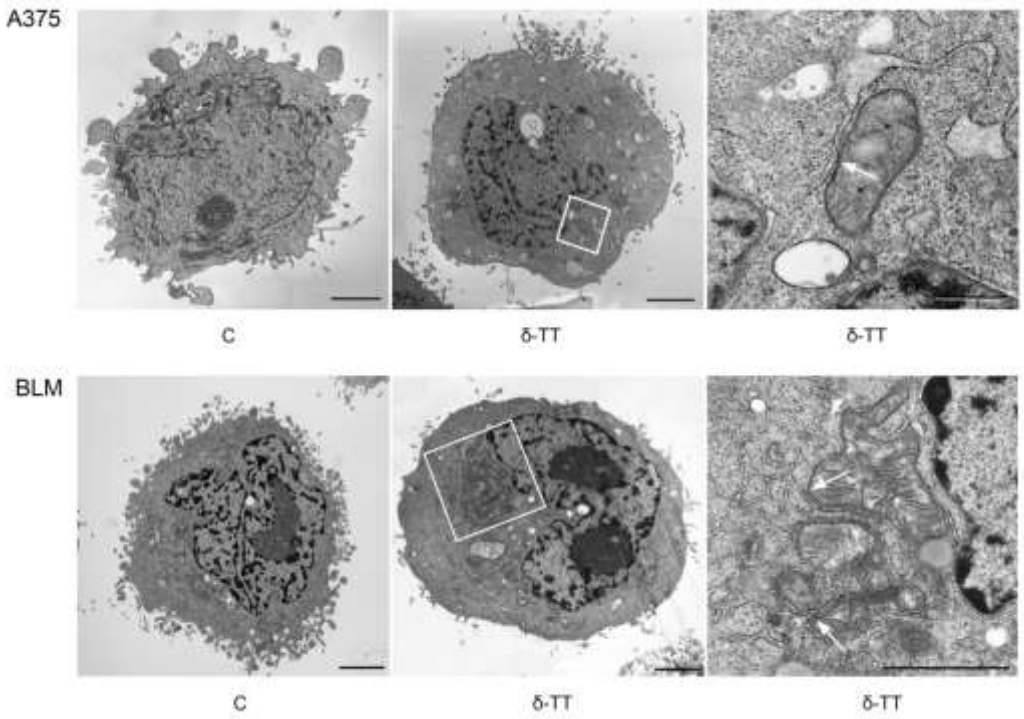

Fig. 20 Formation of MAMs is observed after δ-TT treatment

A375 and BLM cells were treated with δ-TT (15 µg/mL, 12 h) and then analyzed by electron microscopy. Boxed areas in central panels show the formation of mitochondria-associated ER membranes (MAMs) after δ-TT treatment. Scale bars are 2 µm. Boxed areas are enlarged in the right panels and MAMs are highlighted by arrows. Scale bars are 0,5 µm.

Ca^{++} overload and ROS generation are tightly interconnected

In literature it has been shown that Ca^{++} efflux from ER and its influx into mitochondria is correlated with ROS production [353, 379]. Ca^{++} overload occurring during paraptosis stimulates and increases ROS production in cancer cells and the inhibition of Ca^{++} release from ER could counteract redox homeostasis dysregulation.

To analyze the interconnection between these two mechanisms in the anti-cancer activity of δ-TT we pretreated A375 and BLM cells with 2APB (20 µM, 2 hours) and DIDS (75 µM, 2 hours) before the treatment with our compound 15µg/mL.

By flow-cytometry we measured mitochondrial ROS and we observed that mitochondrial ROS levels in melanoma cells pretreated with 2APB and DIDS were significantly lower than ROS measured in cells treated with δ-TT alone (Fig 21).

These data demonstrate that the inhibition of Ca^{++} release from ER and the blockade of Ca^{++} influx into mitochondria prevents mitochondrial ROS generation in A375 and BLM melanoma cells treated with δ-TT, suggesting a close interconnection between Ca^{++} overload and mitochondrial ROS production in the mechanisms underlying the anti-cancer activity of δ-TT.

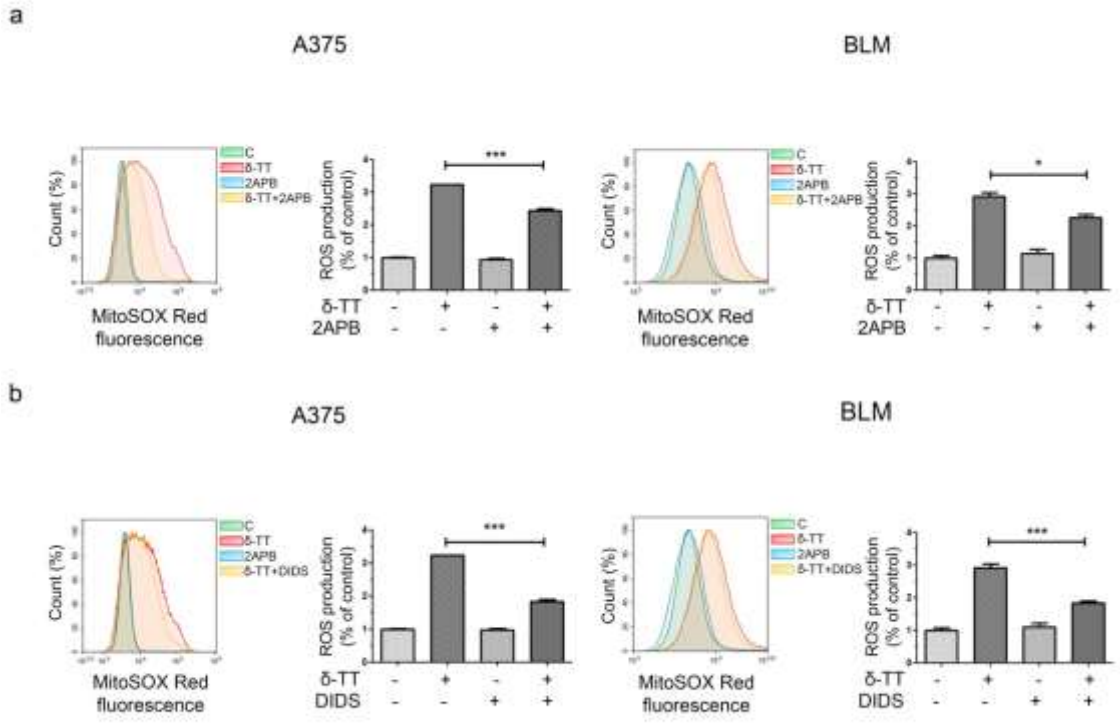

Fig. 21 δ-TT-induced Ca^{++} overload causes mitochondrial ROS overproduction in melanoma cells

(a) A375 and BLM cells were pretreated with the IP3R inhibitor 2APB (20 μM, 2 h) and then with δ-TT (15 μg/mL, 12 h). After treatment, cells were stained with MitoSOX Red (5 μM, 10 min) fluorescent probe. Mitochondrial ROS generation was measured by flow cytometry. One representative experiment of three performed is shown. Data represent mean values ± SEM and were analyzed by one-way analysis of variance followed by Bonferroni's test. ∗P < 0.05 vs C, controls (vehicle); ∗∗∗P < 0.001 vs C, controls (vehicle). (b) A375 and BLM cells were pretreated with the VDAC blocker DIDS (75 μM, 2 h) and then with δ-TT (15 μg/mL, 12 h). After treatment,

cells were stained with MitoSOX Red (5 μM, 10 min) fluorescent probe. Mitochondrial ROS generation was measured by flow cytometry. One representative experiment of three performed is shown. Data represent mean values ± SEM and were analyzed by one-way analysis of variance followed by Bonferroni's test. ***P < 0.001 vs C, controls (vehicle).

ABCG2+ A375 cells are characterized by higher mitochondrial mass

The identification of a specific marker for CSCs is necessary to recognize and target this aggressive subpopulation, responsible for resistance to therapy and tumor relapse. Recent evidence reported that in different tumor types CSCs are coupled with metabolic alterations and mitochondrial mass could be considered a new metabolic biomarker for stem-like cancer cells [235]. Other studies proposed ATP Binding Cassette Transportes as markers for CSCs and recently it has been demonstrated that also melanoma SCs deriving from the A375 cell line (grown as melanospheres culture) are characterized by the expression of ABGC2 transporter and high autofluorescence, probably for the accumulation of riboflavin in intracellular vesicles [163].

Based on these observations, by flow cytometry we analyzed mitochondrial mass with MitoTracker Green FM staining (10 nM, 30 min), a fluorescent probe that enters into mitochondria independently of membrane potential. We could observe that, in the A375 cell line, ABCG2-positive cells are characterized by higher mitochondrial mass compared with ABCG2 negative cells (Fig. 22).

This result suggests that in A375 human melanoma cell line, ABCG2-positive subpopulation shows an altered metabolism, with an increased mitochondrial mass. This is in line with data reported in literature, suggesting that also melanoma stem cells undergo a rewiring of mitochondrial metabolism.

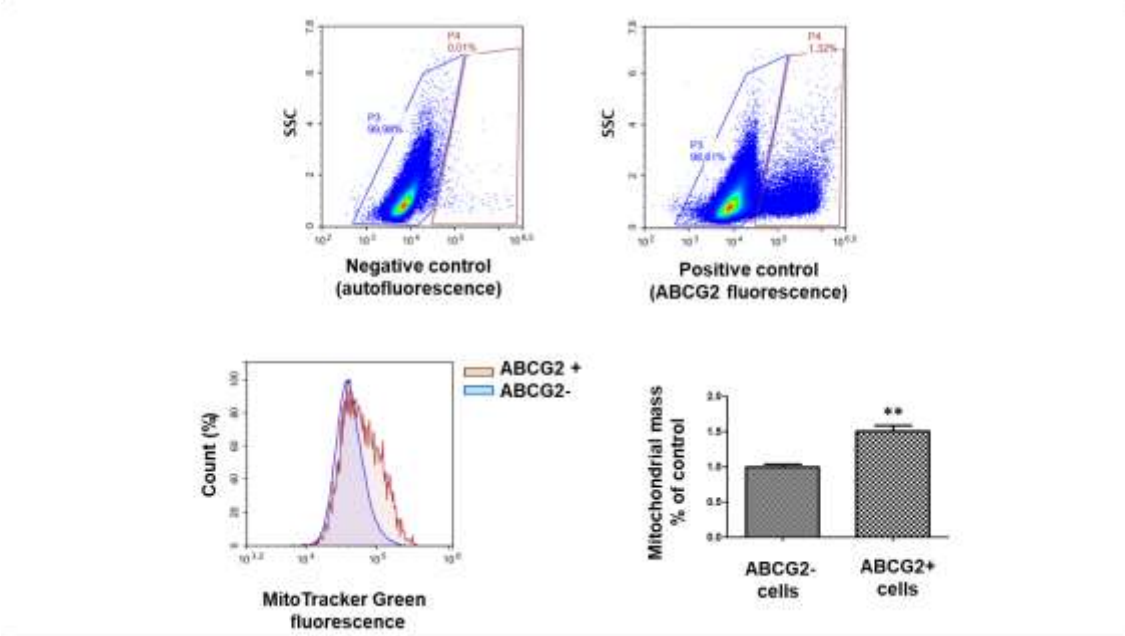

Fig. 22 ABCG2+ A375 cells display higher mitochondrial mass

(a) A375 cells were stained with MitoTracker Green FM (10 nM, 30 min) fluorescent probe and then with ABCG2 fluorescent antibody (5 µM, 30 min). Mitochondrial mass was measured by flow cytometry in ABCG2 -positive and -negative cells. One representative experiment of three performed is shown. Data represent mean values ± SEM and were analyzed by t-test. $**P < 0.01$ vs C, controls (vehicle).

A375 melanospheres display increased OXPHOS and PGC1α expression

We showed that ABCG2+ A375 cells are characterized by higher mitochondrial mass compared with ABCG2- A375 cells. To confirm this observation also in A375 melanospheres (A375 SCs culture enriched in ABCG2+ cells) and validate this stemness model from a metabolic point of view, we then analysed mitochondrial protein expression in this cell culture model.

Western Blot analysis showed an upregulation of OXPHOS proteins levels in A375 SCs compared with A375 non-SCs (Fig. 23a). In particular, we observed a 2.65 fold increase in complex V (Fig 23b), a 5.3 fold increase in complex III (Fig. 23c), a 6.7 fold increase in

complex II (Fig. 23d), a 9.2 fold increase in complex IV (Fig. 23e) and a 11.6 fold increase in complex I (Fig. 23e).

Moreover, in A375 SCs we observed an overexpression of the metabolism inducer PGC1α, the key regulator of mitochondrial biogenesis (Fig. 23f).

These results confirm that, in A375 melanospheres -the stemness model enriched in ABGC2-positive cells- mitochondrial metabolism is rewired. In particular, A375 SCs display increased expression of ETC complexes and an upregulation of PGC1α. This transcriptor factor is involved in the regulation of mitochondrial biogenesis, respiratory capacity, oxidative phosphorylation and O_2 consumption, to provide a more efficient aerobic energy production.

These results suggest that melanoma SCs are characterized by a severe reprogramming of mitochondrial metabolism and this is in line with data in literature reporting that SCs could be characterized by higher mitochondrial mass. These data suggest that this cell culture model reflects features observed CSCs. Moreover, this metabolic reprogramming also reflects metabolic features observed in vemurafenib-resistant cells. Finally, we could suggest that mitochondrial mass could be considered, in addition to ABCG2 transporter, a new metabolic marker to identify melanoma SCs.

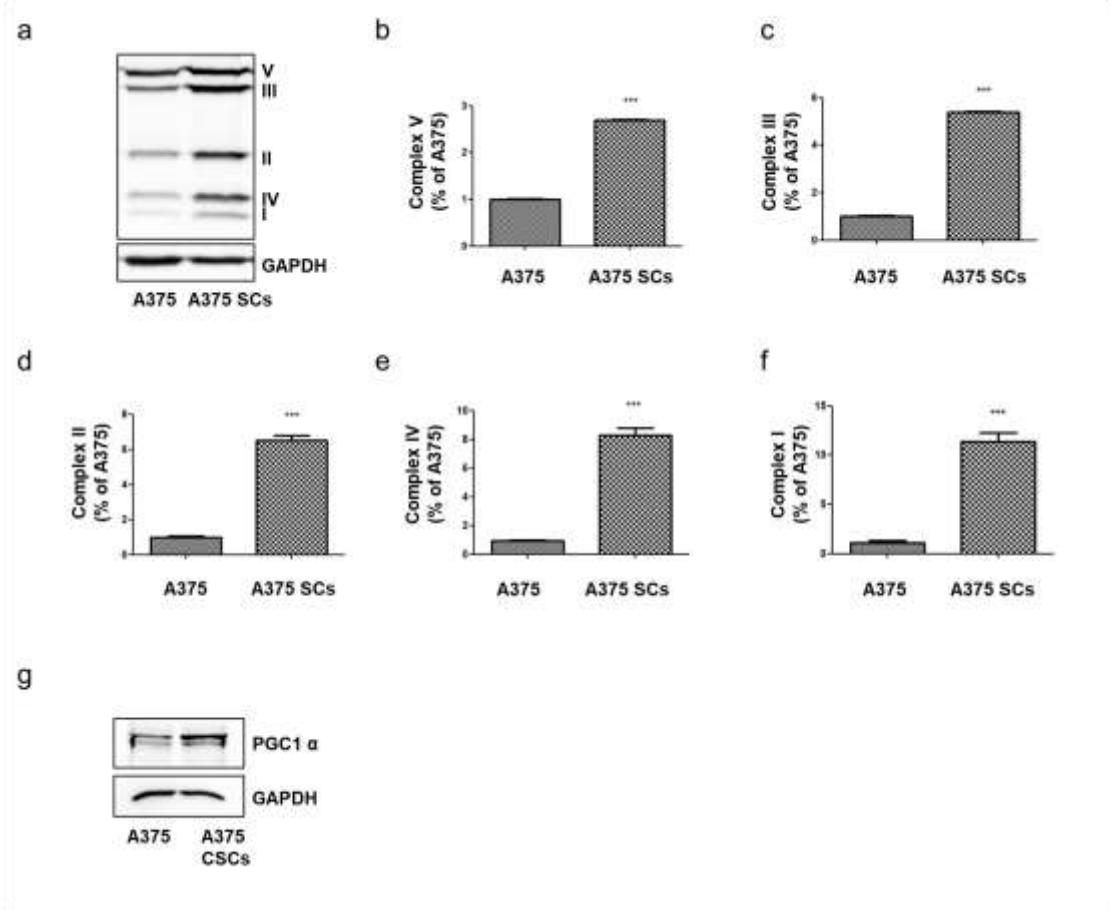

Fig. 23 OXPHOS and PGC1α are overexpressed in A375 SCs

(a) Western Blot analysis of OXPHOS proteins levels in A375 and A375 CSCs. GAPDH was used as a loading control. One representative experiment of three performed is shown; (b) complex V densitometry; (c) complex III densitometry; (d) complex II densitometry; (e) complex IV densitometry; (f) complex I densitometry; (g) Western Blot analysis of PCG1α protein levels in A375 and A375 CSCs. GAPDH was used as a loading control. One representative experiment of three performed is shown.

A375 melanospheres show altered mitochondrial dynamics

It has been reported that melanoma cells resistant to prolonged inhibition of BRAF have been found to be associated with altered mitochondrial dynamics. In particular, BRAF mutated melanoma cells treated with BRAF inhibitors display increased mitochondrial fusion and decreased mitochondrial fission [479].

To evaluate whether these characteristics are intrinsic of stem-like subpopulation independently of the treatment undergone by cancer cells, we investigated mitochondrial dynamics in A375 SCs.

By Western Blot analysis we observed an upregulation of mitochondria fusion markers OPA1 and MFN2 and a downregulation of the fission marker DRP1 in A375 melanospheres compared with A375 non-SCs (Fig. 24).

These results confirm that A375 melanospheres culture model reflects mitochondrial dynamic features observed in vemurafenib-resistant melanoma cells.

These results also suggest that mitochondrial metabolism might be reprogrammed in melanoma SCs and metabolic features observed in BRAF inhibitors-resistant cells might not be due to a metabolic switch occurring during the treatment but, on the contrary, to a selection of an aggressive subpopulation with an altered mitochondrial metabolism.

Moreover, these results suggest that, in addition to standard BRAF and MEK inhibitors, to eliminate this aggressive subpopulation it might be necessary to target this metabolic reprogramming in order to prevent tumor relapse.

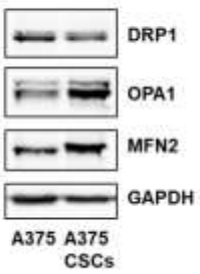

Fig. 24 Mitochondrial dynamic is altered in A375 melanospheres

(a) Western Blot analysis of fission (DRP1) and fusion (OPA1, MFN2) markers proteins levels in A375 and A375 CSCs. GAPDH was used as a loading control. One representative experiment of three performed is shown.

δ-TT impairs mitochondria in melanoma SCs

Recently it has been demonstrated that δ-TT impairs A375 SCs growth, inducing melanoshperes disaggregation and preventing their formation [163]. Here, we have demonstrated that A375 SCs display high mitochondrial mass and increased OXPHOS proteins expression. Moreover, it has been showed that high mitochondrial biogenesis is required for anchorage-independent growth of CSCs spheres [480]. Given these evidences we hypothesized that δ-TT might target A375 SCs affecting mitochondria.

To verify this hypothesis we treated A375 SCs with δ-TT 20 μg/mL for 24 h. Higher dose with respect to A375 non-SCs has been necessary, supporting their aggressiveness.

Western Blot analysis revealed that in A375 SCs δ-TT downregulated ETC complex I and IV proteins levels (Fig. 25a,b,c), PGC1α expression (Fig. 25d) and all proteins involved in mitochondrial dynamics DRP1, OPA1, MFN2 (Fig. 25e), suggesting a severe mitochondrial damage after δ-TT treatment.

Moreover, we found that δ-TT activated the phosphorylation of AMPK (Fig. 25f), demonstrating that the reduction of mitochondrial biogenesis, due to the downregulation of PGC1α and the mitochondrial damage induced by δ-TT, caused energy depletion in A375 SCs.

Taken together, these data demonstrate that δ-TT affects A375 SCs mitochondria impairing mitochondrial biogenesis, downregulating OXPHOS proteins and causing a severe alteration of mitochondrial dynamics. These data suggest that the impairment of mitochondrial function could be responsible for δ-TT-induced inhibition of melanospheres formation.

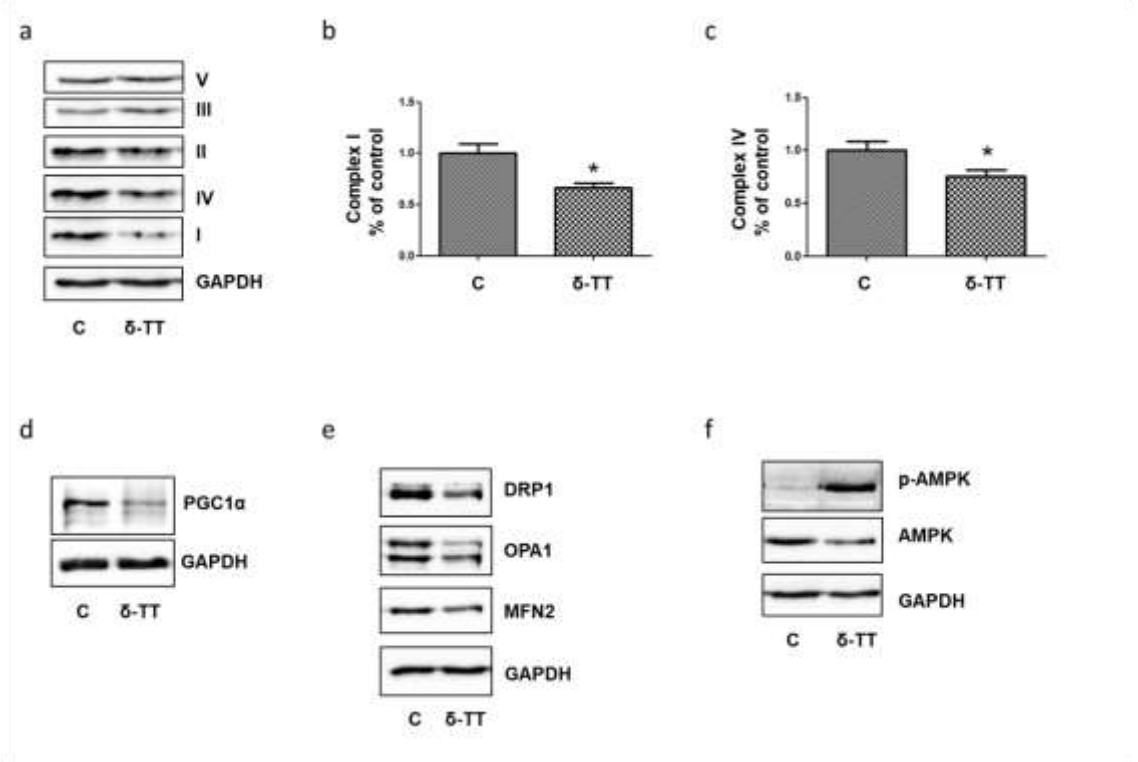

Fig. 25 δ-TT induces mitochondrial damage in A375 SCs

(a) A375 melanospheres were treated with δ-TT (20 μg/mL, 24 h). Western blot analysis was performed to evaluate OXPHOS proteins levels. GAPDH was used as a loading control. One representative experiment of three performed is shown. (b) Complex I densitometry; (c) Complex IV densitometry; (d) A375 melanospheres were treated with δ-TT (20 μg/mL, 24 h). Western blot analysis was performed to evaluate PGC1α protein levels. GAPDH was used as a loading control. One representative experiment of three performed is shown; (e) A375 melanospheres were treated with δ-TT (20 μg/mL, 24 h). Western blot analysis was performed to evaluate DRP1, OPA1 and MFN2 proteins levels. GAPDH was used as a loading control. One representative experiment of three performed is shown; (f) A375 melanospheres were treated with δ-TT (20 μg/mL, 24 h). Western blot analysis was performed to evaluate p-AMPK and AMPK proteins levels. GAPDH was used as a loading control. One representative experiment of three performed is shown.

δ-TT impairs mitochondria in vemurafenib-resistant A375 cells: preliminary data

As discussed above, it is known that melanoma cells escaping antitumor activity of BRAF-inhibitors are characterized by an altered mitochondrial metabolism. In particular, OXPHOS expression, PGC1α expression and mitochondrial fusion have been shown to be induced after BRAF or MEK inhibition [143, 481]. Since we demonstrated that δ-TT impairs mitochondria in A375 cells and in A375 SCs, and given the fact that vemurafenib-resistant melanoma cells are characterized by higher mitochondrial metabolism, we hypothesised that mitochondria could be a target also in melanoma cells resistant to vemurafenib.

First we verified that our model of A375R cells reflected mitochondrial features reported in literature. By western blot analysis, in A375R cells we observed an overexpression of OXPHOS proteins (Fig. 26a), an upregulation of PGC1α (Fig. 26b), an increased expression of OPA1 and MFN2 and a reduced expression of DRP1 (Fig. 26c), in line with data reported in literature.

Then, we treated A375R cells with δ-TT 20 μg/mL for 24 hours. Western Blot analysis revealed that in A375R cells δ-TT downregulated ETC complex I and II proteins levels (Fig. 26d,e,f), PGC1α expression (Fig. 26g) and all proteins involved in mitochondrial dynamics DRP1, OPA1, MFN2 (Fig. 26h), suggesting a severe mitochondrial damage after δ-TT treatment.

Taken together, these preliminary data demonstrate that δ-TT impairs A375R mitochondria, downregulating mitochondrial proteins, affecting mitochondrial biogenesis and mitochondrial dynamics. These data corroborate the data obtained in A375, BLM and A375 SCs, suggesting that the severe alteration of mitochondria could be the mechanism underlying δ-TT anticancer activity in cancer cells, in cancer stem cells and in treatment-resistant cancer cells. Further investigations are needed to better clarify δ-TT activity on cancer cells mitochondria.

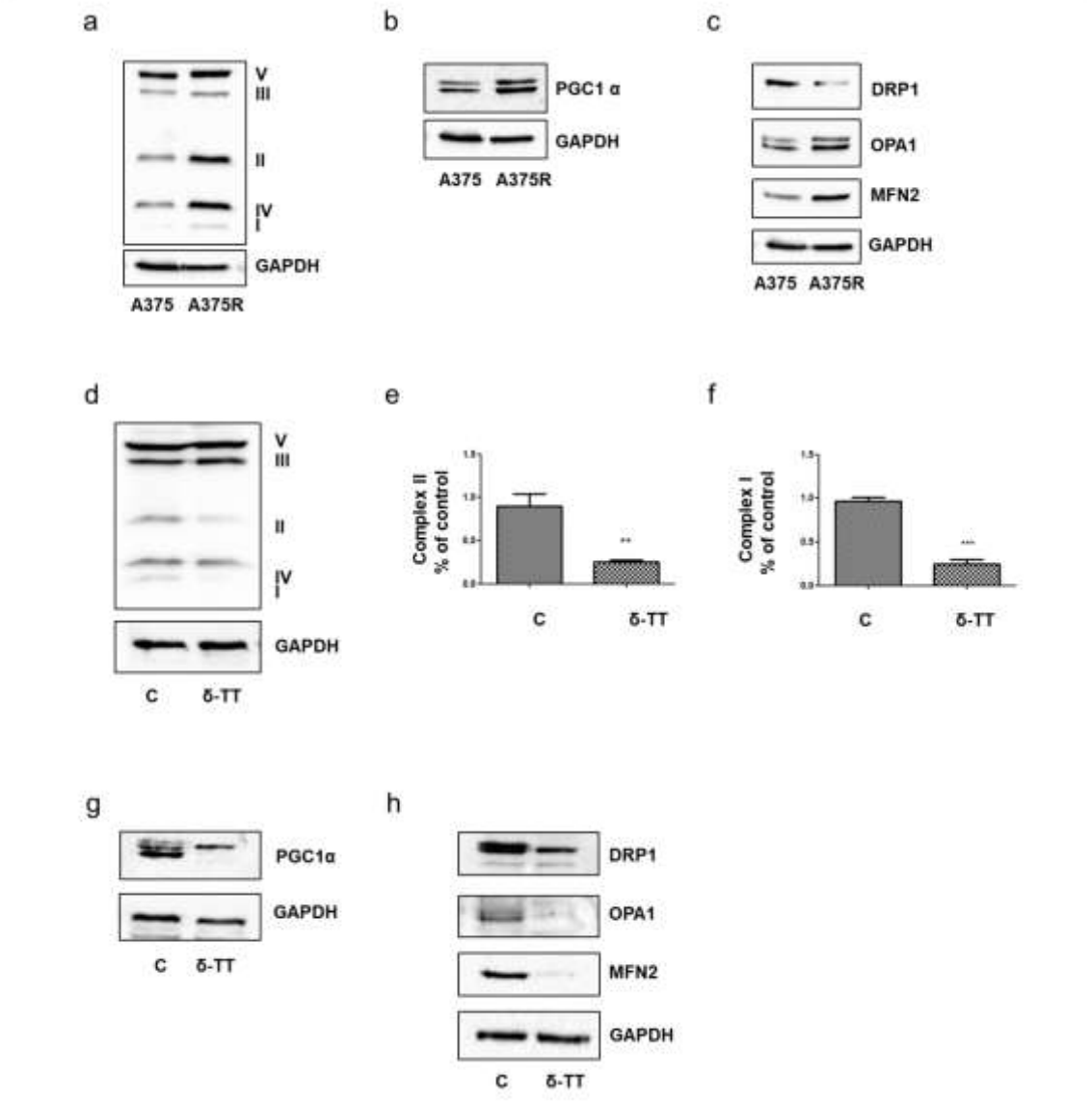

Fig. 26 δ-TT induces mitochondrial damage in vemurafenib-resistant A375 cells

(a) Western Blot analysis of OXPHOS proteins levels in A375 and A375R. GAPDH was used as a loading control. One representative experiment of three performed is shown; (b) complex II densitometry; (b) Western Blot analysis of PCG1α protein levels in A375 and A375R. GAPDH was used as a loading control. One representative experiment of three performed is shown; (c) Western Blot analysis of fission (DRP1) and fusion (OPA1, MFN2) markers proteins levels in A375 and A375R. GAPDH was used as a loading control. One representative experiment of three performed is shown; (d) A375R were treated with δ-TT (20 µg/mL, 24 h). Western blot analysis was performed to evaluate OXPHOS proteins levels. GAPDH was used as a loading control. One representative experiment of three performed is shown. (e) Complex II densitometry; (f) Complex I densitometry; (g) A375R were treated with δ-TT (20 µg/mL, 24 h). Western blot

analysis was performed to evaluate PGC1α protein levels. GAPDH was used as a loading control. One representative experiment of three performed is shown; (h) A375R were treated with δ-TT (20 μg/mL, 24 h). Western blot analysis was performed to evaluate DRP1, OPA1 and MFN2 proteins levels. GAPDH was used as a loading control. One representative experiment of three performed is shown.

DISCUSSION

Malignant melanoma represents one of the most aggressive and treatment resistance form of skin cancers. Despite the availability of several therapy options, melanoma is characterized by large heterogeneity that allows cancer cells to survive. Thus, drug resistance and tumor relapse still represent a problem [143].

In cancer therapy two are the main causes of drug resistance. First, the resistance to apoptosis. In fact, cancer cells are able to escape programmed cell death, de-regulating apoptotic pathways. For this reason, a strategy to overcome this issue could be exploiting the induction of non-canonical cell deaths such as paraptosis, a programmed cell death with different features from apoptosis, characterized by extensive cytoplasmic vacuolization related to ER dilatation and mitochondrial swelling, ROS production, calcium overload and the lack of caspase activation [482].

The second cause of drug resistance is the presence of CSCs. CSCs are a rare subpopulation of the tumor bulk, characterized by the capacity for self-renewal, the ability to generate all heterogeneous tumor cell lineages, giving rise to the tumor mass and to recapitulate continuous tumor growth. They play a key role in drug resistance, contributing to tumor relapse, thus, the identification of specific bio-markers in order to characterize CSCs is necessary to target and eliminate this subpopulation [143].

In the last decade, natural compounds have gained interest due to their anti-cancer properties and tocotrienols, belonging to vitamin E family, demonstrated to exert anti-tumor, anti-metastatic, anti-angiogenetic and pro-apoptotic properties in several cancers. However, the mechanisms and the molecular targets of their activity are poorly clarified [483, 484].

Here, in the first part of this project we investigated the anticancer activity of δ-TT in human melanoma cells, with focus on the induction of paraptotic cell death, mitochondria damage, ROS production and calcium homeostasis dysregulation.

Then, in the second part we focused on melanoma SCs and treatment-resistant melanoma cells, suggesting mitochondrial mass as a marker of stemness and aggressiveness and as a target for the anticancer activity of δ-TT.

Recently it has been demonstrated that δ-TT induces apoptosis triggering ER stress in A375 and BLM cells [163]. Synthetic and natural compounds causing ER stress have

been shown to induce non-canonical cell death such as paraptosis [333, 475, 476, 485-490].

Paraptosis is a programmed cell death and, together with other non-canonical cell deaths (such as necroptosis, mitotic catastrophe, anoikis) has been reported to be induced in apoptosis-resistant tumor cells and mediate the anti-cancer effects of several natural compounds [332, 410, 491]. This type of cell death is characterized by cytoplasmic vacuolization related to ER dilatation, mitochondrial swelling and involvement of JNK, p38, ERK1/2 MAPKs. Moreover, since it requires *de novo* protein synthesis, it is prevented by the protein synthesis inhibitor cycloheximide [325, 327, 332, 410].

Our results demonstrated that δ-TT induces severe morphological alterations in A375 and BLM human melanoma cells, causing a visible cytoplasmic vacuolization. Further analysis associated vacuoles with the dilatation of ER and mitochondria and the induction of paraptotic cell death: indeed, the treatment with the protein synthesis inhibitor cycloheximide blocked the δ-TT -induced cytoplasmic vacuolization. Moreover, molecular analysis showed that this vitamin-E derivative induces the phosphorylation of stress-induced MAPKs JNK, p38 and ERK1/2.

These data are in line with evidence in literature showing that γ- and δ- TT induce paraptotic cell death in colon carcinoma and prostate cancer [454, 455, 489]. Moreover, γ- and δ-TT have been reported to exert anti-cancer activity in different tumor types [178, 484, 492].

ER dilatation and mitochondrial swelling caused by δ-TT suggest that this compound could be responsible for their dysfunction. It has been previously demonstrated that δ-TT affects ER and triggers ER stress in A375 and BLM cell lines and that this signaling pathway is involved in δ-TT toxicity [250]. To get further insight into mitochondrial dysfunction we focused our attention on their integrity and functionality. Recently, mitochondria have been reported as important emerging and promising therapeutic targets in anti-cancer treatment because of their role in cell proliferation and in the induction of cell death [493, 494].

We showed that δ-TT caused the downregulation of OXPHOS complex I proteins levels that leads to the loss of MMP, reflecting a decrease in mitochondrial activity and a reduction in oxygen consumption in both A375 and BLM cell lines. As a consequence, the dysfunction of mitochondrial functionality caused a rapid decrease in ATP production and the activation of the energy stress sensor AMPK. These data demonstrate that δ-TT significantly impairs mitochondrial function, damaging OXPHOS proteins, impairing oxygen consumption capacity and resulting in an extensive mitochondrial membrane depolarization. This mitochondrial damage resulted in mitochondrial dysfunction causing an energy-depleting effect on A375 and BLM cells. These data are supported by increasing evidence that the target of mitochondria is a promising strategy to induce cell death in cancer cells [493-496].

The activity of the mitochondrial respiratory chain is responsible for ROS production. Mitochondrial impairment contributes to ROS overproduction, resulting in uncontrolled oxidative stress. Mitochondria are not only ROS sources but also targets of ROS: indeed, they have been reported to participate in cell death, both canonical and non-canonical, triggering different cell signaling pathways [351, 497, 498].

Different natural compounds have been shown to induce paraptotic cell death through the production of high levels of ROS. In epithelial ovarian cancer morusin triggers paraptosis affecting mitochondria and increasing ROS production [476]; in human breast cancer cell lines, withaferin has been shown to cause ROS-mediated paraptotic cell death [478]; recently, in prostate cancer cell lines PC3 and DU145 δ-TT has been reported to induce both apoptosis and paraptosis, involving ER stress, mitochondrial dysfunction and ROS production [435, 489].

Here we showed that δ-TT induces an extensive generation of mitochondrial ROS in melanoma cells, involved in the cytotoxic activity, in the paraptotic vacuolization and the phosphorylation of MAPKs. These data support that the functional and morphological changes induced by δ-TT in melanoma cells are partially mediated by mitochondrial ROS production and the redox imbalance caused by this vitamin E isoform is responsible for paraptotic cell death in melanoma cells.

Based on the above evidence, we then investigated the potential pathways responsible for the paraptotic cascade observed during δ-TT treatment. It is known that the ER and mitochondria are the main regulators of intracellular calcium homeostasis and that the transfer of this ion between these organelles occurs through ions channels located on the mitochondria-associated ER membranes. Thus, mitochondrial dysfunction is often associated with Ca^{++} homeostasis dysregulation. It has been reported that calcium transfer from ER to mitochondria mediates the cytotoxic activity of celastrol, morusin, curcumin, hesperidin and other several compounds, suggesting that ions homeostasis dysregulation is a mechanisms underlying the anticancer activity of different natural compounds [475, 476, 499, 500].

Thus, we measured calcium levels after δ-TT treatment and we observed that both cytoplasmic and mitochondrial calcium levels were increased in melanoma cells. To study its involvement in the anti-cancer activity of this compound we used the IP3R blocker 2APB the VDAC blocker DIDS, to inhibit calcium release from ER and its influx into mitochondria. The inhibition of calcium flow partially reverted the cytotoxic activity of δ-TT, demonstrating the role of Ca^{++} overload in cell death. Then, we found that both cytoplasmic and mitochondrial Ca^{++} levels were also involved in paraptotic vacuolation and MAPK activation induced by δ-TT in melanoma cells. Moreover, TEM analysis revealed the presence of some sites of tightly communication between ER membranes and mitochondria in both these cell lines, suggesting the existence of calcium fluxes at the ER-mitochondria interface.

Finally, we investigated the relationship between calcium overload and ROS generation in the anti-cancer activity of δ-TT in melanoma cells, demonstrating that Ca^{++} release from ER is directly associated with ROS production.

Notably, many natural compounds have been reported to cause Ca^{++} dysregulation- and ROS- related paraptosis in cancers, but this is the first evidence reconstructing the key role of these molecular interactions in the anti-melanoma effects of TTs.

Then, in the second part of this project we focused on CSCs. Previously it has been demonstrated that δ-TT counteracts melanoma stem cells growth [163], now we

analyzed mitochondrial features of A375 SCs, investigating mitochondria as markers of stemness and we analyzed if δ-TT affects A375 CSCs growth targeting mitochondria.

The identification of specific markers in order to characterize CSCs could provide new targets for cancer treatment. Different cell surface markers, such as CD133 and CD271, have been proposed as CSCs markers in melanoma, but is now accepted that they do not represent a reliable method to detect these cells [143]. Recently, it has been proposed ABCG2 transporter as a new marker for melanoma stem cells grown as melanospheres and resistant to vemurafenib treatment [163]. Interestingly, ABCG2 has also been reported to be associated with enhanced tumorigenic potential in melanoma cells, it has been reported to be expressed in melanoma metastasis and to be correlated with patient survival [143, 174].

Recently CSCs have also been reported to be coupled with metabolic alterations and mitochondrial mass has been proposed as a metabolic biomarker for stem-like cells that are chemoresistant in breast cancer [149, 234, 235].

Moreover, melanoma cells escaping the antitumor activity of BRAF and MEK inhibitors were reported to display an augmented expression of the mitochondrial biogenesis activator PGC1α, increased mitochondrial mass and OXPHOS [143]. Moreover, prolonged inhibition of BRAF was also described to be associated with different mitochondrial dynamics, increased mitochondrial fusion and decreased mitochondrial fission [143, 479, 481].

Given these evidence we investigated mitochondrial features in ABCG2+ subpopulation and in A375 melanosphere model. We observed that ABCG2 positive cells showed higher mitochondrial mass compared with ABCG2 negative cells. We confirmed this observation also in melanosphere model, showing an upregulation of OXPHOS proteins levels and an overexpression of the metabolism inducer PGC1α, the key regulator of energy metabolism. This transcription factor upregulates mitochondrial biogenesis, respiratory capacity, oxidative phosphorylation and O2 consumption, to provide a more efficient aerobic energy production.

Since melanoma cells resistant to prolonged inhibition of BRAF were also reported to be associated with increased mitochondrial fusion and decreased mitochondrial fission

[479], we investigated mitochondrial dynamic in A375 melanospheres, to evaluate whether these characteristics are intrinsic of stem-like cells, independently of the treatment undergone by cancer cells. We observed an upregulation of mitochondria fusion markers OPA1 and MFN2 and a downregulation of the fission marker DRP1 In A375 melanospheres compared with A375 non-SCs.

The upregulation of OXPHOS, PGC1α and fusion markers observed in A375 melanospheres (not treated with BRAF inhibitors, but resistant to vemurafenib as showed before [163]) might suggest that mitochondrial metabolism might be reprogrammed in these cells. This could suggest that the metabolic reprogramming observed in BRAF inhibitors-resistant cells might not be due to a metabolic switch occurring during the treatment but, on the contrary, to a clone selection. Probably, we might suggest that a rare subpopulation characterized by an altered metabolic profile is present in the tumor mass and it survives to BRAF inhibitors treatment. This resistant subpopulation might be responsible for relapse, giving rise to a tumor with a new metabolic pattern.

This could suggest that, in addition to standard BRAF and MEK inhibitors, it might be necessary to target this metabolic reprogramming in order to prevent tumor relapse. Several studies highlight the possibility of selective targeting of CSCs with natural compounds. EGCG, curcumin, genistein, quercetin, resveratrol, apigenin, berberin have been shown to be active against cancer stem cells, modulating the ABC transporters, the WNT/β-catenin pathway, the NOTCH pathway, the Hedgehog pathway and other signaling pathways involved in stemness maintenance [501]. Recently it has become clear that mitochondria could represent a promising target for resistant stem-like cells [232, 502, 503] .

Therefore we investigated if δ-TT counteracts A375 melanospheres growth affecting mitochondria. Experiments showed that δ-TT downregulated ETC complex I and complex IV proteins levels, PGC1α expression and all proteins involved in mitochondrial dynamics: DRP1, OPA1, MFN2, suggesting a significant mitochondrial damage. Moreover, we found that δ-TT activated the phosphorylation of AMPK, demonstrating

that the reduction of mitochondrial biogenesis, due to the downregulation of PGC1α, causes energy depletion in A375 melanospheres.

These data confirm that δ-TT impairs CSCs mitochondria, supporting that its anti-cancer activity in melanoma SCs is mediated by the dysfunction of mitochondrial metabolism.

Finally, to further validate these data we analysed δ-TT activity on mitochondria in A375R cells. First we verified that our model of A375 cells resistant to vemurafenib was characterized by increased OXPHOS, PGC1α and mitochondrial fusion. Then we assessed the effect of δ-TT and we showed that it reduced OXPHOS complexes, PGC1α expression and proteins involved in mitochondrial dynamics (DRP1, OPA1 and MFN1), suggesting a severe structural and function mitochondrial impairment after δ-TT treatment.

Taken together, these preliminary data demonstrate that δ-TT impairs mitochondria also in melanoma cells resistant to vemurafenib and corroborate the idea that the anti-cancer activity of δ-TT in cancer cells, in cancer stem cells and in treatment resistant cancer cells is mediated by the impairment of mitochondrial metabolism. Interestingly, δ-TT mechanism on cancer cells mitochondria is independent of mitochondrial dynamics, since the sensitivity of cancer cells to the anti-proliferative effect of δ-TT is not correlated with higher mitochondrial fusion or fission.

In conclusion, this work demonstrates that δ-TT exerts an anti-cancer activity in A375 and BLM melanoma cells triggering the non-canonical cell death paraptosis. Moreover, we showed that this natural compound affects mitochondria, inducing a significant metabolic dysfunction, ROS production and calcium homeostasis dysregulation. Moreover, we suggested mitochondrial mass as a new metabolic biomarker for the identification and the target of melanoma SCs.

Finally, all these data highlight the key role of mitochondria in cancer: mitochondria are fundamental for cell survival and represent a key player in stemness maintenance and tumor aggressiveness. Our finding support the idea of the importance to target mitochondrial metabolism to eradicate aggressive cancer cells, in order to prevent tumor recurrence, metastasis and drug resistance. Targeting mitochondrial dependency

of cancer cells appears to be promising for the development of novel therapeutic strategies.

Despite all these evidences, data on the effectiveness, the pharmacokinetics and bioavailability of δ-TT *in vivo* and in patients are still incomplete. Thus, animal models and clinical trials to confirm δ-TT efficacy are urgently needed. Moreover, since melanoma standard therapies are associated with high toxicity and a low response rate, the association of δ-TT with standard treatments aimed at reducing drug doses should be investigated, in order to verify the possibility to reduce side effects and prolong overall survival in patients.

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