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THE EFFECT OF SULFORAPHANE IN HUMAN MELANOMA CELLS

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Summary

Melanoma is a malignant tumor that arises from melanocytes present in the basal layer of the epidermis, in hair follicles, more rarely in mucosa eye, inner ear and meninges. Skin is affected in 85% of cases. Human malignant melanoma is an highly aggressive and incurable cancer due to intrinsic resistance to apoptosis, reprogramming proliferation and survival pathways during tumor progression. Several studies have shown that many natural dietary compounds can potently modulate various molecular targets, conducting prevention of cancer initiation, promotion and progression. In particular, dietary fruits and vegetables have been regarded as rich sources of chemopreventive compounds and are widely investigated due to their low toxicity but significant chemopreventive efficacy. Sulforaphane (SFN), an isothiocyanate (ITC) found in cruciferous vegetables, is a common dietary component that has been proven to have a huge cancer chemopreventive potential. It modulates cell death, cell cycle, angiogenesis, susceptibility to carcinogens, invasion and metastasis and possesses antioxidant activities. Pluralities of clinical effects are reported in various experimental diseases as well as human clinical studies. In B16F-10 murine melanoma cell line SFN reduces invasion, inhibits activation of matrix metalloproteinases and, consequently, the developing of lung metastases, and prevents melanin synthesis and tyrosinase expression by affecting the phosphorylation of MAP kinase family. Epidemiological studies have reported association between the consumption of SFN-rich vegetables and reduction in cancer risk at several sites including the bladder, prostate and breast. Moreover, SFN is in phase II trial for prostate cancer.

The aim of this project was to evaluate the effects of sulforaphane on human melanoma cell lines, in order to prove its ability to modulate specific cellular and molecular events involved in this type of tumor. A human primary (A375) and metastatic (501Mel) melanoma cell lines has been used in these studies. I demonstrated that SFN inhibits significantly A375 and 501Mel cell proliferation,

induces cell cycle arrest and apoptosis. Interestingly, SFN-treated melanoma cells accumulated in the G_2/M and sub-G_1 phases of the cell cycle. Moreover SFN showed to induce apoptosis through caspase-9/caspase-3 pathway. As control, human epidermal melanocytes cell lines (HEMa) treated with the same concentration of sulforaphane didn't shown an alteration. Besides its ability to promote tumor cell death, SFN hampers cell migration and invasion. One important process in chemoprotection by SFN involves modulation of the activity of the so-called phase II enzymes, which convert carcinogens to inactive metabolites that are readily excreted from the body, thus preventing their reaction with DNA. Interesting, the results showed that in melanoma cell lines SFN increased significantly the mRNA levels of phase II enzymes such as hemoxygenase-1 (HMOX-1) and NADPH quinone oxidoreductase (NQO1).

To better characterize the molecular events induced by SFN on melanoma, I investigated the expression of nerve growth factor (NGF) receptors, known to be involved in melanoma progression. Using RT-PCR and fluorescence-activated cell sorting analysis, it was found that SFN causes up-regulation of neurotrophin receptors expression (p75NTR and TrkA) in A375 cell lines. Moreover, SFN is able to decrease the A375 migration induced by exogenous β-NGF. These observations led to the hypothesis that SFN induces apoptosis through p75NTR and that p75NTR-dependent apoptosis could represent a homeostatic mechanism to eliminate damaged cells – similar to Fas-dependent apoptosis associated with inflammation – and possibly a 'class effect' of death receptors. Finally, in this study, it was also established SFN-resistant melanoma cell lines in order to elucidate mechanisms leading to drug resistance. All together, these results indicate that, *in vitro*, sulforaphane has a strong antitumor effect on human melanoma cell lines and this raises the possibility that SFN might be a promising candidate for molecular-targeting chemotherapy against melanoma.

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List of Abbreviation

α-MSH	α-Melanocyte Stimulating Hormone/Melanotropin
APS	Ammonium Persulfate
ARE	Antioxidant-Response Element
ARF	Alternative Reading Frame Gene
ASIP=ASP	Agouti signalling protein
ATP	Adenosine triphosphate
BDNF	Brain Derived Neurotrophic Factor
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BSA	Bovine Serum Albumin
CDKN2A	Cyclin dependent kinase inhibitor 2A
CMM	Cutaneous Malignant Melanoma
DMEM	Dulbecco's Modified Eagle Medium
DMSO:	Dimethylsulfoxid
DOPA	Dihydroxyphenylalanine
DTT	1,4-Dithio-DL-threit(ol)
EDTA	Ethylenediaminetetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GCLC	Glutamate-Cysteine Ligase Catalytic Subunit
GCLM	Glutamate-Cysteine Ligase Modifier Subunit
HBSS	Hank's Balanced Salt Solution
HEMa-LP	Human Epidermal melanocytes (isolated from lightly pigmented)
HMGS-2	Human Melanocyte Growth Supplement-2 PMA-Free
HMOX-1	Heme oxygenase 1
KC	Keratinocyte
Keap-1	Kelch-like ECH-associated protein 1
MAPK	Mitogen Activated Protein Kinase
MC	Melanocyte

MCR1	Melanocortin 1 receptor
MM	Malignant Melanoma
Mrna	Messenger ribonucleic acid
NF-Kb	Nuclear Factor-kappa B
NGF	Nerve Growth Factor
NO	Nitric oxide
NQO1	NADP(H) quinoline oxidoreductase-1
NRF2	Nuclear factor, erythroid 2-like 2
NT	Neurotrophin
OCA 1	Oculocutaneous albinism type 1 (tyrosinasenegative),mutated gene: *TYR*
OCA 2	Oculocutaneous albinism type 2 (tyrosinasepositive), mutated gene: *OCA2=P gene*
PI3K	Phosphatidylinositol 3-kinase
PI	Propidium Iodide
POMC	Pro-opiomelanocortin
PS	Phosphatidylserine
p75NTR	p75 Neurotrophin Receptor
ROS	Reactive Oxygen Spices
RPMI	Roswell Park Memorial Institute
SLC45A2	Solute Carrier Family 45, Member 2
SFN	Sulforaphane
TrkA	Tropomyosin related kinase type A
TrkB	Tropomyosin related kinase type B
TrkC	Tropomyosin related kinase type C
TYR	Tyrosinase
TYRP1=TRP	Tyrosinase-related protein 1
UVA	Ultraviolet A radiation (320-400nm)
UVB	Ultraviolet B radiation (290-320nm)
UVR	Ultraviolet radiation (200-400nm)

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Introduction

1. Skin structure and function

Skin represents the body's first line of defense against the external environment. Being the largest organ of the human body, and since it covers the whole body, skin has the widest surface area, precisely of 1.8 m^2 compared to all the other organs; it weighs more than any single internal organ, accounting being about 15% of body weight. Skin is continuous, with the mucous membranes lining the body's surface. The skin surface is not smooth, but there are folds, reliefs, ruts and holes. The thickness, pigmentation, and distribution of the appendages of skin vary in different parts of the body, depending one the function and needs of the area [1].

Skin is divided into two layers separated by a membrane (basal membrane or dermo-epidermal junction). The surface layer, of epithelial nature, is called ectodermal epidermis, while the deep layer of mesodermal origin and structure of connective tissue, consists of the dermis and the subcutaneous adipose tissue. In the dermis, the presence of vessels, nerves and skin appendages (pilosebaceous follicles, apocrine glands, eccrine glands) completes the microscopic skin.

The epidermis is the outer layer, serving as the physical and chemical barrier between the interior body and external environment; the dermis is the deeper layer providing the structural support of the skin, below which there is a loose connective tissue layer, the subcutaneous tissue or hypodermis which is an important depot of fat (Figure 1) [1]. The most outer level, the epidermis, consists of a specific constellation of cells known as keratinocytes (KC), which function is to synthesize keratin a long, threadlike protein with a protective role. The epidermis is commonly divided into four layers according to KC morphology and position as they differentiate into horny cells, including the basal cell layer (stratum germinativum), the squamous cell layer (stratum spinosum), the granular cell layer (stratum granulosum), and the cornified or horny cell layer (stratum corneum). It is composed primarily of two types of cells: KC and

dendritic cells, but it harbors a number of other cell populations, such as melanocytes, Langerhans cells, and Merkel cells. The middle layer, the dermis, is an integrated system of fibrous, filamentous, diffuse, and cellular connective tissue elements that accommodates nerve and vascular networks epidermally derived appendages, and contains many resident cell types included fibroblasts, macrophages, mast cells, and transient circulating cells of the immune system; it is fundamentally made up of the fibrillar structural protein known as collagen. The dermis lies on the subcutaneous tissue, which contains small lobes of fat cells known as lipocytes [2]. Skin is a dynamic organ in a condition of change, constantly self-renewing tissue in which a loss of the cells from the surface of the stratum corneum (desquamation) is balanced by cell growth in lower epidermis. Upon leaving the basal layer, the keratinocytes start to differentiate and during migration through the stratum spinosum and stratum granulosum undergo a number of changes in both structure and composition [2]. Skin is a complex metabolically active organ, which performs important physiological functions; it plays an extremely important role, providing a vast physical barrier against mechanical, chemical, and microbial factors that may affect the physiological status of the body [3]. In addition to those functions, skin also acts as an immune network and, through its pigments, provides a unique defence system against UV radiation (UV-R) [4]. Skin is equipped with sensory function, because it possesses sensory innervations by means of which it is capable of perceiving stimuli of mechanical, thermal and pain; it is in communication with the central nervous system and allows the individual to adapt to external environmental conditions. It is able to eliminate catabolites produced by the body. Through the sweat and sebaceous glands, water, carbon dioxide and small amounts of sebum mineral ions (calcium, chlorine, potassium, magnesium and sodium) are secreted. This process increases with the increase in metabolic activity. Skin acts as both a thermal regulator that an insulator, perform the absorption function, increasingly exploited for the transdermal administration of drugs (Table 1.1).

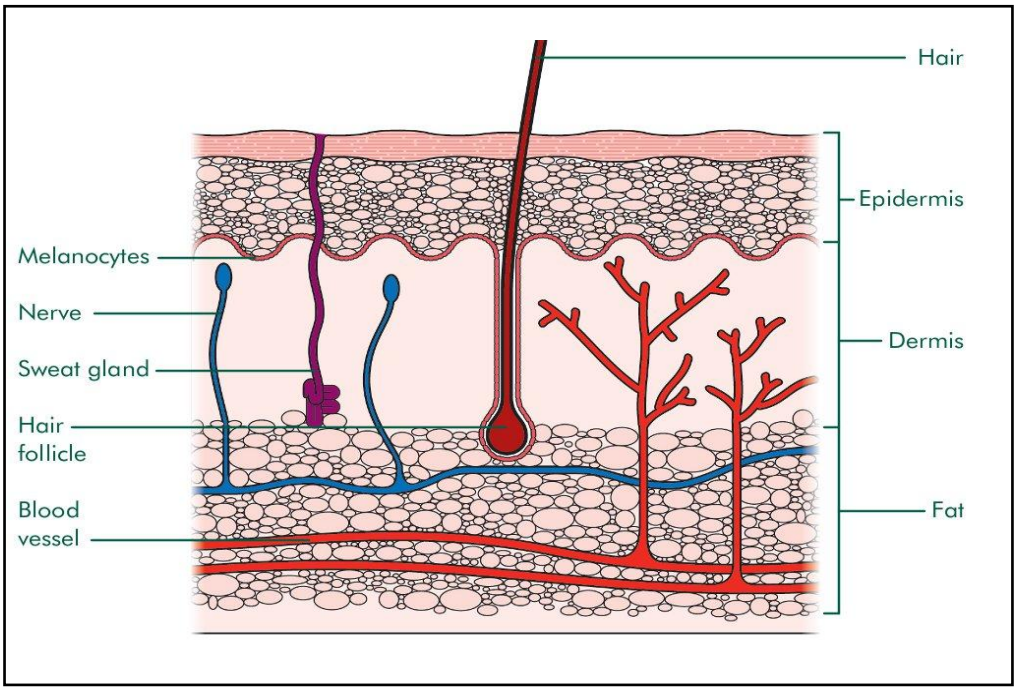

Figure 1. Structure of skin.

There are three structural layers to skin: epidermis, dermis and fat.

Hair, nails, sebaceous, sweat and apocrine glands are regarded as derivatives of skin.

Functions of skin

- Provides a protective barrier against mechanical, thermal and physical injury and noxious agents
- Prevents loss of moisture
- Reduces the harmful effects of UV radiation
- Acts as a sensory organ
- Plays a role in immunological surveillance
- Helps regulate temperature control
- Synthesises vitamin D3 (cholecalciferol)

Table 1. Functions of skin

2. Melanocytes

Human melanocytes of skin are located in the hair follicles (termed follicular melanocytes) and at the epidermal-dermal border, above the basal lamina (termed interfollicular or epidermal melanocytes), besides are localized in the brain, inner ear and choroid layer of the eye (pigmented cell in the retinal pigmented epithelium are not of neural crest origin, rather they develop from locally derived neural epithelium of the optic cup) [5]. On the other hand, mouse melanocytes are generally located in the hair follicles, except for the hairless parts of the body (tail, nose and ears) where they are located in the epidermis.

Melanocytes (MC) are the main responsible cells for skin pigmentation, typically, they are between 1000 and 2500 per square millimeters of skin. Prospective MC, known as melanoblasts, derive from the neural crest beginning in the second month of human embryonic life and migrate throughout the mesenchyme of the developing embryo. They reach specific target sites, mainly the dermis, epidermis, and hair follicles, the uveal tract of the eye, the stria vasculare, the vestibular organ and the endolymphatic sac of the ear, and leptomeninges of the brain. In humans, this migration process takes place between the 10th and the 12th week of development for the dermis and about 2 weeks later for the epidermis [2]. Once established in situ, melanocytes start producing melanosomes, highly organized elliptic membrane bound organelles in which melanin synthesis takes place. The survival and migration of neural crest-derived cells during embryogenesis is highly depends on interactions between specific receptors on the cell surface and their extracellular ligands, precisely it depends on the presence of a tyrosine kinase receptor on their surface (c-kit) and its binding, produced from stem cells. Recently, it was shown that soluble factors secreted by keratinocytes are able to regulate the proliferation of melanocytes, the development of their dendritic extensions and their activity of melanogenesis.

The cytology of the melanocyte varies remarkably. They vary from small to large; although the majority of the cells are bipolar or spindle in shape they may be round, oval, bipolar, spindle, epithelial-like or polydendritic. The amount of the

intracytoplasmic melanin pigment granules that they contain is variable, in actively growing cultures the number of non pigmented cells often exceeds that of pigmented ones, whether or not they contain pigment these cells are all potentially capable of synthesizing melanin. There are also differences in the structure of these cells [6][7]. The bipolar nonpigmented cells contain only nonmelanized premelanosomes, while the large epithelial or polydendritic pigmented cells contain premelanosomes, melanosomes and melanosome complexes. The young and physiologically active MC are usually larger than the propigment cells and their shape may be stellate, bipolar or dendritic. The old melanocytes usually are large, they have much larger cell bodies and shorter processes, in contrast to the small cell body and long and slender processes of the younger melanocytes. They are enzymatically inactive, contain intracytoplasmic melanin granules and usually do not have premelanosomes [7][8].

The function of MC in mammalian skin is to form and maintain dendrites, to synthesize and mature melanosomes with melanin and to secrete these into KC with which they are associated. In the human epidermis, each melanocyte secretes melanosomes to approximately 36 keratinocytes, forming an *epidermal melanin unit* [7]. Melanins are polymers of phenolic compounds that present different characteristic: insolubility, organic superconductivity, photo-protection, stable paramagnetism, affinity to ligands-drugs and metal cautions; melanins, the end-products of complex multistep transformations of L-tyrosine, are represented by eumelanin, pheomelanin, neuromelanin, and mixed melanin pigment. Mammalian melanocytes produce two chemically distinct types of melanin pigments: blackbrown eumelanin and yellow-reddish pheomelanin. They have different functions where eumelanin is photoprotective and acts as a scavenger to Reactive Oxygen Species (ROS), unlike pheomelanin that is probably harmful after UVR exposure, via the generation of free radicals/ROS. Similar to other melanins, neuromelanins are brown/black pigment with stable paramagnetic properties, insoluble in organic solvents and bleached by hydrogen peroxide; neuromelanin existing in substantia nigra, protects against their ability to promote neurodegeneration in fact play a role in Parkinson's and Alzheimer's disease [9]. The variation in skin colour among various races is mainly determined by

the number, melanin content, and distribution of melanosomes produced and transferred by each MC to a cluster of KC surrounding it [10]. The melanin content of human melanocytes is heterogeneous not only between different skin types but also between different sites of the skin from the same individual. This heterogeneity is highly regulated by gene expression, which controls the overall activity and expression of melanosomal proteins within individual melanocytes. In general, highly pigmented skin contains numerous single large melanosomal particles (0.5–0.8 mm in diameter), which are ellipsoidal and intensely melanotic. Lighter pigmentation is associated with smaller (0.3–0.5 mm in diameter) and less dense melanosomes, which are clustered in membrane-bound groups. These distinct patterns of melanosome type and distribution are present at birth and are not determined by external factors (such as sun exposure). The key factor in the production of melanin is the amino acid tyrosine, but tyrosinase is the rate-limiting enzyme for melanogenesis [9]. Tyrosinase catalyzes the conversion of L-tyrosine to Dihydroxyphenylalanine (DOPA) and further to DOPA quinone, which is required for the synthesis of both eumelanin and pheomelanin (Figure 2). The formation of pheomelanin requires the presence of cysteine as well as less tyrosinase activity and less cyclic AMP (cAMP) than does the formation of eumelanin. The activity of tyrosinase is enhanced by DOPA and is stabilized by tyrosinase-related-protein 1 (TRP1), a transmembrane protein of the melanosome [8]. Production of melanin is regulated by various factors via receptor-dependent and -independent pathways, in a hormonal, auto-, para-, or intracrine manner [11][12]. The most important regulators of melanogenesis are melanocortin-1 receptor (MC1R) and its ligands α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH), that are produced by the enzymatic cleavage of proopiomelanocortin (POMC) in MC, keratinocytes and other skin cell types. Binding of α-MSH or ACTH to MC1R on melanocytes stimulates the expression of the melanogenic cascade and eumelanin synthesis [12].

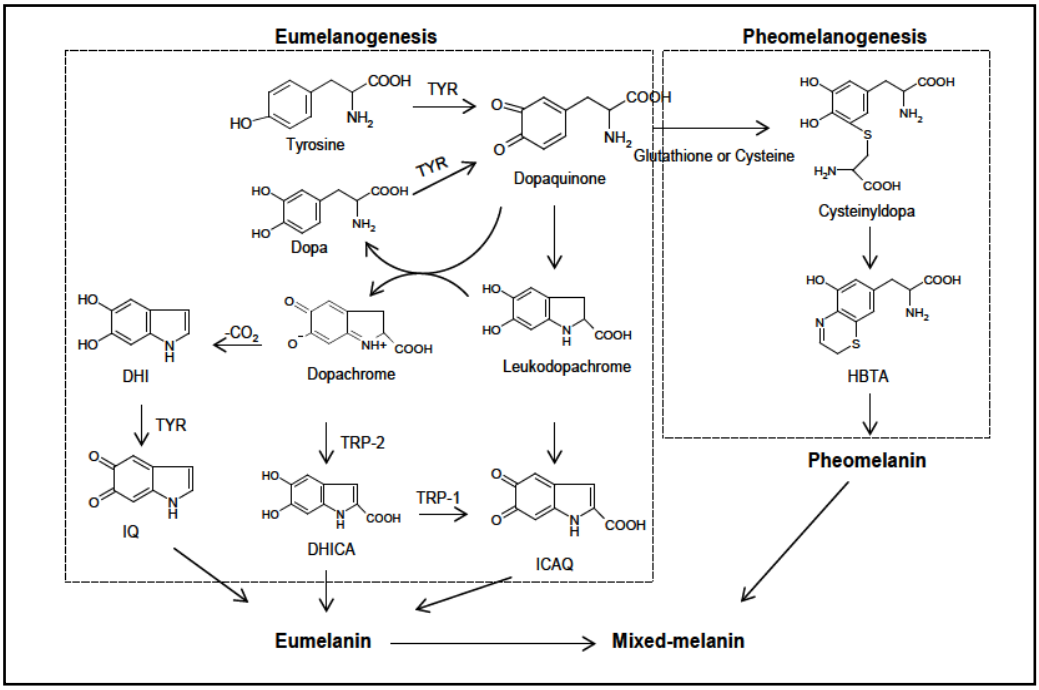

Figure 2. Biochemical pathways leading to the synthesis of melanins.

TRP-1, tyrosinase-related protein-I; DCT, dopachrome tautomerase (also called TRP-2) [12].

The absence or severe dysfunction of tyrosinase and other key pigment enzymes (including P gene, the human homologue of the mouse pink-eyed dilution locus, tyrosinase-related protein 1, TRP1, and membrane- associated transporter protein, MATP) results in oculocutaneous albinism (OCA1–4), which presents with intact melanocytes but inability to make pigment. Beside their ability to distribute melanin to surrounding keratinocytes, MC are also able to produce a wide range of signal molecules, such as cytokines, melanocortin peptides, catecholamines, serotonin, eicosanoids, and nitric oxide (NO). Upon UVR and other stimuli they secrete for example NO as a signaling molecule. Why the melanocytes produce NO is not quite clear, it was shown that NO stimulates melanin production and, as it is released by keratinocytes in response to UV radiation, it could serve as a paracrine factor in UVR-induced melanogenesis [8]. The melanocytes are capable of producing higher amounts of NO than keratinocytes, indicating the possibility that NO acts as an autocrine factor to regulate melanogenesis. However, the main importance of NO in melanocytes

appears not to be its function as an autocrine mediator, but as a signaling molecule linking the melanocyte with other systems in the skin, besides could be related to a phagocytotic property of melanocytes as they produce a number of cytokines. Being the melanocytes susceptible to UVR and oxidative stress, genetic mutations may occur and then subsequent malignant transformations [14].

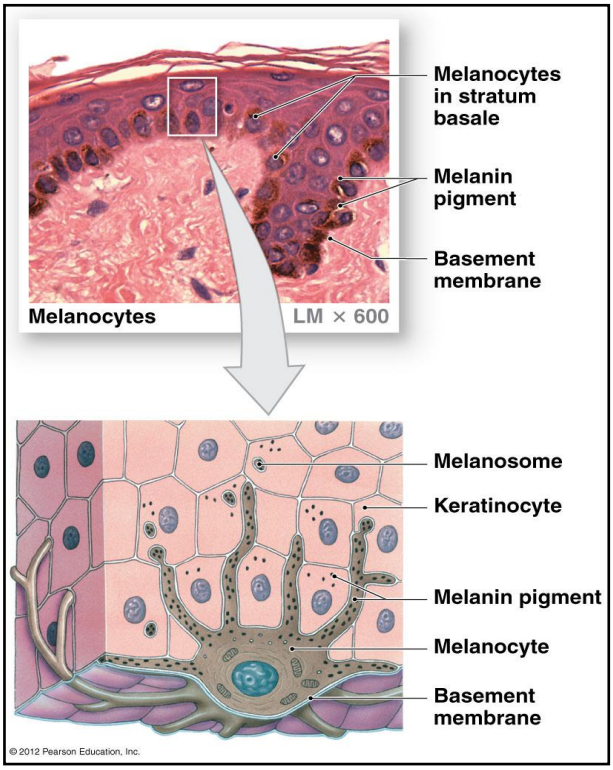

Figure 3. Melanocytes. (Pearson education, 2012)

The anatomical relationship between keratinocytes and melanocytes is known as the "epidermal melanin unit" and it has been estimated that each melanocyte is in contact with ~40 keratinocytes in the basal and suprabasal layers.

3. Melanoma

Skin cancer is the third most common human malignancy. Its global incidence is rising at an alarming rate and its most common forms are basal cell carcinoma, squamous cell carcinoma and melanoma.

Melanoma is a malignant tumor derived from epidermal melanocytes (*cutaneous* malignant melanoma, CMM) and can occur in any tissue that contains these cells, including noncutaneous sites such as the oral mucosa, nasopharynx, paranasal sinuses, tracheobronchial tree, vulva, vagina, anus, urinary tract, central nervous system (leptomeningeal melanoma), and eye. Melanomas can arise de novo in the skin (about 70%) or have a common nevus or a clinically atypical nevus as a precursor lesion (in about 30%) [15].

3.1. Epidemiology, risk factors and prevention

Malignant melanoma (MM) is an aggressive malignancy responsible for nearly 60% of death from skin cancers. There are 2–3 million estimated cases of skin cancer across the world each year, and although melanoma consist of about 132,000 of these (World Health Organization), it is the most dangerous form and it causes most skin cancer deaths [16]. In the United States, nearly 9,500 individuals will die of melanoma in 2013. Malignant melanoma is the 5th most common cause of cancer death in US; in 2013, it is estimated that there will be 76,690 new cases of melanoma of the skin and an estimated 9,480 people will die of this disease [17]. The mortality rates are higher in Australia and New Zealand and lower in South Central Asia, with a 49-fold variation in World AS mortality rates between the regions of the world for males, and a 23-fold variation for females [18]. In Italy, it is estimated that in 2011 nearly 13,000 new diagnoses of melanoma [19], also melanoma ranks high in terms of frequency in subjects aged <50 years, among which is the 8-10% of the total in both sexes, although it represents 2-3% of all cancers (excluding non-melanoma skin) [19]. With increased life expectancy of the elderly population, melanoma will be a public health challenge. If

melanoma is diagnosed early it can be cured by surgical resection, and about 80% of cases are dealt with in this way. Generally melanoma tends to occur at a relatively early age, compared to most other solid tumors, this suggested that only a limited number of events (cellular or genetic) is required for the transformation in MM. Melanoma risk factors include both intrinsic (genetic and phenotype) and extrinsic (environmental or exposure) factors. These associations indicate that genetic factors determine the development of melanoma but, environmental influences, such as sunlight, may increase the risk of developing cancer, as well. While intrinsic factors are inherent to the patient and cannot be modified, it is important to identify the at-risk population of patients; conversely, extrinsic factors, from environment to behaviours, should be examined and minimized as possible, especially for the population with intrinsic increased melanoma risk. Major risk factors for melanoma include ultraviolet radiation exposure, naevi (dysplastic, large number, or giant congenital nevi), increased age, family history, skin pigmentation, immuno-suppression, hormonal factors.

In 1992, the International Agency for Research on Cancer (IARC), identified solar and ultraviolet radiation as a significant environmental risk factor for cutaneous malignant melanoma; subsequently in 2009, an IARC working group classified UV-emitting devices as group 1 carcinogens [20]. Chronic and repeated exposures to UV radiation are the major cause of malignant skin tumors, partially including malignant melanoma (not only after cumulative exposure), via gene mutations and immuno-suppression. Ultraviolet light is divided into UVB (290-320 nm) and UVA (320-400nm). UVC (< 290 nm) is completely absorbed by the atmosphere and is non-relevant for UV induced skin carcinogenesis; UVB is considered the most carcinogenic waveband, inducing erythema, or sunburn, historically associated with skin cancer risk, these radiation, especially, has been reported to be much more mutagenic and carcinogenic in animal experiments than UVA radiation. Most of UVB light is also absorbed by ozone, but 5-10% of it reaches the earth surface, notably, the exposure to the high penetrating UVB radiation leads to DNA lesions, such as the for mation of cyclobutane pyrimidine dimer and pyrimidine pyridine photoproducts. Ultraviolet A light genotoxicity seems to be

induced by indirect mechanisms mediated by reactive oxygen radical, generally causes guanosine to thymine transversions, possibly due to oxidation of DNA bases [21].

Although UVA is the predominant component of sunlight to which humans are exposed, its role in skin cancer, including melanoma, is not nearly as documented as UVB and is currently controversial. UVA exposure can also come from other sources such as sunlamps commonly used in tanning salons. Since UVA has longer wavelengths than UVB, it can penetrate deeper into the skin and, unlike UVB, pass through glass windows. Intermittent sun exposure is also associated with increased numbers of nevi (a potent melanoma risk factor) and nevi located at intermittently exposed body sites [22]. The results of scientific literature have suggested that, for Caucasian population, the number of melanocytic nevi is a good indicator of risk for melanoma and atypical nevi may have an independent role, so the risk of melanoma is low in individuals with few common nevi, while it is higher in individuals with multiple nevi and atypical nevi [23][24]. Nevi are benign clonal proliferations of cells expressing the melanocytic phenotype. Nevi can be either congenital or acquired (common nevi); in congenital nevi, it is presumed that a precursor cell is mutated, possibly by the activation of N-Ras, and an excessive number and daughter cells result. The migrating cells populate subcutaneous, dermal, and epidermal structures, as they attempt to complete normal migration to the skin surface; some of the excess melanocytes completing migration may be discharged through the epidermis as “pagetoid” cells in early congenital nevi. Human skin colours/photo types are mainly dependent on content and type of melanin distributed to the KCs, and factors produced by KCs to regulate pigmentation. An increased risk of melanoma has been associated for long time with characteristics of low Fitzpatrick skin phototype, such as pale skin, blond or red hair, freckles, and tendency to burn and tan poorly. The greatest risk is among patients with red hair or fair complexions, followed by ones who burn easily, tan poorly, and freckle [25].

Dark skin contains the same number and density of melanocytes as fair skin, and the basal pigmentation (constitutive pigmentation) thus depends on the level of the melanogenic activity of the MCs and on the transfer of melanosomes to the surrounding KCs. The size of the melanosomes (smaller in fair skin), the type of

melanin produced (eumelanin/pheomelanin ratio) and the rate of melanosome-degradation of the KCs (smaller degrades faster) are also important elements in the basal pigmentation, in fact eumelanin is present in large amounts in individuals with dark skin and hair whereas pheomelanin predominates in individuals with lower skin types (I-II) and red hair [26]. The genetics of human pigmentation is not wholly understood, but many genes, and polymorphisms within these gene loci, are involved in different ways such as TYR, OCA2, TYRP1, POMC, ASIP, MC1R and SLC45A2 (see Abbreviations), where melanocortin 1 receptor (MC1R) is one of the best studied, the polymorphisms in the this gene have been linked to fair skin and red hair and have been associated with an increased risk of melanoma [27].

Skin type	**Skin Color**	**Characteristics**
I	White; very fair; red or blond hair; blue eyes; freckles	Always burns, never tans
II	White; fair; red or blond hair; blue, hazel or green eyes	Usually burns, tans with difficulty
III	Cream white, olive or fair with any eye or hair color; very common	Sometimes mild burn, gradually tans
IV	Brown; typical Mediterranean Caucasian skin	Rarely burns, tans with ease
V	Dark brown	Very rarely burns, tans very easily
VI	Black	Never burns, tans very easily

Table 2. Fitzpatrick Skin Type Classification Scale.

The Fitzpatrick Skin Type Classification system was developed in 1975 by Harvard Medical School dermatologist Thomas Fitzpatrick, MD, PhD. This system classifies complexions and their tolerance of sunlight. It is used by many skin care professionals in order to determine how someone will respond or react to facial treatments.

About 90% of all melanomas are sporadic and only 10% of all cases of melanoma occur in patients who have a hereditary predisposition for melanoma [28].

However, the clustering of melanoma within families has been thoroughly documented in numerous epidemiological studies. Melanoma appears to be twice as common in persons with an affected parent, three times as common if a sibling is affected, and nine times as common if both a parent and a sibling are affected. Familial melanomas account for ~10% of malignant melanomas and about half of these are associated with known genetic lesions. The hereditary melanoma syndrome may involve mutation in the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene locus, imparting a loss or inactivation of p16 or its alternative reading frame gene (ARF). Cell cycle regulator p16 protein mutations are found in 41% of familial cutaneous melanoma cases [29]. The cyclin dependent kinase 4 (CDK4) enzyme, which is inhibited by p16 binding, is also found to be mutated in small sets of cutaneous melanoma families [30]. Another significant gene, BRAF (V600E), is thought to be crucial to the pathogenesis of melanoma. Several studies implicate that a single mutation in this gene is observed in 50% to 60% of cutaneous melanomas [31]. These mutations are often associated to younger patients (86% between 20 and 30 years old); a predilection for the trunk and extremities; high nevus counts; few freckles; individuals that tan easily; and the superficial spreading subtype [32]. In the high-risk melanoma-prone families several cases of melanoma are present in multiple generations. The affected family members often develop multiple primary melanomas and are diagnosed at a younger age than non-familial sporadic cases, the observed increased melanoma risk in relatives of melanoma cases is probably caused by both genetic factors and shared environmental exposure. However, the majority of familial aggregations show a complex pattern of inheritance that can not be explained solely by the transmission of a single gene. In most cases, therefore, melanoma is a complex trait influenced by genetic and environmental factors and their interactions. Hormonal component is another important factor in melanoma. In fact studies have shown that women have thinner tumors and survive melanoma better than men even after adjustment for Breslow thickness (see alone), ulceration, and body site, which suggests that there may be X linked variable gene expression or hormonal factors affecting melanoma survival [33]. An association between the development of melanoma and

age at menarche, menopausal status, has been observed. Also some studies showed that melanoma is more aggressively during pregnancy; in fact it was considered for many years to have an adverse effect on the course of malignant melanoma; several reports in the 1950s suggested that pregnancy increased the risk of developing melanoma and caused the disease to advance more rapidly and metastasis earlier, especially in patients with a history of melanoma. However, a meta-analysis study that examined data on over 5000 female participants showed that risk of melanoma was not affected by pregnancy, oral contraception, or hormone replacement [34]

The immunosuppression is another important factor in the development of melanoma. Individuals with an immune deficiency (e.g. organ transplant recipients with immunosuppressive therapy and individuals with HIV/AIDS) have an increased risk to develop cancer, and the risk of melanoma has been shown to be 1.24 in HIV/AIDS patients and 2.34 in transplant patients. The comparable risk of nonmelanoma skin cancer (NMSC) was 4.11 for HIV/AIDS patients and 28.62 for transplant patients [35]. Melanoma is a major public health problem in terms of morbidity and mortality. Being many risk factors for melanoma manageable, prevention and education play an important role. A social commitment aimed to both primary prevention (risk reduction) and secondary prevention (early detection, which is critical to improve the clinical outcome of this disease) should be undertaken. Early detection remains one of the most important features of disease burden reduction, for this reason it is necessary to inform people regarding the warning signs of melanoma and strongly encourage them to reduce exposure (intermittent) UVB radiation, such as sunlamps and sunbeds. An effective self-examination for the detection of MM is a prerequisite for a successful secondary prevention. Aiming at a better public education, in the sixties, emerged the ABCDE concept of early recognition as crucial for the detection of malignant melanoma This concept of early recognition is widely disseminated and is recommended. The ABCD criteria are evidence based guidelines established to remind physicians that Asymmetry, Border irregularity, Color variegation, and Diameter larger than 6 mm are features characteristic of melanoma.

3.2. Stadiation

Clinical and histological studies have resulted in defining relatively distinct steps of melanoma development and progression. As in any neoplastic system, melanomas can skip steps in their development, appearing without identifiable intermediate lesion. The progression from each stage to the next is strongly associated to specific biological changes, and this staging is based on experimental models and clinical and histopathological observations. The transition from mature melanocytes to the formation of a nevus can be characterized by an interruption of the cross-talk between melanocytes and keratinocytes that leads to loss of control of the keratinocytes on the melanocytes. Cells then presenting cytological atypia may separate from the basal membrane without undergoing apoptosis, generating architectural disorders in the lesion and hence transformation of normal melanocytes or common acquired nevi into dysplastic nevi or melanoma with radial growth phase (Figure 4). Melanoma does not have the ability to form a tumor mass or to proliferate in dermis, in this phase; it is *in situ*, when it remains confined to the epidermis, or microinvasive, when it shows an initial infiltration of the papillary dermis.

These melanoma cells in radial growth phase have biological properties that are sort of "hybrid" between benign and malignant. The possibility of metastasis is very rare in melanoma with radial growth, however there can be a low mortality rate even in these tumors [6]. In the tumorigenic phase of melanoma progression, the lesional cells have acquired capacity for proliferation in the dermis which occurs "*vertically*", or perpendicular to the direction of the epidermis, thus it has therefore been named the "vertical growth phase" (VPG)[6][16]. In this phase, melanoma cells can form aggregates or nodules that extend to the reticular dermis and subcutaneous tissue (Figure 4). A high level of genetic instability and phenotypic plasticity are the major characteristics of metastatic cells. They are highly motile, independent of growth factors, and capable of invasion of other tissues or organs.

The distinct steps of melanoma development and progression:

<u>Step 0: normal melanocytes.</u>

<u>Step 1: common acquired and congenital nevi with structurally normal melanocytes.</u>

Step 2: dysplastic nevi with structural and architectural atypia.

Step 3: melanoma *in situ* and radial growth phase.

Step 4: vertical growth phase, tumorigenic primary melanomas with competence for metastasis.

Step 5: metastatic melanoma.

The prognostic models are a valid tools to assign patients to different risk categories according to the expectations of survival, this division plays a particular important rule in the clinical of cancer. Microscopically determined attributes that have been associated to metastasis, in addition to the newer concepts of tumorigenicity and mitogenicity, include Clark's level of invasion [36], Breslow's thickness measurement [37], ulceration [38], mitotic rate [39], tumor-infiltrating lymphocytes or TILs [40], angiolymphatic invasion [41], and others.

The level of invasion as defined by Dr. Wallace Clark (called the Clark level) is one of the staging system used for microscopic staging of primary melanoma. The Clark level of a melanoma uses a scale of I to V to describe the thickness of a melanoma in relation to its penetration into the skin (Table 3). It is important not to confuse Clark levels with the TNM (Tumour, Node, and Metastases) stage or number stage (described). The Clark levels only look at the depth of melanoma cells in the skin. Primary tumour thickness was introduced as a prognostic factor by Alexander Breslow in 1970 and has subsequently been validated in multiple studies. For the Breslow scale, pathologists measure the thickness of the melanoma with a small ruler, called a micrometer.

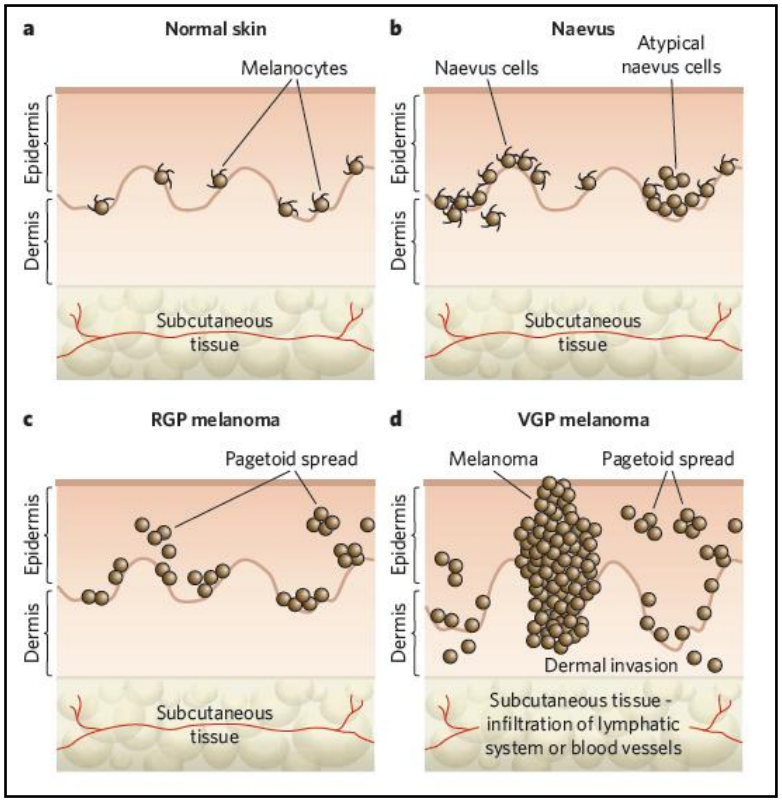

Figure 4. Biologic events and molecular changes in the progression of melanoma [16].

a) Normal skin. This shows an even distribution of dendritic melanocytes within the basal layer of the epidermis. b) Neavus. In the early stages, benign melanocytic naevi occur with increased numbers of dendritic melanocytes. According to their localization, naevi are termed either junctional, dermal or compound. Some naevi are dysplastic, with morphologically atypical melanocytes. c) RGP melanoma. It indicates that the melanoma is growing horizontally or radially, within a single plane of skin layer.

d) VGP melanoma. This is the first stage that is considered to have malignant potential and leads directly to metastatic malignant melanoma, the most deadly stage. Pagetoid spread describes the upward migration or vertical stacking of melanocytes that is a histological characteristic of melanoma.

Doctors use a scale called the primary tumour thickness scale, or the Breslow thickness. It measures in millimetres (mm) how far the melanoma cells have reached down through the skin from the surface. The Breslow thickness is used in the TNM staging system for melanoma; in fact this staging system describes the size of a

primary tumour (T), whether any lymph nodes contain cancer cells (N) and whether the cancer has spread to another part of the body (M). The T part of the TNM describes the thickness of the melanoma (primary tumour) according to the Breslow scale. The TNM Staging System is another way of staging cancer in general, and counts for one of the most (if not the most) commonly used staging systems, it is also used for melanoma. Each cancer type has its own classification system, letters and numbers not always meaning the same thing for every kind of cancer (Figure 5). Once the T, N, and M are determined, they are combined, and an overall "Stage" of I, II, III, IV is assigned. Sometimes these stages are subdivided as well, using letters such as IIIA and IIIB6.

Breslow thickness, ulceration, and Clark s level IV invasion are the attributes in use in the current the American Joint Committee on Cancer (AJCC) staging system [42]. New staging criteria by AJCC published in 2009 allow increased accuracy and precision for clinicians managing malignant melanoma compared with the 2002 guidelines [43](Figure 6). The AJCC 2009 guidelines reiterated the importance of several prognostic factors including tumour thickness and mitotic rate (histologically defined as the number of mitoses/$mm^{2)}$, and significantly the mitotic rate now replaces previous Clark's level of invasion for criteria in defining stage T1b melanomas.

On the basis of a multivariate analysis of patients with distant metastases, the two dominant components in defining the M category keep being the site of distant metastases (nonvisceral v lung v all other visceral metastatic sites) and an elevated serum lactate dehydrogenase level.

T classification	Thickness	Ulceration Status/Mitoses
Tis	N/A	N/A
T1	≤ 1.0 mm	a: w/o ulceration and mitosis <1/mm²
		b: with ulceration or mitoses ≥ 1/mm²
T2	1.01 - 2.0 mm	a: w/o ulceration
		b: with ulceration
T3	2.01 - 4.0 mm	a: w/o ulceration
		b: with ulceration
T4	> 4.0 mm	a: w/o ulceration
		b: with ulceration
N classification	**# of Metastatic Nodes**	**Nodal Metastatic Mass**
N0	0 nodes	N/A
N1	1 node	a: micrometastasis*
		b: macrometastasis**
N2	2-3 nodes	a: micrometastasis*
		b: macrometastasis**
		c: in-transit met(s)/satellite(s) without metastatic nodes
N3	4 or more metastatic nodes, or matted nodes, or in-transit met(s)/satellite(s) with metastatic node(s)	
M classification	**Site**	**Serum LDH**
M0	0 sites	N/A
M1a	Distant skin, subcutaneous, or nodal mets	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

Figure 5. TNM Criteria for Cutaneous Melanoma [43].

*Micrometastases are diagnosed after sentinel lymph node biopsy and completion lymphadenectomy.

**Macrometastases are defined as clinically detectable nodal metastases confirmed by therapeutic lynphadenectomy or when nodal metastasis exhibits gross extracapsular extension.

CLARK LEVEL	INVASION LEVEL
I	Intraepidermal growth/in situ
II	Cells reaching the papillary dermis
III	Cell occupation and expansion of the papillary dermis
IV	Cells invading the reticular dermis
V	Cells invading the subcutaneous fat

Table 3. Clark levels.

Factor	6th Edition Criteria	Recommended 7th Edition Criteria	Comments
Thickness	Primary determinant of T staging	Same	Thresholds of 1.0, 2.0, and 4.0 mm
Level of invasion	Used only for defining T1 melanomas	Same	Used as a default criterion only if mitotic rate cannot be determined
Ulceration	Included as a secondary determinant of T and N staging	Same	Signifies a locally advanced lesion; dominant prognostic factor for grouping stages I, II, and III
Mitotic rate per mm^2	Not used	Used for categorizing T1 melanoma	Mitosis $\geq 1/mm^2$ used as a primary criterion for defining T1b melanoma
Satellite metastases	In N category	Same	Merged with in transit lesions
Immunochemical detection of nodal metastases	Not included	Included	Must include at least one melanoma-associated marker (eg, HMB-45, Melan-A, MART-1) unless diagnostic cellular morphology is present
0.2 mm threshold of defined N+	Implied	No lower threshold of staging N+ disease	Isolated tumor cells or tumor deposits < 0.1 mm meeting the criteria for histologic or immunohistochemical detection of melanoma should be scored as N+
Number of nodal metastases	Primary determinant of N staging	Same	Thresholds of 1 *v* 2-3 *v* 4+ nodes
Metastatic volume	Included as a second determinant of N staging	Same	Clinically occult (microscopic) nodes are diagnosed at sentinel node biopsy *v* clinically apparent (macroscopic) nodes diagnosed by palpation or imaging studies, or by the finding of gross (not microscopic) extracapsular extension in a clinically occult node
Lung metastases	Separate category as M1b	Same	Has a somewhat better prognosis than other visceral metastases
Elevated serum LDH	Included as a second determinant of M staging	Same	Recommend a second confirmatory LDH level if elevated
Clinical *v* pathologic staging	Sentinel node results incorporated into definition of pathologic staging		Large variability in outcome between clinical and pathologic staging; sentinel node staging encouraged for standard patient care, should be required prior to entry into clinical trials

Abbreviation: LDH, lactate dehydrogenase.

Figure 6. Differences Between the 6th Edition (2002) and the Recommended 7th Edition (2009) of the Melanoma Staging System [43].

3.3. Diagnosis

The diagnosis of malignant melanoma depends on the detection of a concerning lesion and pathologic confirmation through skin biopsy. The principal sign of a skin lesion proven to be a melanoma is some change over a period of months. The main initially observed changes of an increase in size and colour changes occur in about 70% of patients. Increase in height, itching and ulceration or bleeding usually occur in more advanced lesion [44]. When melanomas grow thicker, can occur bleeding and ulceration, besides around the lesion a reddish discoloration may be seen. In general, the ability to detect concerning lesions by patients (and their family members) or primary care providers relies on the ABCDE criteria of melanoma detection.

The ABCDE concept of early recognition, introduced in the sixties, is widely disseminated and is recommended:

A = Asymmetry

B = Border irregularity

C = Colour variegation, two or more colours exist within the tumour border

D = Diameter generally greater than 6 mm

E = Evolving

The ABCDE mnemonic device was designed to enhance detection of cutaneous melanoma. Although very useful for patients and certain physician groups, the sensitivity of the ABCDE criteria in detecting cutaneous melanoma is limited. A thorough physical examination must be performed when a patient presents with a lesion arousing suspicions of melanoma. Skin and subcutaneous tissue around the primary lesion and between it and the regional nodal basin have to be examined for satellite and in-transit metastases. The skin of the entire body must be examined for concurrent primary melanomas as they occur in 1% of cases. Dermoscopy (epiluminescence microscopy) is a diagnostic technique that can be used to examine *in vivo* skin lesions with 10- to 20-fold enlargement. This type of instrument employs oil applied to the surface of the lesion (making the dermis more transparent), a glass plate

pressed against the oil (to enhance the *in vivo* evaluation of structures at the dermo-epidermal junction), a light source and magnification. It has been estimated that epiluminescence increases diagnostic accuracy for smaller, clinically borderline lesions by about 20% [45].

Furthermore, digital imaging systems with and without epiluminescence have been studied to determine whether, and to what extent, these devices may augment and/or automate diagnosis. Nevertheless, dermoscopy is recommended to be utilised only by experienced physicians. Moreover, an excisional biopsy is the standard option on a type C basis (the letter c means that there are melanoma cells in small areas of skin very close to the primary melanoma or in the skin lymph channels) as the appropriate diagnostic procedure for a skin lesion suspected to be a melanoma, provided it is anatomically, functionally, and cosmetically feasible. Reliable histology requires examination of the whole lesion, as a resection margin for the diagnostic excision, a distance of 2 mm from the border of the lesion and into the subcutaneous tissue is recommended [46]. When an excisional biopsy is not feasible, for example when the lesion is very large and/or is so anatomically situated that total excision would be mutilating or disfiguring, incisional diagnostic biopsy is unavoidable.

3.4. Types of melanoma

Based upon clinical and pathological characteristics, five major subtypes of primary cutaneous melanoma have been historically distinguished. These include lentigo maligna melanoma (LMM), superficial spreading melanoma (SSM), nodular melanoma (NM), acral lentiginous melanoma (ALM) and desmoplastic melanoma [47].

Tumor growth of melanoma can be biphasic or monophasic, as already said; the biphasic pattern consists of a horizontal or radial initial growth phase (intra-epidermal) followed by a subsequent vertical growth phase corresponding to the infiltration of the dermis and hypodermis. Those melanomas having such biphasic growth pattern are the so-called superficial spreading melanoma and the lentigo maligna melanoma, very often, acral lentiginous melanoma also has such a biphasic growth pattern. The monophasic growth pattern of melanoma consists of tumors having a pure vertical growth which includes mainly the so-called nodular melanoma, this growth phase can include the desmoplastic variant.

Superficial spreading melanoma (SSM): this type of melanoma may develop on any part of the body, and at any age. SSM is the most common form of melanoma, accounting for approximately 70% of all melanomas [47], and it is particularly common on the trunk in males and the lower extremities in females. It begins a small pigmented macule that is asymmetric, has irregular borders, and has color variations (Figure 7).

SSM remains in the flat phase for a shorter period of time than the lentigo maligna type before it penetrates into the deeper levels of the skin. An amelanotic variant has also been reported; rarely, it may clinically simulate a patch of vitiligo.

Lentigo maligna melanoma (LMM): it represents about 10% of all melanomas. LMM arises from lentigo maligna[1] and it is most commonly found on sun-exposed skin in elderly patients (median age is 70 years).

Nodular malignant melanoma (NM): this subtype can present on any location as a nodule with sharply demarcated borders on the skin, often shiny with a slightly

[1] *John Hutchinson first described lentigo maligna in 1890; the disease continues to be called Hutchinson melanotic freckle on occasion. The Hutchinson melanotic freckle was originally thought to be infectious because of its slow yet progressive growth. The lesion has subsequently been characterized as malignant lentigo of elderly people, junctional nevus, and melanoma in situ. Most authors currently refer to it as lentigo maligna when it is confined to the epidermis and lentigo maligna melanoma when it violates the dermis.*

infiltrated base. Epidemiologically, NM is often seen in older patients near the seventh decade of life. The colour, generally darker and more uniform than that of SSM, may vary from black to unpigmented; besides the lesions are characterized by a relatively rapid vertical growth phase (Figure 7).

Acral-lentiginous melanoma (ALM): it can be found on the palms, soles and subungual regions, representing a higher proportion of all melanomas in dark-skinned individuals such as African Americans, Asians and Hispanics. Only 3-5% of melanomas are from the acral lentiginous melanoma type. Lesions are large (3 cm in diameter) with irregular borders, and generally occurs in older individuals (median age is 59 years). ALM often appears as a tan to dark brown macules with an irregular border, but may be ulcerating in more advanced lesions; it is clinical pattern being quite similar to LMM, ALM is though a biologically much more aggressive lesion, with a relatively short evolution to the vertical growth phase.

Desmoplastic melanoma: this is commonly misdiagnosed due to its subtle presentation. It commonly appears as an indurated skin-colored papule, plaque or nodule, and it is demonstrates more sarcoma-like tendencies with increased hematogenous spread when compared with other subtypes. The pathological presentation of desmoplastic melanoma is characterized by a proliferation of spindle cells, which are non-pigmented spindle-shaped melanocytes. The nuclei of these spindle cells tend to hyperchromatic and may be elongated. The cytoplasm is usually scant an increased nucleus to cytoplasm ratio commonly seen in malignant cells [48].

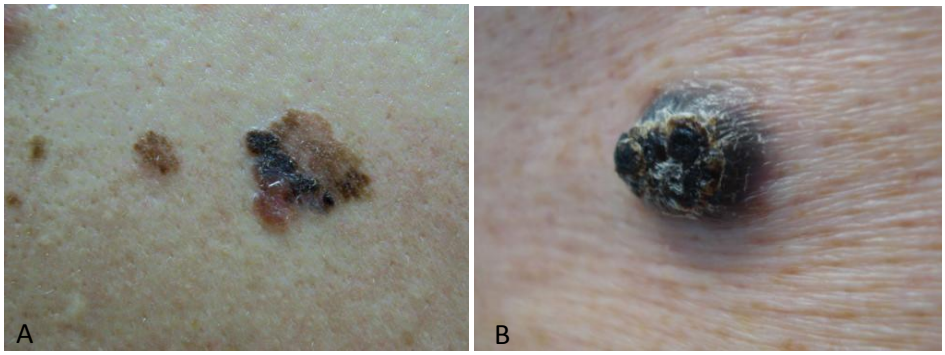

Figure 7. Types of melanoma

A) Superficial spreading melanoma with a well demarcated edge and irregular pigmentation;

B) Nodular malignant melanoma, the lesion presents as a nodule (lump) that has been rapidly enlarging over the previous weeks to months.

3.5. Genetics of melanoma

Oncogenesis is a multistep, complicated process due to multiple acquired or inherited genetic alterations that result in aberrant regulation of the cell cycle. These genetic alterations can affect regulation of the cell cycle, cell proliferation, cell death, and cell differentiation. Broadly speaking, genes responsible for the development of oncogenesis can be grouped into two main categories: oncogenes (ie, the growth promoting variant of a protooncogene) and tumor suppressor genes. Protooncogenes encode to necessary proteins for regulating cell division; when non-mutated and functioning properly, they are regulated very closely to allow for sufficient cellular division while avoiding overproduction of the cells. An activating mutation in only one copy of the proto-oncogene usually is sufficient for oncogenesis to occur—the so-called dominant effect. Tumor suppressor genes function to inhibit cellular proliferation or promote cell death, this class of genes act by inhibiting proteins that control cell cycle progression. Alterations in tumor suppressor genes can lead to cancer through a loss-of-function mutation, it is therefore necessary for both copies of the alleles to be mutated for sufficient inactivation of the tumor suppressor gene to occur, which ultimately leads to oncogenesis. Tumor suppressor genes are therefore retained to act in a recessive manner [49]. In tumours, most mutations are "passengers", and this means that they do not contribute to oncogenesis. Moreover, they provide for information about the various steps leading to the oncogenic transformation of cells, as occurs, for UV-exposure in skin cancer. This kinds of mutation are very useful to study the pathogenesis of cancer. Only a small subset of somatic mutations is made of "driver mutations", which confer the oncogenic footprint to cancer cells, being thus eligible for diagnosis and molecular therapy. To date, more than 300 "driver" genes have been identified across all human cancers; not surprisingly, they all converge over the few key cellular pathways which regulate cell life and differentiation. The development and progression of melanoma is characterized by the acquisition of chromosomal deletions, amplification and gene mutations [50]. Approximately 10% of all melanomas occur in a familial setting. In addition to recognized heritable traits,

such as skin, hair, and eye color, and the presence of large numbers of benign nevi, atypical nevi, or giant congenital nevi, a family history of melanoma is a major risk factor for development of melanoma. The many gene expression profile studies performed up to now have evidenced that melanomas show significant genetic heterogeneity. There is a melanoma genetics consortium, GenoMEL (Genetic and environmental determinants of melanoma: translation into behavioural change), which is an organization with melanoma research groups from 14 countries around the world pooling data, in order to elucidate the genetic field of melanoma and with a special interest for familiar melanoma (www.genomel.org). Important molecules, in this context, are RAS and BRAF, these are molecules that mediate the response of cells to extracellular mitogenic stimuli and play a central role in regulating cell growth, survival and cell proliferation.

The products of the family of genes RAS, small proteins related to the cytoplasmic membrane, are constituted by three tissue specific isoforms: HRAS, KRAS and NRAS. For this last molecule, NRAS, the mutations are the most detected in MM (15 -30% of cases) [51][52]. The stimulation of oncogenic NRAS activates specific cytoplasmic protein kinase function with the downstream RAF and Phosphatidylinositol 3-Kinase (PI3K) [51]. A major achievement in melanoma research was the demonstration of mutations also in *BRAF* in a large proportion of them [53]. BRAF is one of the three human RAF genes (ARAF, BRAF e CRAF), it encodes a kinase that acts in the mitogen-activated protein kinase (MAPK) signal transduction pathway and thus is important for regulation of a variety of cellular processes (growth, survival and migration) [16] (Figure 8). This gene is mutated in 50% to 70% of melanomas, and the most common mutation is a glutamic acid for valine substitution at position 600 (V600E); ^{V600E}BRAF stimulates constitutive ERK signalling, stimulating proliferation and survival and providing essential tumour growth and maintenance functions. Moreover it contributes to neoangiogenesis [53] by stimulating autocrine vascular endothelial growth factor (VEGF) secretion and recent studies have identified several genes in melanoma that function downstream of ^{V600E}BRAF.

To make complex this whole scenery is the observation that, in presence of mutations in the gene NRAS, which have been demonstrated to be exclusive with mutations in the BRAF gene, the signal transduction in the MAPK pathway is diverted to CRAF, which therefore acquires a key role in maintaining the stimulation of cell proliferation in this subgroup of melanomas [54].

Actually, an increased level of expression of CRAF is responsible for the acquisition of resistance to therapy with BRAF inhibitors. These data therefore have implications in the development of new therapeutic strategies based on the combination of treatments simultaneously inhibiting BRAF and CRAF or BRAF and PI3K, other pathways activated by NRAS mutated [55].

As already mentioned, another signalling pathway that is emerging as important in melanoma is the phosphoinositide-3-OH kinase (PI(3)K) pathway. Phosphoinositides are membrane lipids that are converted to second messengers through hyper-phosphorylation by one of a family of PI(3)Ks. PI3K phosphorylates phosphatidylinositols of the cell membrane to produce phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 then recruits the serine/threonine kinase AKT, also known as protein kinase B, to the cell membrane (Figure 8).

The lipid second messengers activate numerous downstream effector pathways, and signalling is terminated by the lipid phosphatase PTEN (phosphate and tensin homologue). PI(3)K signalling regulates cell survival, proliferation, growth (increase in cell mass) and motility, and it is hyperactivated in a high proportion of melanomas. This is partially explained in part by the findings that PI(3)K mutations occur in 3% of metastatic melanomas, PTEN function is lost in between 5% and 20% of late-stage melanomas [56] and the PI(3)K effector protein kinase B (PKB, also known as Akt) is overexpressed in up to 60% of melanomas; notably, BRAF and PTEN mutations are coincident in about 20% of cases [57]. The relationship of these genes has not been shown genetically, but these mutation patterns conform neatly to our current understanding of the pathway.

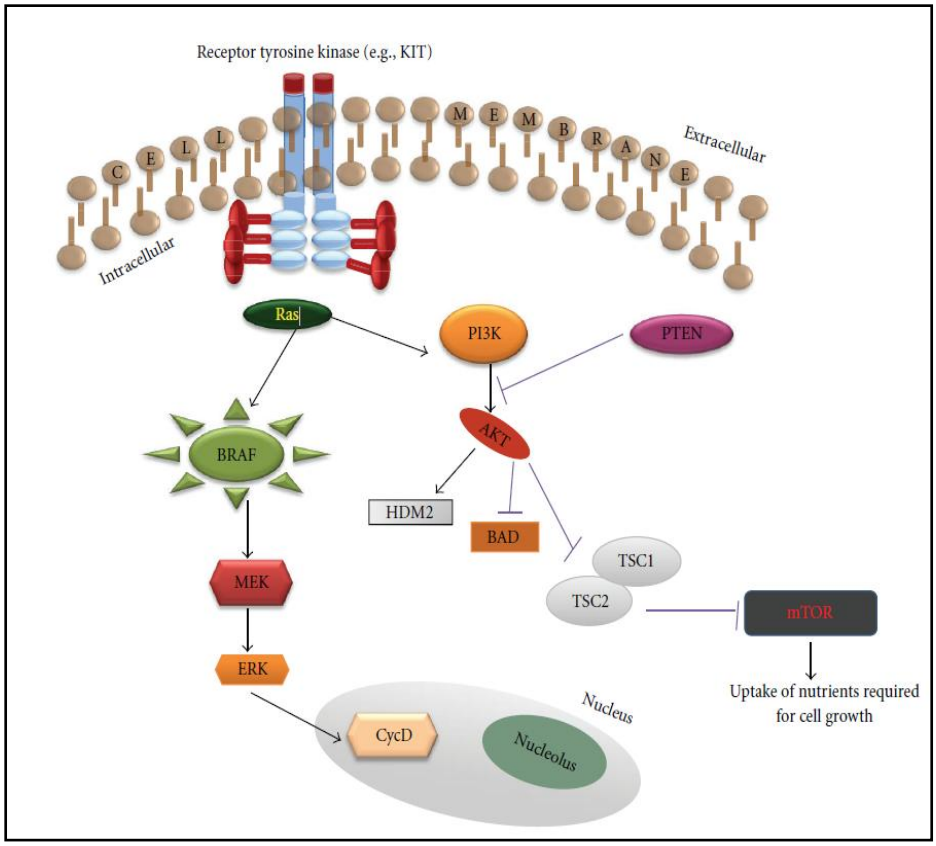

Figure 8. The Mitogen-Activated Protein (MAP) Kinase and Phosphatidylinositol 3' Kinase (PI3K) Pathways [58].

Signals from receptor tyrosine kinases can promote proliferation through the MAP kinase pathway and survival through the PI3 kinase pathway.

The signaling cascade is initiated by growth factor stimulation of membrane-bound receptor tyrosine kinases. Receptor stimulation then activates RAS, a small G protein. On activation of RAS, a complex forms between RAS and one of the RAF serine/threonine protein kinase isoforms. Formation of the complex between RAS and RAF leads to activation of RAF. Once RAF is activated, the signal is transduced through phosphorylation of MEK (MAPK/ERK kinase, also known as MAP2K), which is serine/ threonine kinase present in 2 isoforms (MEK1 and MEK2). Phosphorylation activates the kinase activity of MEK, which phosphorylates the MAPK (also known as ERK) isoforms, MAPK3 (also known as ERK1) and MAPK1 (also known as ERK2). Activated RAS also triggers the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which conducts antiapoptotic signals that complement the proliferative effects of the MAPK pathway.

BRAF and PI(3)K are activated downstream of Ras, so in the presence of oncogenic NRAS additional mutations in BRAF and PTEN are unnecessary, because these components are already activated. Indeed, their mutation in this context might even antagonize tumour progression, because they could stimulate pathway superactivation. On the other hand, since PTEN and BRAF are not mutually activating and these pathways cooperate to stimulate proliferation, separate mutations are required to activate both pathways. Another gene that has an important role in melanoma is CDKN2A gene. Several studies have shown that CDKN2A gene is mutated in 20-40% of melanoma-prone families (with ≥3 members affected by melanoma), as compared to a mutation frequency of about 1-2% in population-based melanoma patients [58]. CDKN2A is the major high-risk melanoma susceptibility gene identified to date. The association was first delineated in the 1990s; since that time, it has been determined that, in aggregate, 25% to 50% of familial melanoma kind reds are affected by a CDKN2A mutation. In smaller studies, up to 10% of patients with multiple primary melanomas (MPMs) have been identified to have a CDKN2A mutation. In a large population-based study, however, reported CDKN2A mutation rates are about 1% for the unselected melanoma patient and about 3% for individuals with MPMs [46]. CDKN2A encodes two distinct proteins translated, in alternate reading frames (ARFs), from alternatively spliced transcripts[2]. The alpha transcript, encodes a low molecular weight protein, p16Ink4a that contains predominantly four ankyrin (Ank) repeats. The p16Ink4a protein inhibits the activity of the cyclin D1-cyclin-dependent kinase 4 (CDK4) or 6 (CDK6) complex [59]. These complexes phosphorylate the retinoblastoma protein, allowing the cell to progress through the G1 cell cycle checkpoint. Thus, p16 acts as a tumor suppressor and negatively regulates cell growth by arresting cells at G_1 [59]. The smaller beta transcript, specifies the alternative product p14ARF, it binds to and prevents human double minute-2 (HDM2) from accelerating the degradation of p53, thus possessing tumour-suppressive effects. Normally, p53 senses genetic damage and allows pause for DNA repair or activates apoptosis if there is too much DNA damage.

[2] *The CDKN2A locus on chromosome 9p21 is composed of 4 exons (E) – 1α, 1β, 2, and 3 – and encodes 2 tumor suppressors, p16INK4a and p14ARF (termed p19ARF in the mouse), via alternative reading frames. p16INK4a is translated from the splice product of E1α, E2, and E3, while p14ARF is translated from the splice product of E1β, E2, and E3.*

Decreased p53 leads to genetic instability when mutations and other genetic damage are left (Figure 9). A mutated p14ARF does not inhibit HMD2, which in turn accelerates the destruction of p53 and thus enhances growth and survival of altered/damaged cells instead of cell cycle arrest and apoptosis [60].

Cyclin-dependent kinase 4 (Cdk4), which physically and genetically interacts with the p16Ink4a, is another important high-risk locus that that been implicated in melanoma. In cell lines and in cases of familiar melanoma respectively, both somatic and germline mutations of CDK4 have been identified.

This is a rare high-penetrance melanoma predisposition gene, indeed, only three melanoma families worldwide are carriers of mutations in CDK4 (Arg24Cys and Arg24His). It is located at 12q13.6, and which encodes a protein interacting with the p16Ink4a protein. Since the two proteins directly interact, the consequences of p16Ink4a loss and Cdk4 activation are similar; thus, carriers of CDKN2A and CDK4 mutations also share a similar phenotype [61]. The Rb protein, which is downstream of p16Ink4a and Cdk4, is an essential gatekeeper for hereditary retinoblastoma. Hence, mutations in the entire CDKN2A/CDK4/Rb pathway appear to play a significant role in melanoma pathogenesis [62].

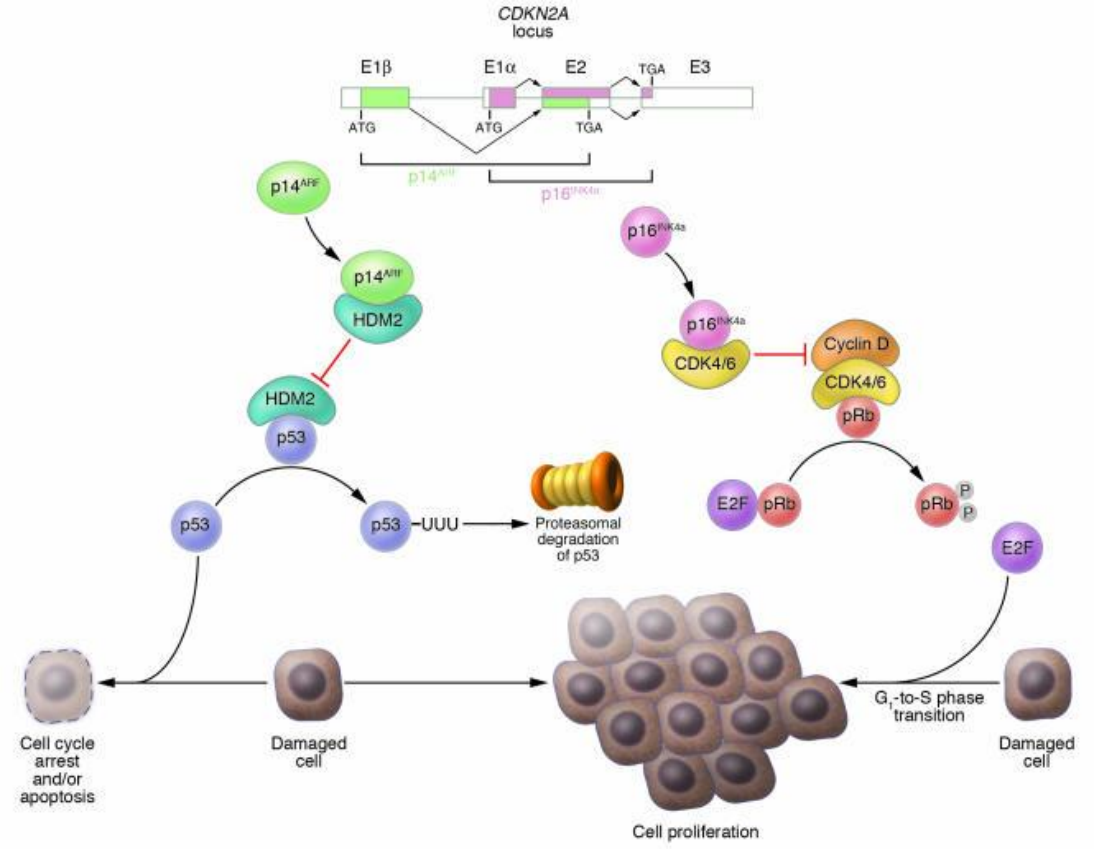

Figure 9. Structure and function of the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene [63].

Although structurally very different, both protein products act as negative regulators of cell cycle progression.

The p16INK4A protein inhibits the activation of CDK4 and CDK6 by cyclin D1 (CCND1), thereby preventing the subsequent phosphorylation of RB1. Underphosphorylated RB1 sequesters the transcription factor E2F and prevents it from inducing the progression from G_1 to S phase of the cell cycle. In contrast, p14ARF binds to human homolog of murine Mdm2 (HDM2), thereby inhibiting the function of HDM2. HDM2 functions in human cells to mark proteins for degradation through ubiquitination, a posttranslational modification of proteins whereby an ubiquitin molecule is attached to the protein. In the absence of p14ARF, HDM2 targets p53 for ubiquitination (UUU) and subsequent proteosomal degradation, and the loss of p53 impairs mechanisms that normally target genetically damaged cells for cell cycle arrest and/or apoptosis, which leads to proliferation of damaged cells.

An increased interest has been focused on the activity of the microphthalmia-associated transcriptor factor (MITF), which is considered to be the "master regulator of melanocytes" since it seems to be crucial for melanoblast survival and melanocyte lineage commitment [64]. MITF has been shown to be amplified in 10–20% of melanoma metastases and to potentially act as a dominant oncogene [65].

The mechanism of action of MITF is however rather complex: low or absent expression of this protein predisposes the cell to apoptosis; intermediate levels of expression of MITF promote the proliferation and cell survival. The differentiation functions of MITF are displayed when the expression levels of this protein are high, indeed, high MITF levels have been demonstrated to exert an anti-proliferative activity in melanoma cells [64]. In this regard, low levels of MITF protein were found in invasive melanoma cells and have been associated with poor prognosis and clinical disease progression. Studies in literature speculated that MITF might be a new prognostic marker in intermediate-thickness malignant melanoma; the retention of MITF expression in the vast majority of human primary melanomas, including non-pigmented tumors, is consistent with this hypothesis and has also led to the widespread use of MITF as a diagnostic tool in this malignancy. Melanoma arising from mucosal, acral infrequently have BRAF mutations, but commonly have amplifications or activating mutations of KIT. It is a type III transmembrane receptor tyrosine, which is recognized as a ligand for a stem cell factor (SCF). Dysregulation of c-KIT plays a role in systemic mastocytosis, acute myelogenous leukaemia, gastrointestinal stromal cell tumors (GISTs) and germ cell tumors.

A critical role for c-Kit for normal neural crest and normal melanocyte development [66], differentiation, proliferation, survival and migration [67] has been recognized, but its function in melanoma remains unclear. KIT is expressed in some melanomas, loss of expression is observed with progression of disease from superficial to invasive to metastatic stages, suggesting that KIT possesses tumor suppressive functions. Monsel et al. demonstrated that c-Kit mutated melanocytes require a specific epigenetic environment to be transformed into melanoma cells. c-Kit mutants cause in fact a strong activation of the phosphatidyl-inositol-3 kinase (PI3K) pathway, which, per se, is not sufficient to promote transformation of melanocytes [68]. However, in the chronic

hypoxic skin microenvironment, and/or when a constitutively active form of hypoxia-inducible factor 1alpha (HIF-1alpha) is coexpressed, c-Kit mutants activate also the Ras/Raf/Mek/Erk pathway, transforming the melanocytes, (Monsel et al., 2010). This scenario is extremely interesting, considering that KIT mutations are mutually exclusive with BRAF and NRAS, to identify a subset of melanomas arising from a distinct molecular mechanism of transformation, which may be specifically targeted by KIT inhibitors [68].

3.6 Treatment of melanoma

Melanoma remains unique among solid tumours in that its treatment primarily is surgical. Radiation is only of limited benefit, and chemotherapy has been disappointing in both the adjuvant and metastatic settings. Melanoma can metastasize to almost every major organ and tissue. The most common initial sites of distant metastases are the skin and soft tissue, and lymph nodes; the most common sites of visceral metastases are the lung, brain, liver. Disseminated melanoma show survival rates of less than 5% over 5 years. The primary treatment method, especially for non-metastatic melanomas, remains the surgical excision. A total excisional biopsy is preferred to incisional biopsy, when possible, to ensure minimal sample selection and proper Breslow thickness assessment. Nevertheless, incisional biopsies are still valuable in large, mucosal, or clinically atypical lesions and are shown to have no impact on prognosis. Several expert studies demonstrate that excision for invasive melanoma should include a minimum of 1 cm margins and no more than 2 cm around the primary tumor. Excision with 0.5 to 1 cm margins is appropriate for in situ tumors. Lesions less than 2 mm in thickness can be excised with 1cm margins and any tumor more than 2 mm thickness probably warrants excision with 2 cm margins [69].

Melanoma cells have low levels of spontaneous apoptosis in *vivo* compared with other tumour cell types, and are relatively resistant to drug-induced apoptosis in *vitro*. Most chemotherapeutic drugs function by inducing apoptosis in malignant cells, so resistance to apoptosis is likely to underlie drug resistance in melanoma, and this extraordinary resistance to chemotherapy, radiotherapy and immunotherapy is a major barrier to successful treatment of melanoma. Melanoma has historically been refractive to chemotherapeutic treatments. Although a number of agents have been assessed in clinical trials, dacarbazine (DTIC), until recently, has been the standard approved treatment option for patients with advanced (stage IV) melanoma [70]. Dacarbazine (also known as imidazole carboxamide) is an antineoplastic chemotherapeutic drug used in the treatment of various cancers, such as Hodgkin lymphoma or malignant melanoma. Its exact mechanism is not known, but two main hypotheses have been proposed. First, dacarbazine belongs to the family of alkylating

agents, which are drugs attaching an alkyl group to DNA. They stop tumor growth by cross-linking guanine nucleobases in DNA double-helix strands; this makes the strands unable to uncoil and separate. Secondly, dacarbazine inhibits DNA synthesis by acting as a purine analog, impairing DNA and RNA replication. Both mechanisms blocked by drug are necessary for DNA replication, and consequently the cells can no longer divide; this drug acts nonspecifically, but as cancerous cells usually proliferate more than normal cells, they are consequently more sensitive to DNA damage [70]. Furthermore, it interacts with sulfhydryl groups, inhibiting protein synthesis and is not specific phase, a small quantity exceeds (in 15% of cases) the blood-brain barrier. Studies have shown that dacarbazine (DTIC) exerts immunostimulatory effects by inducing local activation of natural killer (NK) and T cells, suggesting that upon treatment the tumor participates in the initiation of an immune response [70].

Another drug used for melanoma is temozolomide, a novel oral alkylating agent that spontaneously converts to the active metabolite of DTIC 5-(3-methyltriazen-1-yl) imidazole- 4-carboximide (MTIC), unlike DTIC which requires metabolic activation. Temozolomide has 100% bioavailability with extensive tissue distribution including penetrating the blood-brain barrier into the cerebral spinal fluid (CSF). In a phase II study, the objective response rate was 21%; in a phase III trial temozolomide demonstrated efficacy equal to that of DTIC with improved health related quality of life (QOL). Median survival was 7.7 months and 6.4 months for the temozolomide and DTIC groups, respectively [73].

Other systemic therapeutic strategies, after chemotherapy, include bio-chemotherapy, immune adjuvants, cancer-specific vaccines, monoclonal antibodies. Another chemiotherapic drugs such as carmustine (antineoplastic alkylating agent), paclitaxel (mitotic inhibitor; works by interfering with normal microtubule growth during cell division by hyperstabilizing the structures of these microtubules) and cisplatin[3] (mitotic inhibitor; works by cross-linking with DNA in different ways, thus interfering with cell

[3] *The salts of platinum, cisplatin and carboplatin, act as alkylating forming bonds intra-and cross-stranded DNA, deforming the structure and preventing cell replication. Cisplatin has a half-life plasma of 58-73 hours. Both cisplatin and carboplatin have mediocre activity as single agents: about 15% of responses with a median duration of 3 months.*

division and triggering DNA repair mechanisms, including apoptosis) have shown single-agent mediocre activity in metastatic disease.

Many classes of targeted drugs are in development, such as monoclonal antibodies, small molecule tyrosinekinase inhibitors, and antisense oligonucleotides. Theoretically, a targeted therapy or target should be more effective and less toxic because it is more selective, although unfortunately is not always true.

Because NRAS and BRAF are validated therapeutic targets in melanoma, it is not surprising that drugs that target this pathway are of considerable interest. The first drugs targeting this pathway to enter the clinic were the Ras farnesyl transferase inhibitors[4], which were designed to block an essential post-translational modification on Ras; it is thought that these agents block Ras activation through inhibition of the enzyme farnesyl transferase, resulting in cell growth arrest. The results, however, led to a number of questions; first, the promising results in preclinical models were not confirmed in the clinic, unexpectedly, tumors containing non mutated Ras were also sensitive to farnesyl transferase inhibitors. Furthermore, it is questioned whether, in dose finding studies of biochemical modulators like FTIs, the drug target or its biochemical effect would be a better pharmacodynamic end point than the classic study end point of clinical toxicity.

The first RAF inhibitor to be clinically tested in patients with melanoma was sorafenib, but this drug showed little efficacy: only 1 response of 39 patients with melanoma treated [74].

Sorafenib as a monotherapy or in combination with chemotherapy is of limited use. It is a non-selective inhibitors, precisely, it is an multi-kinase inhibitor that inhibits tumor proliferation by targeting multiple kinases including the vascular endothelial growth factor receptors (VEGFR1, VEGFR2, VEGFR3) and the platelet-derived growth factor receptor (PDGFR), and it targets tumor progression by inhibiting FLT3, C-Kit and BRAF [75]. The first real selective BRAF inhibitor to be developed in the clinical setting is vemurafenib (PLX4032). Vemurafenib is a small molecule inhibitor that binds potently

[4] *Farnesyl transferase inhibitors are a new class of biologically active anticancer drugs. The exact mechanism of action of this class of agents is, however, currently unknown. The drugs inhibit farnesylation of a wide range of target proteins, including Ras.*

to and selectively inhibits the BRAF V600 oncogenic mutation. Preclinical studies of vemurafenib in cell lines that were positive for the BRAF mutation demonstrated vemurafenib's ability to inhibit extracellular signal regulated kinase (ERK) activation, arrest the cell cycle, selectively inhibit cell growth and proliferation, and induce apoptosis leading to cell death [70][76]. Efficacy has been documented only for vemurafenib monotherapy and in patients with metastatic melanoma harboring V600 BRAF mutations. Approximately 90% of patients experience some degree of tumor regression early in the course of therapy. In a disease as aggressive as metastatic melanoma it is not surprising that vemurafenib has demonstrated a survival advantage compared to historically minimally effective single-agent chemotherapy. Compared to dacarbazine, vemurafenib reduced the risk of death by 63% ($p < 0.001$) [77].

On May 29, 2013, the Food and Drug Administration (FDA) approved dabrafenib (made by GlaxoSmithKline). Dabrafenib acts as a BRAF inhibitor and it was approved for the treatment of patients with advanced melanoma that contains the V600E mutation of BRAF. It is seen as being a next-generation product but is in the same class as the first BRAF inhibitor to reach the market, vemurafenib. Dabrafenib is highly efficacious in melanoma patients with BRAF V600E mutations, with response rates of approximately 50% and progression-free survival of 6 months. There is also early data to suggest that dabrafenib is effective in controlling metastases in the brain [78].

Dabrafenib is not indicated for the treatment of patients with wild-type BRAF melanoma because of the potential risk of tumor promotion. Concurrent with this action, FDA approved the THxID BRAF assay for detection of BRAFV600E mutations. The approval of dabrafenib was based on demonstration of improved progression-free survival (PFS) in a multi-center international open-label randomized (3:1), active-controlled trial. This trial enrolled 250 patients with previously untreated, histologically confirmed, unresectable stage III or stage IV melanoma determined to be BRAFV600E mutation-positive based on centralized testing. Patients were randomly assigned to receive either dabrafenib or dacarbazine. The assessed response rates were 52% for patients treated with dabrafenib, which included a 3% complete response rate, and 17% for patients treated with dacarbazine [79].

On the same day, trametinib has been approved (made by GlaxoSmithKline), for the treatment of patients with unresectable or metastatic melanoma with BRAF V600E or V600K mutation. Trametinib has a related but slightly different mechanism of action to dabrafenib. It acts as a mitogen-activated, extracellular signal-regulated kinase (MEK) inhibitor in the mitogen-activated protein kinase (MAPK) signalling pathway, which mediates cell growth and survival. In phase 1 and 2 trials, trametinib showed evidence of tumor regression and disease stabilization in patients who had melanoma with a V600E or V600K BRAF mutation [80]. There was no evidence of anti-tumor activity with trametinib in patients who had received prior BRAF inhibitor therapy. This was evaluated in a single-arm, multicenter, international trial enrolling 40 patients with BRAF V600E or V600K mutation-positive, unresectable or metastatic melanoma, all of whom had received prior treatment with a BRAF inhibitor. None of these 40 patients achieved a confirmed partial or complete response, as determined by the clinical investigators [81]. Host immune function has been widely observed to play a role in the development and regulation of melanoma growth. In an effort to exploit this interaction, potential immunotherapies have been developed such as interleukin-2 (IL-2), interferon-α (IFN), and most recently, ipilimumab a fully humanized monoclonal antibody which is specific against CTLA-4.

IL-2 is a lymphokine that stimulates T-cell proliferation and function; augments natural killer cell proliferation and cytotoxic activity; and triggers the release by activated lymphocytes of cytokines such as interferon gamma, tumor necrosis factor, and others. High-dose bolus IL-2 (HD IL-2) was approved by the Food and Drug Administration (FDA) in 1998 for the treatment of metastatic melanoma due to the potential for durable complete responses in a small number of patients. The overall response rate of 270 melanoma patients enrolled in clinical trial at the National Cancer Institute and that were treated with IL-2 was 16% with 6% complete responses and median survival of 11.4 months [82].

Treatment with INF-a to date has not demonstrated significant improvement in survival either as a single agent or combination therapy and also holds an increased side effect profile.

Other cytokines such as IL-12, IL-18, and granulocyte macrophage colony-stimulating factor have been tested as monotherapy or in combination with other agents in advanced stage melanoma, but the response rates have been disappointing[83][84].

In 2011, the Food and Drug Administration (FDA) approved a breakthrough melanoma treatment called Yervoy (ipilimumab). Not only this is the first melanoma drug to receive FDA approval in 13 years, but it's the first therapy proven to extend overall survival for advanced stage melanoma patients. Ipilimumab is a recombinant, human monoclonal antibody which upon binding to the cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) on the surface of T cells blocks the effects of the negative T-cell regulator CTLA-4. This causes an enhanced T-cell activation and proliferation which leads to increased T-cell-mediated activity against tumor cells. The approved dosing schedule is 3 mg/kg intravenously every 3 weeks for 4 doses. Ipilimumab earned FDA approval following the results of 2 phase III trials. It was shown to prolong OS by approximately 3.6 months when given alone or in combination with a vaccine to patients with previously treated metastatic melanoma. In a separate study, compared to dacarbazine and placebo, ipilimumab is given in combination with dacarbazine increased OS by 2.1 months [85].

Ipilimumab has not only been shown to increase median OS relative to other treatments at a study population level, but it has also induced unusually long-lasting survival in individual patients. A recent report in 36 heavily pretreated patients with metastatic melanoma found that 3 out of 30 patients achieving complete remission with ipilimumab 10 mg/kg had ongoing remission at 36, 34, and 41 months [86].

In another analysis of 177 patients, all but one of the 15 complete responders had ongoing responses at 54 to 99 months [87]. Responses have also been achieved with ipilimumab in patients with brain metastases from melanoma, particularly when metastases are small and asymptomatic [88].

One mechanism by which melanoma is thought to evade the immune system is through tumor expression of programmed death ligand 1 (PD-L1). PD-L1 is a negative regulator of the immune system that acts through binding of the PD-1 present on activated lymphocytes and PD-L1/PD-1 interaction causes immune tolerance through

apoptosis of the activated lymphocyte. MDX-1106 is a genetically engineered fully human immunoglobulin G4 monoclonal antibody specific for human PD-1 [89]. A phase 1 study of anti-PD-1 antibody, MDX-1106, demonstrated single agent responses in a variety of previously treated, refractory solid tumors including melanoma with few treatment related immune toxicities [89]. The clinical experience with anti-PD1 treatment is limited but encouraging and a dose escalation study of the combination of MDX-1106 and ipilimumab is currently recruiting. In addition to antibodies that target PD-1, phase 1 trials of anti-PD-L1 are also underway. Immunotherapy is the only treatment strategy that has been shown to provide, even if in a small subgroup of patients, long and lasting survival. As part of this therapy can also include specific adoptive cell therapy (ACT) this consists in *vitro* identification of autologous lymphocytes extracts from the tumor (TILs, Tumor-Infiltrating Lymphocytes[5]) with antitumor activities, then the expansion of these cells and in their infusion in the patient. This therapeutic strategy has a number of advantages compared to other therapies such as anti-tumor specificity, but has the limit of the incapacity of the cells to persist for a long time *in vivo* in the host. ACT therapy theoretically allows for the selection of the most highly avid antitumor lymphocytes, their expansion in the absence of endogenous regulatory mechanisms, and the manipulation of the host immune environment in their absence. Although recent reports have documented the potential clinical efficacy of ACT, the generation of large numbers of highly active anti-tumor T cells is a significant technical challenge and remains a serious impediment to the wider use of T-cell transfer as a standard cancer therapy. In one study, T-cell populations derived from tumor infiltrating lymphocytes were reported to induce objective clinical responses in ACT therapy for melanoma patients; in a series of 86 patients treated with TIL in combination with high-dose IL-2 therapy, 29 patients exhibited objective clinical responses although many were of short duration. In that clinical trial, TIL were administered based solely on their expansion to a target cell number, irrespective of their tumor recognition. Subsequent attempts to improve on

[5] *Tumor-infiltrating lymphocytes are cells that actively attack and destroy the cells that make up a tumor. These lymphocytes are the body's natural defense against cancer and can often completely destroy tumors without the assistance of additional cancer therapies. In patients who must undergo therapy, including radiation or chemotherapy, tumor-infiltrating lymphocytes are still an important factor in recovery.*

ACT for melanoma patients have focused on more reliable methods of generating cultures that specifically recognize tumor cells. Adoptive cell transfer therapies depend on reliable and reproducible methods for the generation of tumor-reactive T lymphocytes. Recently, one approach that has been developed in an effort to speed up the process and avoid terminal differentiation of T cells is to limit the time TIL are initially expanded from excised tumors with high-dose IL-2. Usually it takes between 4 to 5 weeks to expand enough TIL from tumor fragments (about 50 million) with IL-2 to proceed with the next step of culture which is the REP generating the final TIL infusion product [90].

The new approach, called “young TIL”, reduces this initial expansion period in IL-2 down to a few weeks before the cells are subjected to the rapid expansion protocol (REP) [91]. In the “young TIL” protocol, TIL are immediately isolated by enzymatic digestion of tumors and expanded rather than having to wait for them to migrate out of tumor fragments in culture. This results in more TIL being immediately accessible for expansion and a shorter time needed to reach minimal numbers needed for the REP as well as higher success rates (>80%) in reaching these minimal cell numbers needed from accrued patients. Although TIL therapy has shown great potential to treat metastatic melanoma, a number of issues have emerged that need to be addressed to bring it more into the mainstream of melanoma care. First, was been reached the point where a pivotal phase II or phase III trials are needed in an attempt to gain regulatory approval of TIL as standard-of-care. Second, improvements to expand TIL in therapy are needed, that minimize the time the T-cells when they are in culture and improve the memory and effector characteristics of the T cells for longer persistence and enhanced anti-tumor activity *in vivo*. Third, there is a critical need to identify surrogate and predictive biomarkers in order to better select suitable patients for TIL therapy in order to improve response rate and duration [92].

The modest antitumor activity of the chemotherapeutic agents, led to investigation of combinations of these agents to improve outcomes. Single-institution studies suggested that combination chemotherapy might lead to an increase in the response rate and possibly survival.

The most widely used of these is known as the “Dartmouth Regimen” and consists of quadruple drug therapy with DTIC, cisplatin, carmustine and tamoxifen. This combination was reported to give a higher response rate than that reported for single agents or combinations previously tried. Experience summarized from studies of 197 patients treated in 5 centers indicates an overall response rate of 47% [93]. Overall, with combination chemotherapy can be obtaining a greater number of responses, but at the price of an increase significant toxicity without improved survival [94].

Interferon-α was tested in combination with monochemotherapy using dacarbazine, temozolomide, or cisplatin, with overall response rates of approximately 25%. These regimens, however, were not superior to single-agent dacarbazine or multi-agent chemotherapy [94].

As with IFN-α, IL-2 has been combined with dacarbazine, dacarbazine and cisplatin, or dacarbazine, cisplatin, and tamoxifen in phase II clinical studies, but showed no clinical benefit, as it did not improve survival compared with multiagent chemotherapy or other biochemotherapy combinations used in other studies.

Moreover, pre-clinical work has suggested that BRAF inhibition leads to an increased tumor recognition by T-cells providing a rational for the combination of BRAF inhibitors with agents that stimulate the immune system such as ipilimumab. In addition, BRAF inhibitors and other targeted therapies will likely be combined with ipilimumab, IL-2, anti-PD-1, and other immunotherapies that are currently being tested. Another approach is to use agents that target several pathways together. The molecular chaperone heat-shock protein 90 (Hsp90) regulates folding and function of newly synthesized proteins, among which are many protein kinases, including CRAF, BRAF, Cdk4 and Cdk6. Among the many targeted agents designed to block the activity of oncogenic proteins, inhibitors of the heat shock protein 90 (Hsp90) molecular chaperone are of special interest [70]. These agents cause the simultaneous depletion of many oncogenic chaperone ‘client’ proteins, thus leading to the parallel blockade of several oncogenic pathways and multiple key cancer cell functions including proliferation, invasion and angiogenesis. Several Hsp90 inhibitors have been identified, including the benzoquinone ansamycin derivative 17-allylamino-17-

demethoxygeldanamycin (17-AAG, tanespimicin); it has shown promising antitumor activity in numerous preclinical models, and is currently undergoing clinical testing with evidence of activity in trastuzumab-refractory breast cancer [17-19]. In addition, preclinical studies indicated that 17-AAG has therapeutic activity against melanoma cells, most likely via effects on the RAF family of oncoprotein.

Early clinical studies revealed evidence of Hsp90 target modulation and some signs of biological activity (stable disease) in melanoma patients that may potentially be associated with the presence of BRAF or NRAS mutation. Interestingly, mutant BRAF, which is present in almost 70% of all melanomas, shows marked dependency on Hsp90 and is degraded by 17-AAG treatment, while WT BRAF activated by NRAS is also Hsp90 dependent [20]. Furthermore, melanoma cells, regardless of their BRAF or NRAS mutation status, are sensitive to the growth inhibitory effects of 17-AAG, consistent with the action of Hsp90 inhibitors on multiple oncoproteins, including CRAF [95].

Treatment of metastatic melanoma remains a challenge. While surgery and radiation therapy may play a role in the palliation of symptoms from local tumor growth, systemic therapy is the mainstay of treatment for metastatic melanoma. Treatment with HD IL-2 may induce durable responses in a small subset of patients and should be considered in eligible patients. Chemotherapeutic approaches may have a palliative benefit. Single-agent chemotherapy is usually well tolerated. Combination chemotherapy and biochemotherapy have not improved survival but may lead to increased response rates at the cost of higher toxicity. Many novel therapeutic approaches are promising, and participation in clinical trials should be considered the standard of care.

4. Natural compounds and cancer

Natural products can exhibit many beneficial effects on human health. The health-beneficial effects of plants or their extracts and chemical structure diversity represent the fount of inspiration for new ways in cancer chemoprevention and treatment. As far as cancer is concerned, naturally occurring compounds have been reported to prevent tumorigenesis and also to suppress the growth of established tumors.

This substances play a relevant role in cancer therapy today with substantial numbers of anticancer agents used in the clinic being either natural or derived from natural products from various sources such as plants, animals and microorganisms. The anticancer drug discovery and the screening programs such as those promoted by the National Cancer Institute (NCI) have played an important role in the development of anticancer natural compounds. During the last few years, natural product-based drug discovery is increasing due to the use of new technologies [96]. Natural compounds that, not only have a therapeutic potential but also a strong chemopreventive. Safety is always a primary consideration in studies involving human subjects, particularly patients without evidence for overt cancer, in fact an ideal chemopreventive agent should be nontoxic, effective at lower doses, economical, and easily available.

In recent years, cell cultures and animal studies have suggested the cancer preventive potential of several nutritional compounds, including those found in green and yellow vegetables, citrus fruits, and spices.

Natural products can be broadly divided into four categories:

- Microbial word
- Animal sources
- Marine sources
- Plants sources

Microorganisms were not explored as a source of potential drug candidates until the discovery of penicillin in 1929. Since then, a large number of terrestrial and marine microorganisms have been screened for drug discovery. Microorganisms have a wide

variety of potentially active substances and have led to the discovery of antibacterial agents like cephalosporins , and anticancer agents like epirubicin. New compounds derived from microorganisms include rapamycin and geldanamycin. Rapamycin (sirolimus) is amacrolide compound obtained from Streptomyces hygroscopicus. Rapamycin is a potent immunosuppressant and also possesses antifungal and antineoplastic properties. Rapamycin acts as a specific inhibitor of m-TOR (mammalian target of rapamycin) that is a downstream mediator of PI3K/Akt [97]. Geldanamycin, an analogue of rapamycin, is a benzoquinone ansamycin natural fermentation product from the same microbial source that binds to, and inhibits the 90 kDa heat-shock protein HSP90 [98]. Animals have also been a source of some interesting compounds that can be used as drugs. Precisely, epibatidine, obtained from the skin of an Ecuadorian poison frog, is ten times more potent than morphine. Venoms and toxins from animals have played a significant role in designing a multitude of cures for several diseases [99].

Although marine compounds are yet underrepresented in routine clinical practice, aquatic environment may become a potentially valuable source of novel compounds. During the past 30 years, thousands of new compounds with different biological activities varying from anticancer to antiviral have been isolated from marine sources [100]. Marine compounds, for instance, can interfere with very relevant intracellular targets such as signal transduction, microtubule stabilisation or new forms of interaction with DNA. They can be extremely potent in culture, with IC_{50}S in tumour cell lines in the nanogram range. Probably they need potency and rapid penetration in cellular membranes to efficiently protect themselves in an aquatic environment that rapidly dilute their poisons [101, 102]. Currently, over 20,000 natural marine products are isolated and identified from various organisms, including sponge, algae, coral and ascidian [103]. The use of plants as medicines has a long history in the treatment of various diseases. The earliest known medical document is a 4000-year-old Sumerian clay tablet that recorded plant remedies for various illnesses. During the ancient Egyptian civilization, a great wealth of information already existed already on medicinal plants. Among the many remedies prescribed were mandrake for pain relief,

and garlic for the treatment of heart and circulatory disorders. This information, together with hundreds of other remedies, was preserved in the Ebers papyrus about 3500 years ago. After the arrival of Columbus, many New World plants became available to Europeans, and by the time of Henry VIII in England (1491-1547), an entire European or Western medicalsystem that blended plant use and astrology had developed. For centuries, medicine in the West meant herbal medicine. The scientific literature is rich in epidemiological studies that support significant differences in the occurrence of cancers between oriental and occidental populations. Generally, populations that consume a high level of natural herbal products have a reduced incidence of cancer. An example is the low incidence of colon cancer in Asian countries with high consumption of soybean products [104]. There is lately a great interest in screening for plants to be used in cancer prevention and treatment. For this reason, extracts from different plants have been extensively studied; nowadays, 35.000-70.000 plant species have been screened for their medicinal use. Herewith some examples of the most important ones will be reported. Surely one of the most studied and promising is curcumin, which is well-known to inhibit the initiation and promotion in skin cancer [105]. Curcumin is a main component of the spice turmeric, commonly used in Indian and Southeast Asian foods; it is isolated from the roots (rhizomes) of the plant *Curcuma longa*, is the major yellow pigment present in turmeric, widely used as a spice. Although turmeric and its chemical components have been used in traditional medicine for thousands of years, it was only in the 1980s that curcumin attracted much attention thanks to its anti-tumorigenic activity; in fact, Kuttan et al reported that turmeric extract inhibited the growth of Chinese hamster ovary cells, and was cytotoxic to lymphocytes and Dalton's lymphoma cells in *vivo* [106]. Furthermore, several studies demonstrated that curcumin interrupts the carcinogenesis process by inhibiting the initiation step or suppressing the promotion and progression stages in animal models [107].

Considerable importance possesses the resveratrol, a polyphenol found in numerous plant species, including mulberries, peanuts and grapes. It is potential chemopreventive and chemotherapeutic activities have been demonstrated in all

three stages of carcinogenesis (initiation, promotion, and progression), in both chemically and UVB-induced skin carcinogenesis in mice, as well as in various murine models of human cancers [108]. Evidence from numerous *in vitro* and *in vivo* studies have confirmed its ability to modulate various targets and signalling pathways [109].

To continue this overview there is the tea, which is one of the most widely consumed beverages and is rich in substances with antioxidant properties. Different processing techniques yield different types of tea. Although both black tea and green tea have been studied for their chemopreventive potential, green tea showed higher promise and greater efficacy against multiple types of cancer.

Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in green tea and has gained the most attention with respect to anticarcinogenic activity [110]. In a UV-induced mouse skin carcinogenesis model, the administration of EGCG increased the number of wild-type p53[6]-positive, p21-positive and apoptotic sunburn cells, demonstrating *in vivo* up-regulation of tumor suppressor and cell cycle regulator genes by this polyphenol. Moreover, the chemopreventive activity of EGCG through blockade of cell cycle in different stages, showing that of EGCG causes in G_0/G_1 arrest and another in G_2/M phase [111][112][113].

Besides the mentioned dietary agents, other natural compounds are being actively investigated for their chemopreventive or chemotherapeutic potential, many of which show strong promise. These include carotenoids (lycopene, lutein, alpha-carotene, beta-carotene) genistein, isothiocyanates, ellagic acid, some triterpenes (such as lupeol, betulinic acid, ginsenosides, oleanolic acid), polyunsaturated fatty acids (PUFAs), ginkolide B and many more. Nature represents an impressively huge 'database' of different and diversified molecular scaffolds.

The advancement in new screening systems allowing the analysis of large libraries of isolated naturally occurring compounds offers new important and fast tools for the selection of promising antimetabolic compounds especially from dietary origins with reduced side effects. Moreover low costs for their extraction/production in large

[6] *The tumor suppressor gene p53, as the 'guardian of the genome', protects cellular DNA from a variety of carcinogenic insults by blocking cell proliferation, stimulating DNA repair and eliminating damaged cells by promoting apoptosis*

amounts make them interesting for commercial objectives and represent a good basis for chemical modifications that may further improve their anticancer activities and facilitate their pharmacological use and efficiency.

5. Sulforaphane

5.1. *Cruciferous* vegetables glucosinolate and isothiocyanates: chemical structure and proprieties.

As previously discussed, a connection between vegetable consumption and health has long been established. Furthermore, evidence has shown that cruciferous vegetables particularly are more strongly associated with the reduction of cancer risk than vegetable consumption in general and have been suggested as protective against different types of tumor. Vegetables of the *Cruciferae* family are in the botanical order Capparales, which includes the *Brassicas* genus. Cruciferous vegetables include the Brassica genus, which includes cabbage, kale, broccoli, cauliflower, Brussels sprouts, kohlrabi, rape, black and brown mustard, and root crops such as turnips and rutabagas (swedes). The chemopreventive effect of cruciferous vegetables is thought to be partially due to their relatively high content of glucosinolates (β-thioglucoside N-hydroxysulfates), which distinguishes them from other vegetables.

Glucosinolates can be divided into three classes based on the structure of different amino acid precursors [114]:

1. aliphatic glucosinolates derived from methionine, isoleucine, leucine or valine;
2. aromatic glucosinolates derived from phenylalanine or tyrosine;
3. indole glucosinolates derived from tryptophan.

The glucosinolates are not bioactive and seem to have no chemopreventive effects unless they are converted to isothiocyanates (ITCs) and indole-3 carbinols by hydrolysis catalyzed by myrosinase[7]. The loss of cellular integrity as a result of wounding, insect or pathogen attack activates the binary glucosinolate-myrosine system and leads to the generation of thioglucose, sulfate and an unstable intermediate which rearranges spontaneously into several degradation products [115]. Chemical conditions such as

[7] *Myrosinase is a dimeric protein with a molecular weight in the range of 62–75 kDa per subunit. Purified and characterized enzymes have shown to be highly glycosylated and are characterized by varying degrees of ascorbic acid activation. Distribution of myrosinase isoenzymes seems to be both organ-specific and species-specific [115].*

pH, availability of ferrous ions and presence of myrosinase- interacting proteins determine the final composition of the product mix which can include isothiocyanates, oxozolidine 2-thiones, nitriles, epithionitriles, and thiocyanates (Figure 10) [114].

An isothiocyanate is a compound with the structure R-N=C=S, where R is an alkyl or aryl group. Isothiocyanates are reactive compounds, particularly with respect to nucleophilic attack at the electron-deficient central carbon atom. The central electrophilic carbon of isothiocyanates (-N= C= S) undergoes rapid addition reactions with biological nucleophiles, in particular, amines and thiols. Isothiocyanates react with amines to generate stable thiourea derivatives, whereas reaction with thiols generates labile dithiocarbamate adducts. Seminal studies by Drobnica et al. characterised the reactivity of a variety of isothiocyanates with small molecules, peptides and proteins, and showed that isothiocyanates react up to one thousand times faster with thiol groups than with amino groups, rendering proteins with functional and structural cysteine residues particularly sensitive targets for modification.

In contrast to their glucosinolate precursors, the isothiocyanates are typically lipophilic and may be highly reactive, volatile, malodorous, taste sharp or bitter, and some of them can be hepatotoxic, antibacterial,antifungal, antiprotozoal, nematocidal, allelopathic, or produce goitrogenic degradation products.

Clearly an understanding of the bioavailability, transport and metabolism of glucosinolates after consumption of Brassica vegetables as food is a prerequisite for understanding the mechanisms of their protective effects in humans. The structural diversity and chemical reactivity of glucosinolate breakdown products, as well as the milieu from which they have to be isolated, have long inhibited progress in this field but the improvements in analytical methods for detecting and quantifying isothiocyanates and their excretory metabolites are now transforming this situation.

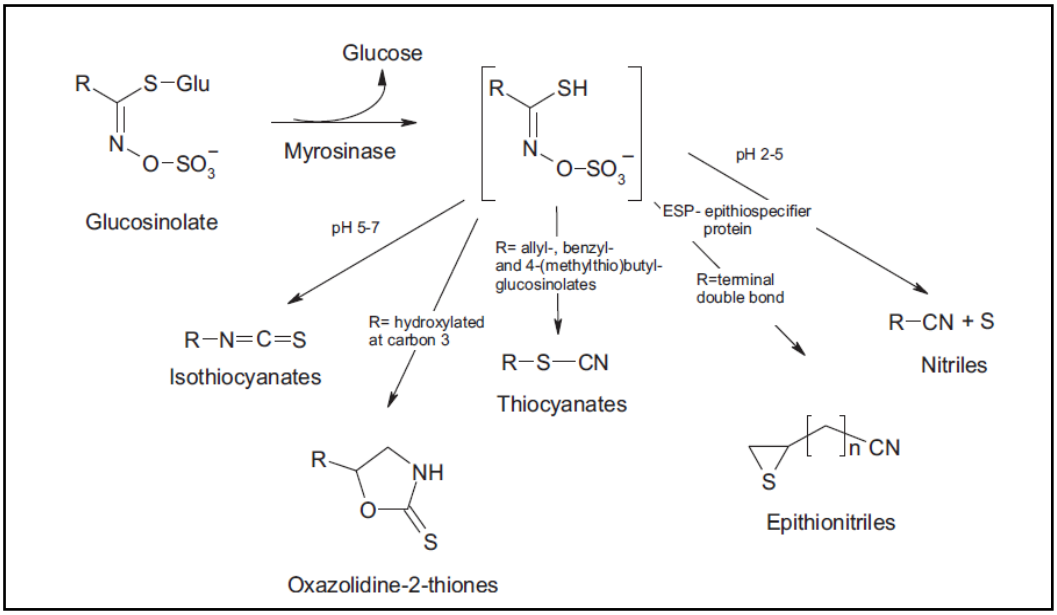

Figure 10. Glucosinolates.

The most characterized ITC compound is sulforaphane (-)-1-isothiocyanato-(4R)-(methylsulfinyl)butane [CH3-SO-(CH2)4-NCS, the hydrolysis product of glucoraphanin, generally found in high concentrations in broccoli (Figure 11)[116]. Glucoraphanin is either converted to sulforaphane (SFN) by the endogenous myrosinase, as described, or once consumed, if the myrosinase has been denatured by cooking, by microbial thioglucosidases in the gut; SFN is then absorbed and conjugated with glutathione before being metabolized via the mercapturic acid pathway prior to excretion from the body in the urine. Because of it is lipophilicity [logP (octanol/water) 0.72] [117] and molecular size (mol. wt. 177), sulforaphane is likely to passively diffuse into the enterocytes [118]. In detail, after absorption, SFN is conjugated with glutathione (SF-GSH) by glutathione-S-transferase (GST) leading to maintenance of a concentration gradient and facilitating a fast passive absorption into the cell. It is metabolized via the mercapturic acid pathway, producing predominantly cysteinylglycine (SFN-CG), cysteine (SFN-Cys), and N-acetyl-cysteine (SFN-NAC) conjugates that are excreted in the urine. Among these conjugates, sulforaphane-N-acetylcysteine is the most prevalent.

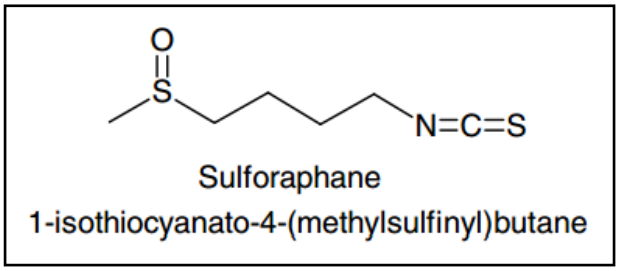

Figure 11. Structures of sulforaphane.

Upon absorption into the bloodstream, sulforaphane readily accumulates in tissue and exerts anticarcinogenic effects. In one human study, a single 200 µM dose of sulforaphane from broccoli sprouts yielded peak plasma concentrations between 0.943 and 2.27 µmol/L at one hour post feeding; the half life of sulforaphane was 1.77 ± 0.13 hours [119]. The means by which SFN may confer beneficial effects are well investigated and it has been demonstrated that SFN has many possible mechanisms of action.

The effect of SFN on carcinogenesis has in particular been intensely studied, the evidence from which suggests SFN is able to both help prevent initiation and slow progression of the disease. The many modes by which sulforaphane acts and pathways it alters can often overlap making defining its precise role difficult to determine. Here the main mechanisms by which sulforaphane in protecting health, namely suppression of oxidative stress, regulation of phase I and II enzymes, reduction of inflammation, initiation of the heat shock response, inhibition of histone deacetylases, suppression of the cell cycle, induction of apoptosis, inhibition of angiogenesis, and reduction of migration and metastasis, are discussed (Figure15).

5.2. Pharmacokinetics

The ability of SFN to be distributed throughout the body and reach target tissues has been investigated *in vitro*, in mouse models and in human subjects.

In study with rats, following a 50 μmol gavage of SFN, detectable SFN was evident after 1h , showing a half life of approximately 2.2h [120]. Human perfusion experiment showed that 74±29% of SFN from broccoli extracts can be absorbed in the jejunum and that a portion of that returns to the lumen of the jejunum as SFN-GSH. Pharmacokinetic studies in both rats and humans also support that SFN can be distributed in the body and reach μM concentrations in the blood.

Moreover, in a pilot study, in human mammary tissue, an a oral dose of broccoli sprout preparation containing 200 μmol SFN one hour prior to tissue removal, showed mean accumulation of 1.45±1.12 pmol/mg in the right breast and 2.00±1.95 pmol/mg in the left [121]. This suggest, that following oral dosing, sulforaphane metabolites are readily measurable in human breast tissue enriched for epithelial cells, providing a strong rationale for evaluating the protective effects of a broccoli sprout preparation in clinical trials of women at risk for breast cancer.

The last aspect of SFN pharmacokinetics concerns its excretion. When efficient metabolism of glucoraphanin occurs, SFN-NAC is the primary SFN metabolite excreted in the urine. In the humans, SFN and its metabolites were excreted with the first-order kinetics and most data indicate that SFN and it metabolites are cleared from the body within 72h of dosing. From these data it can be extrapolated that maintenance of SFN concentrations in the body can be achieved by consuming recommended servings of cruciferous vegetables once a day. Collectively, the published data indicate that SFN can be absorbed, reach μM concentrations in the blood, accumulate in tissues, and be maintained to achieve the anticancer effects.

5.3. Biological processes modulation by Sulforaphane

5.3.1. Modulation enzymes of phase I and phase II

Induction of phase II enzymes by phytochemicals represents an important mechanism for achieving protection against cancer. Although phase II enzymes have been traditionally recognized as those catalyzing the conjugation of endogenous ligands, glutathione and glucuronic acid, to endo- and xenobiotic substrates, this classification is expanding to include proteins that catalyze a wide variety of reactions that confer cytoprotection against the toxicity of electrophiles and reactive oxygen species (ROS). Together with ROS, other molecules, including foreign chemicals from the environment and food, are capable of causing cellular damage.

Foreign chemicals are metabolised by phase I cytochrome P450 (CYP450) enzymes that catalyze their conversion to a more hydrophilic form, allowing their conjugation to endogenous ligands, a process catalyzed by phase II detoxification enzymes, in order for them to be excreted from body.

Sulforaphane acts as an indirect antioxidant regulating cellular defence mechanisms notably activating the nuclear factor (erythroid-derived)-like 2 (Nrf2) pathway. Nrf2 is a transcription factor that plays a key role in regulating the enzymes and other molecules that help protecting the cell from oxidative stress [122]. Under normal circumstances Nrf2 is sequestered in the cytoplasm by kelch-like ECH-associated protein 1 (Keap1) which targets it for degradation. During oxidative stress Keap1 is modified causing a conformational change that leads to the release and activation of Nrf2. It translocates to the nucleus where it binds to promoters containing the antioxidant response element (ARE) upregulating the transcription of cytoprotective enzymes. Regulatory mechanisms may collaborate with Keap1 in regulation of Nrf2 and ARE-mediated genes. Although largely unclear, multiple stress-related signaling pathways, such as the mitogen-activated protein kinase (MAPK), p38 MAPK, phosphotidylinositol 3-knase (PI3K), c-Jun-N-terminal kinase (JNK), endoplasmic reticulum (ER)-resident kinase (PERK), have been documented to mediate Nrf2 activation and/or regulation of ARE responsive genes [122]. Extensive studies during

the past decade have proven the notion that the transcription factor NF-E2-related factor 2 (Nrf2) is an essential element for the regulation of the ARE. The proteins encoded by the genes that possess ARE between the sequences enhancer include enzymes associated with the biosynthesis the glutathione redox proteins with sulfhydryl groups and enzymes of drug metabolism. The first evidence of a metabolic pathway mediated by ARE for gene regulation has been that some xenobiotics are able to modulate the activity of enzymes of phase I and II, involved in drug metabolism.

Exposure to sulforaphane allows Nrf2 to escape the degradation dependent on Keap1 (Figure 12), thereby increasing its nuclear localization and the activation of Nrf2-dependent genes [123]. Nrf2 occupies a pivotal role in the maintenance of GSH homeostasis by transcriptionally regulating key enzymes particularly glutamate cysteine ligase modulatory and catalytic subunits (GCLM/GCLC) and glutathione peroxidase (Gpx). The regulation of other GSH enzymes such as GSH synthetase and GSH reductase was also suggested to be Nrf2-dependent. The heterodimer GCLM and GCLC subunits have an essential role in GSH homeostasis by catalyzing the first-step, ratelimiting enzymatic reaction in GSH biosynthesis. GCLC or the heavy subunit (~73 kDa) is the catalytic engine which adjoins glutamate and cysteine into γ-glutamylcysteine followed by a final step of adding glycine via the GSH synthetase at the completion of GSH biosynthesis. GCLM is the smaller (~28 kDa) and modulatory subunit which increases efficiency and substrate affinity of GCLC, and desensitizes negative feedback of GSH on GCLC.At molecular level several Nrf2-dependent genes have been discovered and can be induced by SFN. Analysis of gene expression profiles, which took advantage of the series of oligonucleotide U74Av2 mouse genome, have identified 26 genes Nrf2-dependent; these genes are inducible by SFN and among them there are those coding for xenobiotic metabolizing enzymes such as NAD(P)H dehydrogenase, quinone 1 (NQO1), glutathione S-transferase (GST), gamma-glutamyl cysteine synthetase (GCS) and for biosynthetic enzymes glutathione [124]. These studies, therefore, showed that among the products of genes induced by SFN, across the street Nrf2-dependent, are included xenobiotic metabolizing enzymes,

antioxidants, proteins that regulate the adhesion, growth and cell cycle, transport proteins and transcription factors (Figure 12).

Sulforaphane can modulate phase I metabolism through direct interactions with cytochrome P450 enzymes (CYP) or regulating their transcript levels within the cell. Phase 1 enzymes, as said before, usually involve oxidation, reduction, or hydrolysis and generally lead to detoxification, but are also involved in converting procarcinogens to carcinogens; inhibition of phase I enzymes is thought to be an important step in blocking chemically-induced carcinogenesis [122].

The ability of SFN to inhibit the enzymes of phase I is linked to that of counteract the formation of DNA adducts caused by compounds carcinogenic. A wide range of data is available, which demonstrates that SFN may inhibit DNA-adduct and chemical carcinogenesis through alteration of the level of certain CYP isoforms in rodents via a competitive mechanism as well as by a direct covalent modification. For example, SFN decreased enzyme activities in rat hepatocytes associated with CYPs 1A1 and 2B1/2, namely ethoxyresofurin-O-deethylase and pentoxyresofurin-O-dealkylase, in a dose-dependent manner; similarly in human hepatocytes, sulforaphane decreased the activity of CYP3A4, the major CYP in human liver, by decreasing it is mRNA levels [124].

In vitro studies have indicated SFN as a potent inhibitor of mutagenesis induced by heterocyclic amines (HCA). Exposure to HCA, resulting from the cooking of meat, has been implicated in the etiology of certain cancers, such as breast, colon and prostate cancer. The treatment with SFN significantly reduced the level of DNA adducts formed by 2-amino-1-methyl-6-fenilimidazo [4,5 b] pyridine, the most abundant type of HCA in the hepatocytes and in human HepG2 cells, in order dose-dependent manner [125]. Sulforaphane was also shown to protect against benzo(a)pyrene (BaP)-induced single-strand DNA breaks in the comet assay, with concentrations as low as 5 μM SFN, in the human mammary ephitelian cell line MCF-10F [126].

The ability of SFN to activate these Nrf2 driven detoxifying genes is well documented both in *vitro* and *in vivo*. Sulforaphane causes responses dose and time dependent, but the level of induction and/or the type of enzyme induced vary according to the cell lines, as demonstrated in comparative study, that valuated the effect of SFN 25 μM of

enzyme activity of aldo-keto reductase (AKR), NQO1, GST and glutathione reductase (GR) in seven cell lines: HepG2, HepG2, MCF7, MDA-MB-231, LNCaP, HeLa e HT-29 [127]. NQO1 exemplifies a protein with multiple protective roles that include and extend beyond its catalytic function. It is a widely-distributed FAD-dependent flavoprotein that catalyzes the reduction of quinones, quinoneimines and nitroaromatics. The classical direct antioxidant role of NQO1 is inherent in its catalytic mechanism: the obligatory two-electron reduction of a broad array of quinones to their corresponding hydroquinones by using either NADPH or NADH. Because of the numerous deleterious effects of quinone compounds, such as the ability to generate reactive oxygen species through redox mechanisms cyclic, the removal of a quinone from a biological system through the it is considered a reaction of detoxification.

More recent *in vivo* work in the F-344 rat model confirmed phase II enzyme induction in the prostate and colon after dietary supplementation of a broccoli or SFN diet; precisely, in one study where the rats were fed with a broccoli diet, it was observed a 4.5 fold induction of NQO1 activity in the rat colon. In another study, rats gavaged with SFN had increased NQO-1, total GST activities in the prostate, liver and bladder [128][129]. Sulforaphane is also a very potent inducer of the enzyme heme oxygenase-1 (HO-1) that catalyses the conversion of heme to biliverdin which in turn is reduced enzymatically to bilirubin. These widespread reactions are involved in the continuous breakdown of haemoglobin. Bilirubin is a very potent and versatile antioxidant, especially at low oxygen tensions such as prevail in tissues. Direct evidence has now been obtained in cells where heme oxygenase-1 activity has been overexpressed by trasfection of the cognate cDNA are indeed more resistant to oxygen toxicity. It therefore seems likely that heme oxygenase-1 induction conferes antioxidant protection; it displays anti-apoptotic, and anti-inflammatory effects and appears to have a complex role in angiogenesis [130]. Recently it has been demonstrated that, SFN increased the expression of Nrf2 and of downstream targets HO-1 and NQO-1 in mouse Neuro2a neuroblastoma cells and the sciatic nerve of diabetic animals [131]. Sulforaphane was also effective in counteracting oxidative stress induced by

antipsychotic drugs in human neuroblastoma SK-N-SH cells, increasing GSH levels and inducing NQO1 activity [132].

These data describe that sulforaphane and ITC are able to protect the cells from excessive production of free radicals, not by acting as donors or electron acceptors, but modulating the expression of phase II enzymes and modifying intracellular levels of GSH. The ITC in the cell are conjugated with GSH and this leads to a decrease of the intracellular pool of such a compound, which is compensated by the activation of γ-glutamylcysteine synthase, the rate limiting enzyme in the synthesis of GSH. All together these reports provide for evidence to protection through sulforaphane against cancerogen-DNA damage *in vivo* and *in vitro* and they suggest a role for both inhibition of certain phase I enzymes and modulation of the xenobiotic-metabolizing enzyme systems; all this shifts the balance of cancerogen metabolism towards detoxification, that is an important mechanism of chemopreventive activity.

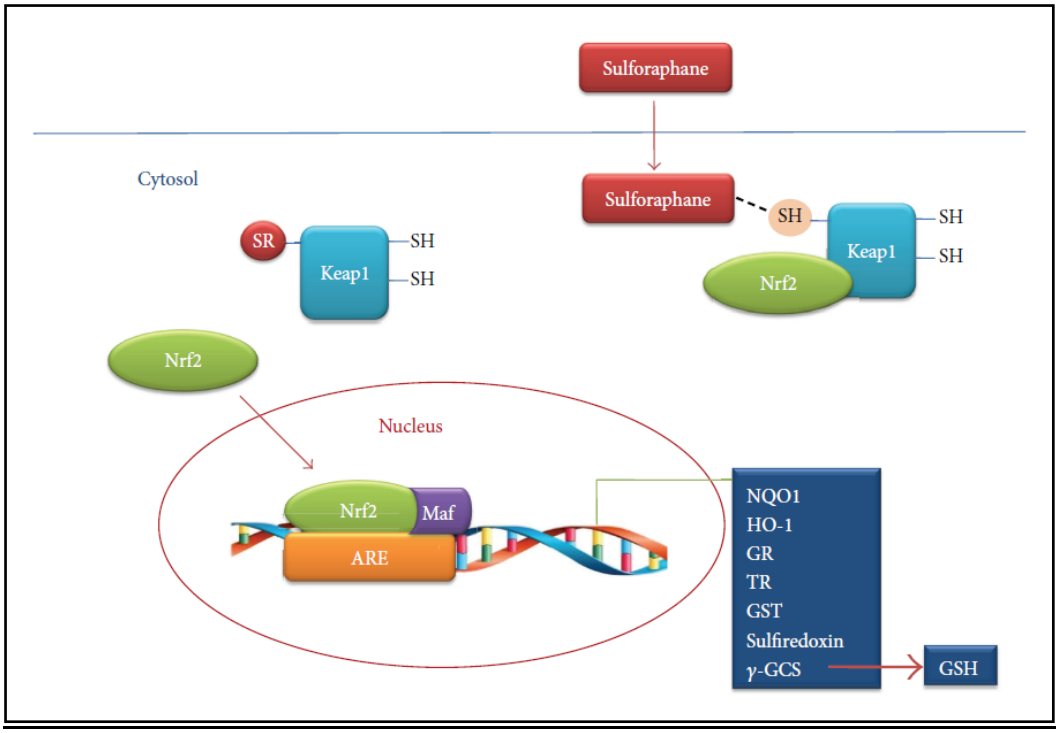

Figure 12. Mechanism of ARE-mediated antioxidant enzymes induction by sulforaphane [123].

5.3.2. Cell Cycle

The progression of cell cycle through the four stages, G_1, S, G_2 and M, is highly regulated by a number of checkpoint mechanisms that are essential cytoprotective responses to stress including DNA damage and abnormal mitogenic signals. Two of the most important proteins involved in the cell cycle machinery are cyclin-dependent kinases and cyclins; a variety of cyclin/cdk complexes are in fact able to guide the cdks to appropriate substrates and activate their catalytic activity (Figure 13). A normal cell growth is maintained through a balance between the cyclin/CDK complexes that promote cell growth and CDKIs that promote cell cycle arrest. There is accumulating evidence that sulforaphane can arrest cell cycle at different stages of its progressions, a mechanism by which it can inhibit the proliferation of cancer cells and exert its anti-carcinogenic effect. Block of G_0/G_1, G_2/M, and S phase by SFN treatment has been

documented in breast, bladder, colon, prostate, and T cells depending on the cell type, with several proposed mechanisms [124][133][134][135].

The ability of sulforaphane to induce cell cycle arrest is due to different mechanisms. For example, results of several *in vitro* studies suggest that in a concentration and time-dependent manner SFN treatment (10 μM – 20 μM) may cause reduction in cell viability and a G_2/M cell cycle arrest in both prostate and colon cancer cells; furthermore the blockage is caused by down-regulation of cyclin B1, Cdc25B and Cdc25C levels [124]. In human colon carcinoma cell (HT-29), G_1 arrest at other phases of the cell cycle occurred concomitant with an increase in p21, and a decrease in cyclin D1, cyclin A, and c-myc [135].

The tumor suppressor and cell cycle inhibitor protein p21 seams to play an important role in SFN-induced cell cycle arrest. An induction of p21 is consistently observed regardless of cell type and p53 status. Finally, a block in mitotic phase in breast cancer cells has been shown, due to inhibition of tubulin polymerization [136]. Tubulin-containing microtubules play a pivotal role in cell division, motility and intracellular trafficking in all eukaryotes. Therefore, anti-microtubule drugs that disturb tubulin polymerization and interfere with mitosis have gained much attention in the cancer drug discovery.

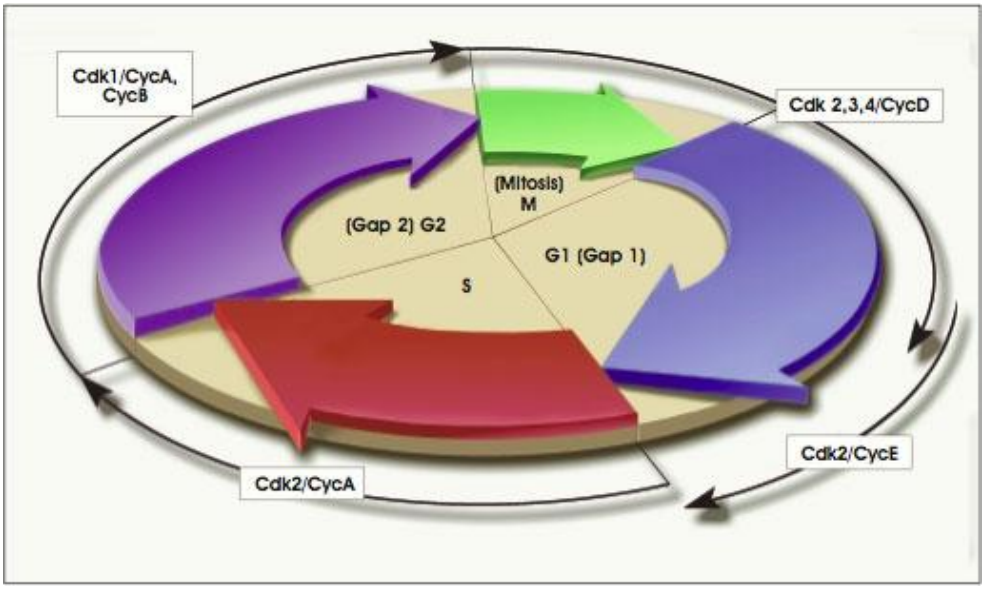

Figure 13. Cell Cycle.

The division cycle of most eukaryotic cells is composes of discrete phases: M, G_1, S, and G_2. In most cells, this coordination between different phases of the cell cycle is dependent on a system of checkpoints and feedback controls that prevent entry into the next phase of the cell cycle until the events of the preceding phase have been completed. Several cell cycle checkpoints function to ensure that incomplete or damaged chromosomes are not replicated and passed on to daughter cells. Loss of cell cycle regulatory mechanisms in tumor cells increases the chance of rapid proliferation. The phosphorylation status of the various components is important for regulating CDK complexes that govern cell cycle progression while CDK inhibitors govern cell cycle arrest.

5.3.3. Induction of apoptosis

Apoptosis, or programmed cell death, is a highly regulated process that occurs under a range of physiological and pathological conditions as part of the cellular mechanism. Apoptosis plays important roles in the development and maintenance of homeostasis and in the elimination of cells that are damaged or no longer necessary for the organism. Inappropriate regulation of apoptosis may cause serious disorders, such as neural degeneration, autoimmune diseases and cancers. The morphological features of apoptosis include cell shrinkage, chromatin condensation and fragmentation of the cell

into compact membrane enclosed structures, called “apoptotic bodies” that are engulfed by macrophages and removed from the tissue in a controlled manner. These morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes, which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles.

Caspases are essential elements in cells for the process of apoptosis, and have been called executioner proteins. The death-receptor caspase cascades and the mitochondrial caspase cascades are the two caspase pathways that can control apoptosis. Moreover, several hallmarks of apoptosis are the cytochrome C release from the mitochondrial membrane, the changes in Bcl-2[8] protein family ratios, the poly (ADP-ribose) polymerase (PARP[9]) cleavage and the cytoplasmic histone associated DNA fragments. A major well-researched activity of sulforaphane is the induction of apoptosis, which has been recorded in a number of different cell lines. In human colon carcinoma cells (HT-29), SFN-induced condensation of nuclear chromatin and cell surface expression of the phospholipid phosphatidylserine, both hallmarks of apoptosis [135].

Furthermore, SFN increased intracellular free Ca2+, upregulating calpain, activating caspase-12 and subsequently caspase-9. Sulforaphane also induced Bax, and inhibited Bcl-2, triggering the release of cytochrome c from the mitochondria leading to the activation of caspase-9 and then caspase-3 [137].

One way that SFN induces apoptosis is by interfering with the mitochondrial integrity. Mitochondrial play an important role in apoptosis because the permeabilization of

[8] *The Bcl-2 family is the best characterized protein family involved in the regulation of apoptotic cell death, consisting of anti-apoptotic and pro-apoptotic members. The anti-apoptotic members of this family, such as Bcl-2 and Bcl-XL. In contrast, pro-apoptotic members of this family, such as Bax and Bak, trigger the release of caspases from death antagonists via heterodimerization and also by inducing the release of mitochondrial apoptogenic factors.*

[9] *Poly(ADP-ribose) polymerase (PARP-1) is a nuclear enzyme that catalyzes the transfer of ADP-ribose polymers onto itself and other nuclear proteins in response to DNA strand breaks. During apoptosis, cleavage of PARP-1 in fragments of 89 and 24 kDa has become a useful hallmark of this type of cell death. This cleavage is well studied and is generated by the caspases 3 and 7, proteases activated during apoptosis.*

their outer membrane and subsequent release of mitochondrial proteins into the cytosol commits the cell to apoptosis. Decrease in mitochondrial potential in response to SFN has been identified in prostate and bladder cancer cell [138].

In recent studies, SFN has demonstrated potent anti-tumor activities also in human neuroblastoma SH-SY5Y cells; precisely treating cells with sulforaphane resulted in the depletion of mitochondrial membrane potential (ΔΨ), which in turn increased caspase 9, caspase 3 and the up-regulation of phosphorylated MEK/ERK without generating reactive oxygen species [139].Furthermore, always in recent studies, it was demonstrated that SFN, in ALL (acute lymphoblastic leukemia) leukemic cells, induced in dose-dependent apoptosis, which was associated to the activation of caspases (3, 8, and 9) and inactivation of PARP. Interestingly, sulforaphane also inhibited the AKT and mTOR survival pathways in most of the tested ALL cell lines by lowering the levels of both total and phosphorylated proteins [140]. SFN may also induce apoptosis through mitogen-activated protein kinase (MAPK) signal transduction. These proteins are important signaling components that convert extracellular signals into intracellular responses through a series of phosphorylation events. There are three distinct but parallel MAPK cascades identified in mammalian cells, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. Each of them consists of three kinases, a MAPK kinase kinase (MAPKKK) which phosphorylates and activates a MAPK kinase (MAPKK), which in turn phosphorylates and activates one of the three MAPKs. Literature data state that, sulforaphane activated all three MAPKs in liver hepatocellular HepG2 cells, JNK and ERK in human prostate cancer PC-3 cells, suggesting it is activity in these pathways.

Subsequently, the classical hallmarks of apoptosis such as chromatin condensation, traslocation of phosphatidylserine across the plasma membrane and DNA fragmentation were detected in SFN-treated cells from brain [141], breast [133], colon [135], lung [142], ovary [143], and prostate [144]. As previously reported to date several reports mainly using *in vitro* models demonstrate that SFN mediates apoptosis by regulating multiple targets in the apoptotic pathway.

5.3.4. Action on the angiogenic and metastatic process.

Angiogenesis, the process where new blood vessels are formed from preexisting ones, can be classified as either physiological or pathological. Physiological angiogenesis provides a driving force for organ development in ontogeny, is necessary for ovulation, and is a prerequisite for wound healing; pathological angiogenesis occurs during tumor growth at primary and metastatic sites.

The signaling pathway governing tumor angiogenesis is exceedingly complex, involving various angiogenic mediators. The major signaling mediators include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), epidermal growth factor (EGF), angiopoietins, endothelins, integrins and cadherins [145]. During angiogenesis, endothelial cells migrate, proliferate, organize into tube-like structures, and play an active role in tissue remodeling. Substantial evidence also suggests that stroma cells adjacent to the cancer cells, including fibroblasts and inflammatory cells such as macrophages, neutrophils, and lymphocytes, can interact with cancer cells and express angiogenic factors. More recent studies demonstrate that sulforaphane is also capable of inhibiting angiogenesis and metastasis. Sulforaphane was shown to interfere with essential steps of neovascularization from proangiogenic signaling and basement membrane integrity to endothelial cell proliferation, migration, and tube formation.

In literature it is reported that, SFN inhibited tumor growth and markers of angiogenesis and metastasis in an model of prostate cancer, all this, through FOXO transcription factors, these proteins have also been implicated in the negative regulation of signaling by the hypoxia-inducible factor 1 (HIF-1) during vascular development, raising the possibility these suppress not only tumor formation but also tumor [146]. Moreover, another study demonstrated that SFN can inhibit, in an orthotopic model of prostate cancer, the activation of NFκB and its gene products such as VEGF, HIF-1·, MT1-MMP, MMP-2, MMP-9, COX-2, IL-6 and IL-8 [146]. Some of these molecules can cooperate to degradation of the basement membrane, liberate growth factors for blood vessel endothelial cells, which then, show pleiotropic responses that facilitate cell migration, proliferation, tube formation, and survival. Moreover,

sulforaphane also inhibited the proliferation and tubular formation on matrigel of human umbilical vein endothelial cells *in vitro*, and was responsible for suppression of MMP-9 activity and invasiveness of human MDA-MB-231 breast cancer cells [147].

Sulforaphane interferes with all essential steps of neovascularization from proangiogenic signaling and basement membrane integrity to endothelian cell proliferation, migration and tube formation. Sulforaphane was a potent inhibitor of B16F-10[10] melanoma-induced pulmonary metastasis in C57BL/6 mice by the inhibition of activation of matrix metalloproteinases (MMPs), thereby inhibiting lung metastasis. In animals treated with SFN there is a significant reduction of the marker of pulmonary fibrosis and markers of cell proliferation, in addition to an increase in the survival of metastases bearing animals [148].

The ability to metastasize is one of the characteristics distinctive features of malignant tumors, if there were no metastases, the majority of tumors would already be curable. It is on the front of these "tumors at a distance" that there is the most important challenge to change the fate of those who fell ill with cancer.

5.3.5. Anti-inflammatory activity

An established risk factor for many cancers, chronic inflammation has been accepted as playing a key role in the aetiology of 20% of all cancers including cancers of the stomach, liver and intestine. In normal tissue, after an inflammatory response anti-inflammatory cytokines and proteins are produced to end the inflammation but in chronic inflammation this homeostasis does not occur and the inflammation persists. Studies on the association between inflammation and cancers have identified several key molecules mediating the inflammatory process in tumor formation and promotion. One of these key molecules is the nuclear factor-kB (NF-kB) that is found to be constitutively activated in many human cancers, and leads to induction of cell proliferation, blockade of apoptosis, and promotion of metastasis. Nuclear factor (NF)-kB proteins are present in the cytoplasm in association with inhibitory proteins called

[10] *B16F-10 melanoma cells are highly metastatic and form colonies of tumor nodules in the lungs when administered through the tail vein, promoting lung fibrosis and collagen deposition.*

inhibitors of kB (IkBs). Degradation of IkBs allows traslocation of NF-kB into the nucleus and bind to their cognate DNA binding sites to regulate the transcription of large numbers of genes including antimicrobial peptides, cytokines, chemokines, binding sites to regulate the transcription of large numbers of genes including antimicrobial peptides, cytokines, chemokines, stress response proteins and anti-apoptotic proteins. Inhibition of NF-kB strongly enhances the apoptotic potential of the chemotherapy treatment, an observation that indicates that inhibition NF-kB could be a new adjuvant approach in chemotherapy. Inhibitors of NF-kB seem to become an important weapon in the anticancer therapy. Moreover, several studies claim that, inflammatory cytokines such as interleukin-1 (IL-1), tissue necrosis factor (TNF-α), overproduction of nitric oxide (NO) and prostaglandins (PGE2) have been also associated with chronic inflammation and cancers [149].

Lately SFN has been implicated in protection against inflammation; for example, in rat microglia cells, it has been shown that, SFN significantly reduces lipopolysaccharide (LPS)-induced expression of proinflammatory cytokines IL-1B, interleukin-6 (IL-6) and TNFα, inhibits NO production and attenuates NF-κB p65 translocation to the nucleus. Additionally in the murine BV2 microglia cell line, sulforaphane inhibits LPS-induced activator protein 1 (AP1) and NFκB mediated reporter activity [150].

Another study, *in vivo*, sulforaphane was effective at increasing production of two anti-inflammatory cytokines thought to reduce cancer risk, IL-2 and interferon-γ (INFγ), in tumor-bearing Balb/c mice; moreover, sulforaphane inhibits LPS-induced expression of cyclooxygenase-2 (COX-2) mRNA and protein in macrophages. These effects are mediated, at least in part, by inhibition of NF-κB, CREB and JNK activation. The major transcriptional factors (NF-κB, CREB and AP-1) of COX-2 gene regulation are negatively regulated by sulforaphane which is important because these transcription factors play a critical role in the regulation of a variety of genes involved in inflammatory responses. Cyclooxygenase (COX) is a key enzyme catalyzing the rate-limiting step in the biosynthesis of prostaglandins from arachidonic acid. COX-2 is undetectable in most normal tissues but is rapidly induced by oncogenes, growth factors and cytokines. In effect, growing evidence indicates that COX-2 plays a key role in several

biological processes, such as inflammation and tumorigenesis [151]. This is because the targeted inhibition of COX-2 is a promising approach to inhibiting inflammation and carcinogenesis as well as to prevent cancer and sulforaphane may be an important determinant of clinical response in inflammatory diseases.

5.4. Sulforaphane and melanoma

Melanoma, as well as other cancers, has a long-term and multistep process such as initiation, promotion, and progression stages of carcinogenesis. Theoretically it can be targeted and interrupted along these different stages.

Previous studies have demonstrated that SFN has may physiological effects including anti-cancer, anti-oxidantion, and detoxification in many neoplastic cells (Figure 15). Suggesting that, SFN acts on the process of carcinogenesis by affecting the three phases tumor initiation, promotion and progression phases, and suppressing the final steps of carcinogenesis (angiogenesis and metastasis)(Figure 14).

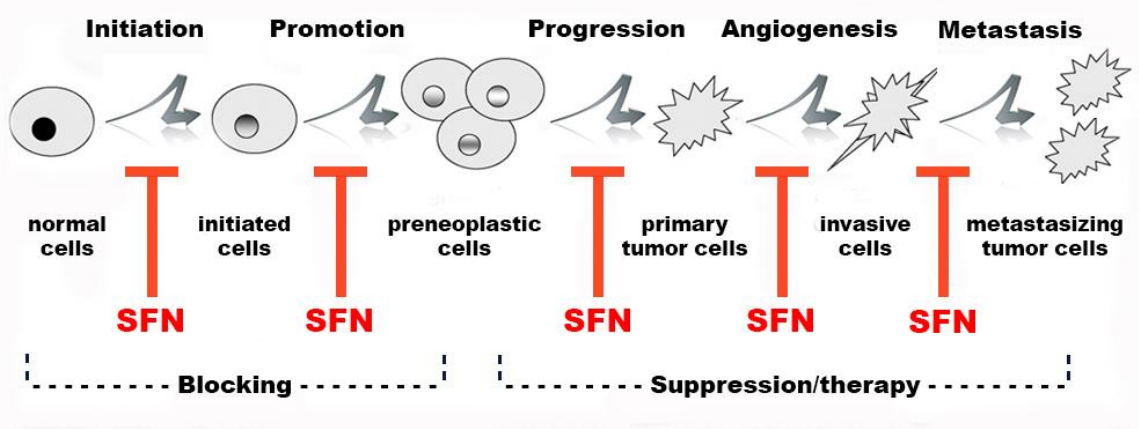

Figure 14. Sulforaphane and process of carcinogenesis.

Several studies have linked the role of SFN to melanoma tumorigenesis and progression. So far, the obtained data are results of studies performed in mouse models of melanoma *in vivo* and *in vitro;* the treatment of SFN causes diverse responses induction of apoptosis in B16F-10 melanoma cells [152], such as presence apoptotic bodies, and by nuclear DNA fragmentation, activation of caspases 3 and 9, Bax, and p53 and the down-regulation of Bcl-2, caspase-8, Bid, and NF-kB. It was demonstrated the involvement of pro-inflammatory cytokines, tumor necrosis factor-

alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-12p40, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in SFN-induced apoptosis [152].

In a murine model, have been studied the inhibitory effects of sulforaphane on the occurrence of lung metastases induced by B16F-10 melanoma cells; in animals treated with SFN it is noted a significant reduction of markers of cell proliferation (metalloproteinases 2 and 9), in addition to an increase in the survival of animals bearing metastases [148]. Cell culture studies performed using, still, B16 and S91 murine melanoma cells showed that sulforaphane inhibited growth and proliferation of cancer cells by downregulating deacetylation enzymes. In addition, have been investigated polymeric drug delivery systems to increase its therapeutic efficacy and to minimize its potential side effects, by albumin microspheres encapsulating SFN, showing that albumin based polymeric delivery system was efficacious and has the potential to enhance the therapeutic effect and anticancer activity of SFN [153]. Other studies demonstrate that SFN inhibited also melanogenesis and tyrosinase expression by affecting the phosphorylated MAP kinase family, showing like an effective skin-whitening agent [154].

Further, it was reported for the first time a combination of a plant-derived flavonoid, quercetin, and sulforaphane on proliferation and migration of melanoma; these compounds in combination significantly suppressed melanoma growth as compared to their individual use in a mouse model [155].

In human skin, in order, topical broccoli sprout extracts (BSE) decrease UVR erythema response and may protect against UVR DNA damage; a pilot study [156], still in progress, is evaluating BSE-SFN in relation to melanoma progression biomarkers, and expression of STAT proteins of atypical melanocytic nevi. Moreover, clinical trials, in progress as well, evaluating SFN in atypical nevi-precursor lesions [157], see if oral administration of freeze dried, powdered broccoli sprouts have any effect on whether moles end up becoming melanoma.

Sulforaphane demonstrated anticancer effects also in melanoma; the findings shown previously, provide for a strong rationale for continuing to investigating the properties of chemoprevention and chemotherapy of SFN in human melanoma. Besides,

experiments of skin cancer but not of melanoma, demonstrated a role of sulforaphane in inhibition of the transcription factor activator protein-1 (AP-1), these data underline that SFN may be an important molecular mechanism in chemoprevention also in squamous cell carcinoma [158].

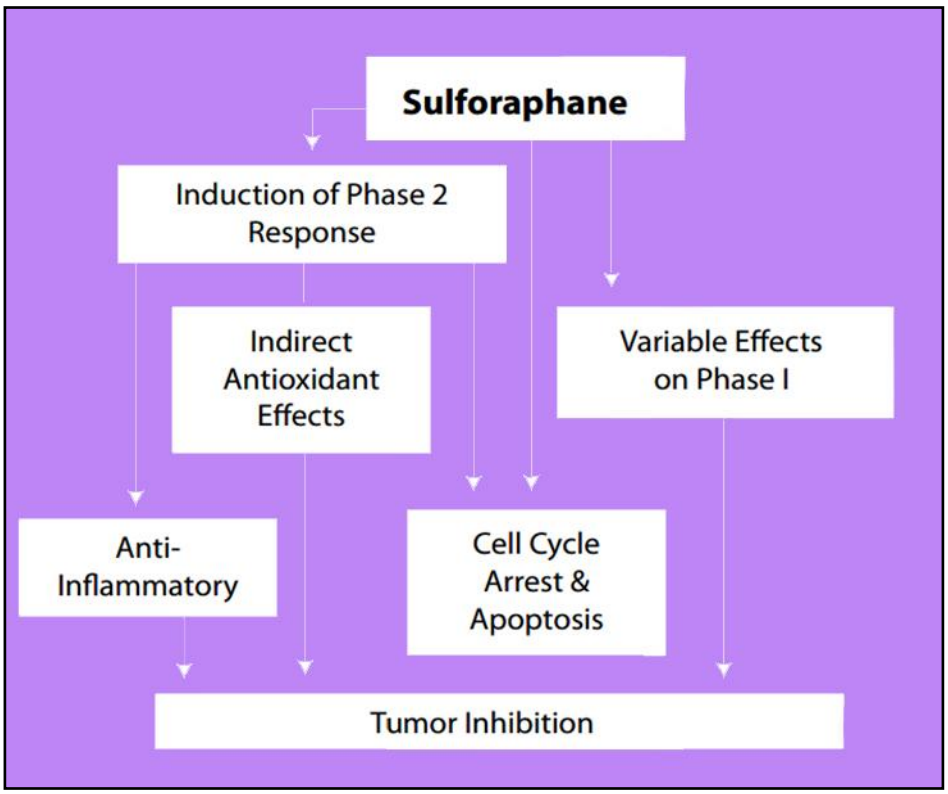

Figure 15. Sulforaphane activity.

6. Neurotrophins and their receptors

The family of growth factor proteins, neurotrophins (NTs) play an important role in influencing several important cellular activities such as proliferation, differentiation and cell growth [159]. In the 1940s, the discovery by Rita Levi-Montalcini of the first growth factor pioneered the field of growth factor research. She identified NGF as a substance secreted from mouse sarcoma tissue that stimulated neuronal survival and neurite outgrowth from chicken ganglia. This provided some of the first evidence of paracrine signaling, whereby cells in one tissue secreted a protein which readily diffused to another tissue to elicit cellular changes in the target tissue [160].

NGF is the prototypic member of the small family of neurotrophins, which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) [161]. Structurally these important proteins are all similar, existing as homodimers and having a duplicate site for binding to receptors 5 [162]. Occasionally a fifth protein is included in this family, namely novel neurotrophin 1 (NNT-1). However NNT-1 is, technically, a cytokine and as it bears no structural resemblance to the other family members (5).

The history of NGF and neurotrophin research is tightly woven into the field of neuroscience, where NGF has been shown to promote survival and differentiation of neurons, outgrowth of neurites, while BDNF is also involved in learning and memory [163]. A critical role for neurotrophins in development of the nervous system has been revealed by gene targeting in the mouse.

All the neurotrophins are initially produced as 30-35 kDa precursor proteins, the prepeptides of the precursor proteins work as target signals for the translocation of the neurotrophin precursor into the lumen of the endoplasmatic reticulum and are thereafter cleaved. The remaining pro-neurotrophins are cleaved intracellularly by furin, a serine protease, or proconvertases at a highly conserved dibasic amino acid cleavage site, releasing the biologically active 12-14 kDa C-terminal product [164]. There are three ultimate fates of intracellular proneurotrophins: intracellular cleavage followed by secretion; secretion followed by extracellular cleavage, or secretion

without subsequent cleavage; uncleaved proneurotrophins signal by binding and activating p75 neurotrophin receptor (p75NTR). The ligand–receptor complex is presumably transported retrogradely, but, so far, this process has not been investigated (Figure 16).

The mature form of NGF is a symmetrical dimer composed of two 12.5 kDa monomers which associate via hydrophobic interactions [165]. Each monomer is composed of approximately 118 amino acids which fold into four distinct loop regions and two b-pleated strands [165]. The loop regions of NGF are largely composed of polar and charged amino acids which face the aqueous external environment, and the majority of the structural diversity, from the other three members of the neurotrophin family, depends on these regions [166]. The mature human neurotrophins share significant structural homology, as approximately 50% of the amino acids are identical for all the members of the protein family and they therefore share the same biochemical characteristics [167]. The structure based alignment of the four human neurotrophins, outlines the sequence similarity of their sequences.

Neurotrophin expression is controlled initially through cellular interactions in tissues that determine sites and levels of neurotrophin gene expression. Each of the neurotrophin genes is regulated through multiple promoters and enhancers, multiple extrinsic stimuli, including Wnt[11] and TGF-b family members, thyroid hormone, steroids and inflammatory cytokines have been shown to control expression from neurotrophin genes [168].

Functionally NGF was originally established as playing a vital role as a growth factor in nerve proliferation and survival. The elevated expression of neurotrophic factors in injured nerves and targets is believed to be essential for successful survival and regeneration of injured neurons. However it was then found to play an important role as a mediator in airway inflammation [169]. The non-neural functions of NGF have gained significant attention in recent years and NGF has been shown to have roles in

[11] *The Wnt signaling pathway is a conserved pathway in metazoan animals. The name Wnt is resultant from a fusion of the name of the Drosophila segment polarity gene*wingless *and the name of the vertebrate homolog,* integrated or int-1

development of the male and female reproductive systems, the endocrine, cardiovascular and immune systems.

Neurotrophins operate in a number of non-neuronal tissues including skin where a complex NT network exists with various cells that are either the target or the source of NTs, thus playing autocrine and paracrine functions [170]. NGF, which is synthesized in the epidermis, is retrogradelly transported to the ganglia to stimulate the release of neuropeptides in the skin, thus favoring cutaneous neurogenic inflammation [171]. Moreover, null mutations of genes in the neurotrophins and their receptors lead to loss/reduction of specific neurons in sensory ganglia; on the other hand, cutaneous overexpression of NTs results in skin hyperinnervation and increase in the number of sensory neurons innervating the skin. Not only NGF is neurotrophic at the skin level, but it possesses a number of biological effects also in cutaneous cells; normal human keratinocytes synthesize and release NGF that can act as a growth factor for these cells [172]. Besided neurotrophins are important for melanocyte migration, viability and differentiation together with other paracrine signalling molecules [173]. Normal human melanocytes are a target of the NTs skin network, because they express all the NTs receptors both *in vitro* and *in vivo* [174]. NGF is implicated in melanocyte survival [175], migration and dendricity, and its synthesis and secretion are enhanced by UV irradiation.

6.1 Members of the neurotrophin receptor family

Neurotrophins mediate their different functions through two structurally distinct classes of transmembrane receptors; the 'common' p75 neurotrophin receptor (p75NTR) which binds all of the neurotrophins with approximately equal affinity, and specific receptor tyrosine kinase receptors called tropomyosin related kinases (Trks) that exhibit specificity in neurotrophin binding.

Whereas p75NTR binds all neurotrophins with low affinity (KD[12] = 10^{-9}M) and specificity, Trk receptors bind neurotrophins with higher affinity (KD = 10^{-11}M) and specificity [176][177]. The three Trk receptors (TrkA, TrkB and TrkC) are type one transmembrane proteins, they are glycoproteins possessing an extracellular ligand-binding domain, which conveys ligand-specificity, a hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase domain; while TrkA binds NGF and NT-3, TrkB binds BDNF, NT-3, NT-4/5 TrkC only binds NT-3 (Figure 16). The interaction of neurotrophins with their appropriate Trk receptor produces receptor dimerisation and autophosphorylation of seven intracellular tyrosine residues. The phosphorylated tyrosine residues allow interaction with cytoplasmic signaling molecules that signal cell growth and differentiation through a variety of pathways.

The propensity of neurotrophins to produce diametrically opposing effects on cell survival has led us to propose a 'yin and yang' model of neurotrophin action. In this simple model, the binary actions of neurotrophins depend on both the form of the neurotrophin (pro- versus mature) and the class of receptor that is activated. Although considerable evidence supports the 'yang' action of neurotrophins, few studies have addressed the 'yin' aspect of the model, in which proneurotrophins, acting through p75NTR, have effects opposite to those of the mature form. It therefore remains to be established whether the yin and yang actions of neurotrophins are equally prevalent. Neurotrophins are known to have different and complex actions, and it will be

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[12] *K_d=[DR]/ [D][R]; K_d is the equilibrium dissociation constant and is the reciprocal of the affinity. K_d is widely used to describe the binding of drugs to a receptor. There is an inverse relationship between K_d and affinity. The smaller is the K_d, the greater is the affinity.*

interesting to determine whether the yin–yang model can be extended to other functions. It should also be noted that the effect of mature neurotrophins is not limited to one direction [178].

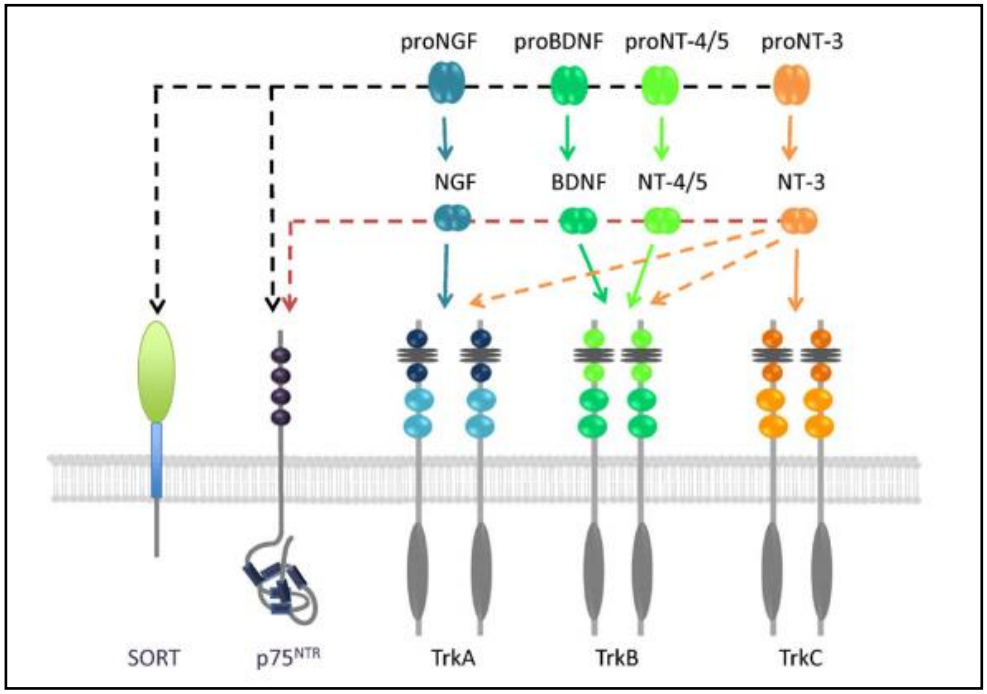


Figure 16. Neurotrophin–receptor interactions [179].

Whereas NGF, BDNF, NT-3, and NT-4/5 as well as their respective precursors (proNGF, proBDNF, proNT, and proNT-4/5) all bind to p75NTR, Trk receptors bind neurotrophins with different specificities and sortilin binds only the precursor forms. Sortilin is a member of the family of Vps10p domain-containing transmembrane proteins and binds mature NGF, proNGF, proBDNF and proNT-3. If sortilin is coexpressed with p75NTR and associates with it, then the affinity of this receptor complex to proNGF is increased. Nothing is known about the signalling pathways triggered by sortilin. It is not clear whether sortilin acts only as a co-receptor of p75NTR facilitating its binding to proNGF or if it can independently trigger a signalling cascade. If such independent function of sortilin exists, it has most probably a proapoptotic character.

6.1.1 TrkA

The cellular responses regulated by neurotrophin-mediated activation of tropomyosin-related kinase (Trk) tyrosine kinase receptors include proliferation and survival, axonal and dendritic growth, assembly and remodeling of the cytoskeleton, membrane trafficking and fusion, synapse formation, function and plasticity [180]. In TrkA, NGF binds to the D5 extracellular domain of the receptor through two areas: a specificity patch that confers ligand specificity, and a conserved binding patch that is found in all Trk receptors.

Human TrkA receptor is encoded by a gene of 23kb located on chromosome 1q21-q22; this gene contains 16 introns of 70bp to 3.3kb and 17 exons of 18 to 394bp, with the 9 first exons encoding for the extracellular part of the receptor [181][182]. The TrkA protein contains 790 amino acids with a molecular weight of 140 kDa [183], and it is composed of an intracellular domain containing a tyrosine-kinase intrinsic activity, a unique transmembrane helix, and an extracellular domain dedicated to NGF binding; this extracellular domain is highly glycosylated, which is essential for the activation of TrkA signalling pathways [184]. NGF binding leads to dimerization of the receptor, resulting in activation of it is intrinsic tyrosyn-kinase activity through trans-phosphorylation of tyrosine residues 670, 674, 675 (in the activation loop of the kinase domain) and, subsequently, tyrosines 490, 751, 785 (outside the kinase domain), which result in activation of downstream signalling pathways [183].

TrkA actives multiple downstream cascades in neurons, including the Ras/MEK/ER pathway, the phosphatidylinositol 3-kinase (PI3 kinase) pathway, the phospholipase C (PLC) pathway, and Src kinase signaling (Figure 17). These pathways have also been studied largely in the context of NGF activation of TrkA and extrapolated to the other Trk receptors. In the last few years, several new proteins downstream of Trk receptors have been described that may contribute to the biological functions elicited by neurotrophins. NGF activation of the TrkA expressed on neurons can activate signaling pathways close to the nucleus through a specific mechanism called retrograde transport.

Once activated by NGF, the TrkA is internalized, mainly through activation of three mechanisms: clathrine-dependent internalization, caveolae-dependent internalization, or macroendocytosis. All these mechanisms are involved in TrkA internalization and depend on : i) the cell type studied, ii) the concentration of NGF, and iii) the amplitude of the signal generated by TrkA activation. Once internalized, only a few number of TrkA receptors are transported close to the nucleus, using early endosomes characterized by expression of the small G protein Rab5 and it is effector EEA1 (Early endosome antigen 1) [185]. Most of internalized TrkA receptors are either degraded through targeting to lysosomes or to the proteasome after ubuquitination, or recycled at the cell membrane [186].

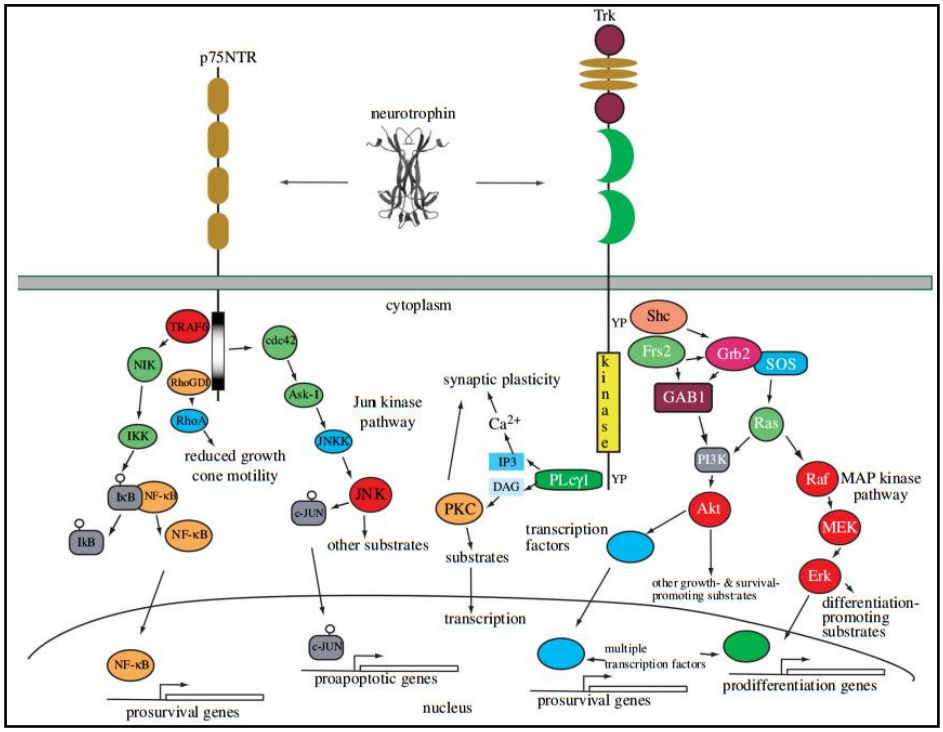


Figure 17. Representation of intracellular pathways mediated by Trk receptors and p75NTR [187].

The Trk receptors control three main ways: 1) Ras activation that involves the activation of MAP kinases that promote neuronal differentiation including the growth of the axon; 2) activation of PI3K through Ras or Gab1 that promotes the survival and growth neurons; 3) activation of PLC-γ which involves the activation of protein kinase Ca2+ dependent (PKC) that promotes synaptic plasticity. Specifically, NGF binds to the extracellular domain of the TrkA receptor and induces its dimerization thereby activating its intracellular tyrosine kinase domain. The phosphorylated tyrosine residues are recognized by proteins through their SH2 (Src homology domain 2) domains. The adapter protein Shc (Src homology 2-containing protein) interacts with the phosphorylated Y490 residue, phosphatidyl-inositol 3-kinase (PI3K) interacts with the phosphorylated Y751 residue, and phospholipase Cγ (PLCγ) interacts with the phosphorylated Y785 residue, thereby initiating three main signaling pathways that have been widely studied in particular in neuronal cells. This extends to driving invasion, metastasis and autophagic cell death in certain cancer cell types.

The p75 regulates three main ways: 1) activation of NF-kB that determines the transcription of several genes, including many promoters of cell survival; 2) activation of Rho, which controls the growth of the growth cone; 3) activation of pro-apoptotic signal mediated by p75ntr which requires the presence of sortilin.

6.1.2 p75NTR

p75NTR (CD271) was the first of the neurotrophin receptors to be identified and it was originally proved that it acted as a low affinity receptor for NGF. Since then it was demonstrated that p75 was able to bind all members of neurotrophin family and it is generally accepted that all neurotrophins are bound with similar (low) affinity with a fast dissociation rate [188]. Neurotrophin binding to p75NTR promotes survival of some cells and apoptosis of other cells, while it also affects axonal outgrowth in neurons both *in vivo* and *in vitro*.

p75NTR is a 75 kDa glycoprotein with four extracellular cysteine rich repeats required for ligand binding, it is a type I transmembrane receptor, with an intracellular domain[13] containing a juxtamembrane region and a death domain (DD) sequence; p75NTR is a member of the death-promoting tumor necrosis factor receptor (TNF-R) superfamily, which also includes the characteristic death receptors TNF-R apoptosis-inducing ligand (TRAIL)-R and Fas/CD95. Although p75NTR binds dimeric neurotrophin ligands, there is some controversy over the oligomeric status of p75NTR and evidence indicates that it may signal as a monomer or as a dimer. Recently, it was demonstrated that p75NTR can form covalent homodimers through a disulphide bond in the transmembrane region. The role of p75NTR is still controversial: in the presence of Trk, p75NTR increases high-affinity NT binding, thereby enhancing the ability of Trk to promote survival. By contrast, in the absence of Trk, p75NTR can induce apoptosis, via it is own signal transduction. These opposing effects are mediated by the association of p75NTR with a number of different receptor partners. Firstly, in absence of Trk neurotrophin receptor, as already mentioned, expression p75NTR can induce apoptosis and cell death; in the absence of TrkA, it has a rapid rate of ligand association. The signaling pathways from the CD271-dependent apoptotic response are incompletely understood but are thought to involve activation of JNK and further downstream, also through p53, Bad and Bim proteins, events such as release of cytochrome c and activation of

[13] *The intracellular domain (ICD) contains a palmitoylation site at cysteine 279, two potential TRAF-binding sites, a Type II death domain, a potential G protein activating domain, and a PDZ domain binding motif.*

caspases 9, 6 and 3. A number of intracellular proteins have been shown to activate JNK and promote apoptosis; these include neurotrophin-receptor interacting MAGE (melanoma-associated antigen) homologue (NRAGE), neurotrophin-associated cell death executor (NADE), TNF (tumour necrosis factor)-receptor-associated factors 2 and 6 (TRAF2 and TRAF6), and neurotrophin-receptor-interacting factor (NRIF). Two different regions of p75NTR have been required for cell death induction, the juxtamembrane region named 'chopper' and the fourth and fifth a-helices of the six a-helices comprising the death domain in p75NTR (Figure 18) [189]. In fact, while the deletion of the death domain does not reduce the cell death, the absence of the 'chopper' domain prevents, death domain would act so as to regulate or modulate the apoptotic pathway initiated by the domain chopper, rather than promote itself. It was demonstrated that the proform of NT, proNGF, binds p75NTR, in association with its co-receptor sortilin (Figure 16). More specifically, sortilin, a member of the vps-10 protein family and a recently described coreceptor with p75NTR for pro-neurotrophins, binds the 'pro' region of NGF, whereas p75NTR binds mature NGF.

The p75NTR-sortilin complex couples with proNGF to induce apoptosis by the activation of an as yet unidentified pathway. In the absence of sortilin, on the other hand, the proNGF is activated to NGF, which then goes to stimulate the receptor TrkA [189][190]. The intracellular domain (ICD) of p75NTR can be released and translocated to the nucleus in response to both NGF and other neurotrophins. Upon binding of NGF to p75NTR, the release of ICD involves two sequential cleavages of the transmembrane protein. The first cleavage occurs in the extracellular part of the protein and is mediated by the metalloproteinase α-secretase, while the second cleavage occurs in the intramembrane region and is mediated by a γ-secretase. The final result is the release of the intracellular domain, which traffics to the nucleus and acts as a transcriptional regulator. It has been shown that the release of ICD does not require a previous internalization step and that it can take place directly at the cell surface and does thereby represent a novel neurotrophin-mediated signaling pathway (Figure 18) [191].

p75NTR signaling can also lead to downstream activation of nuclear factor-kappa B (NF-κB) which promotes cell survival through upregulation of anti-apoptotic genes such as cFLIP, which interferes with the activation of initiator caspase-8, the Bcl-2 family member Bcl-XL, and inhibitor of apoptosis proteins (IAPs) XIAP and cIAP1/2. The activation of NF-κB requires several proteins, including TRAF6, p62, interleukin-1 receptor-associated kinase (IRAK), and receptor-interacting protein-2 (RIP2). Upon activation in response to neurotrophins, NF-κB translocates to the nucleus and triggers the expression of Hes1/5 to modulate dendritic growth [193]. Another co-receptor for p75NTR is the glycolipid-anchored Nogo receptor (Nogo-R) which binds myelin based growth inhibitors, including Nogo-A (Nogo isoform), myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMGP) and restricts axonal regeneration by promoting growth cone collapse of injured neurons.

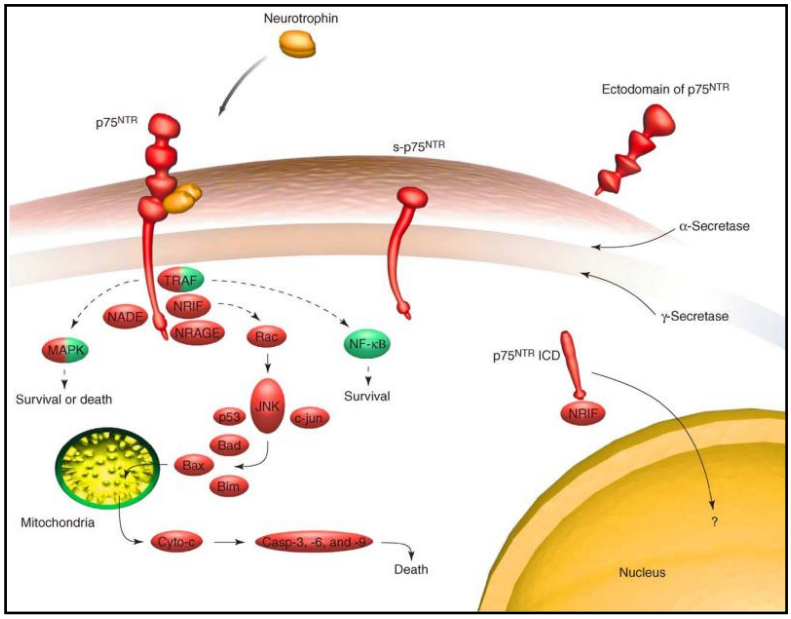


Figure 18. p75NTR activates signaling pathways regulating neuronal death and survival [192].

The short p75NTR variant (s-p75NTR) lacking of a ligand binding domain. p75NTR arises from alternative splicing of exon III of the p75NTR locus, this variant, which is expressed in several neuronal tissues, is unable to bind NTs.

This effect is known to necessitate co-expression with p75NTR and the transmembrane protein LINGO-1 because both are required for a response to myelin. Upon ligand binding, p75NTR binds to the Rho guanine dissociation inhibitor (Rho-GDI), relieving RhoA from its inhibition, an activation cascade distinct from that in pro-apoptotic stimulation [190]. The ability of Trk and p75NTR receptors to have different binding sites and affinities to particular neurotrophins determines both their responsiveness and specificity. The ratio of receptors is important in dictating the numbers of surviving cells, and interactions between p75NTR and Trk receptors provide greater discrimination between different neurotrophins. When the two receptors are co-expressed, the rate at which NGF can associate with TrkA increases by about 25-fold [194], resulting in the generation of high-affinity binding sites. Consistent with this, Trk activation that occurs in response to low concentrations of neurotrophin is enhanced in the presence of p75NTR. The p75NTR receptor binds physically to the Trk receptors; it has been reported that upon binding of NGF to p75NTR a bend of the entire NGF molecule is induced, making it difficult for another p75NTR-molecule to interact with the NGF dimer. The TrkA binding site on NGF, composed of the central β-sheet, is unaffected by the conformational changes induced by p75NTR, since these changes are localized to the distal loops of NGF (Figure 19) [196].

Importantly, although p75NTR is widely expressed during development, it is down regulated in many cells of the adult organism and only re-expressed in conditions involving neuronal injury, such as neurodegenerative disease states. Several neurological diseases, deficits and syndromes have been related to p75NTR expression; these include Alzheimer's disease, amyotrophic lateral sclerosis, neural crest tumors, stroke, ischemia and excitoxicity, cerebellar Purkinje cell degeneration, schizophrenia, bronchial asthma and some autoimmune disorders [190]. The expression of p75NTR in various types of cancer have also been widely studied. The largest study involved 1150 tumours and fetal, adult and normal tissue; although p75NTR expression was not correlated to a cancerous phenotype, it was discovered to be a useful marker in specific non neural mesenchymal tumours such as dermatofibrosarcoma and rhadomyosarcoma. In addition, in some cancers, such as

prostate and bladder carcinoma, p75NTR acts as a tumour suppressor and progression from benign to metastatic tumours is associated with a decrease in CD271 expression. Literature data suggest that p75NTR has a role as a tumour suppressor in pancreatic cancer. In a study of 56 human primary pancreatic cancers it was found that the expression of p75NTR was associated with a longer overall survival. In studies of pancreatic cancer cell lines it was found that NGF exhibited both stimulatory and inhibitory effects, depending on the expression levels and ratio of TrkA to p75NTR [196].

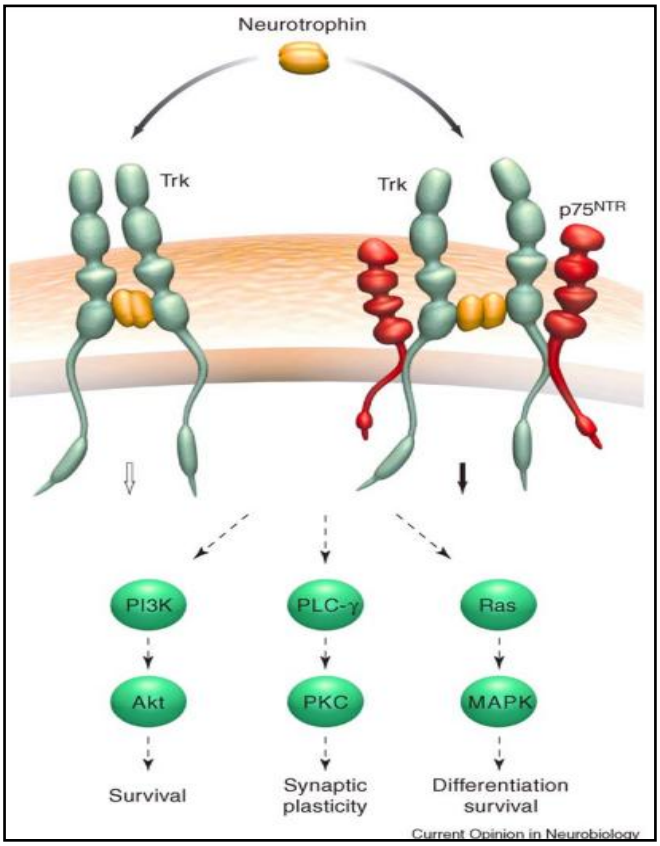


Figure 19. Trk and p75NTR.

Coexpression of p75NTR with Trk refines ligand specificity and affinity, and modifies the association with cytosolic adaptor proteins. As a consequence, p75NTR–Trk dimer complexes might possess signaling activities that are different from those of a Trk dimer.

6.2 p75NTR and melanoma

The relevance of neurotrophins to tumor biology is not well-characterized, although there are clear links: NGF was originally purified from a sarcoma, TrkA was discovered in a human colon carcinoma biopsy and p75NTR was purified from a human melanoma cell line [197][198]. Altered neurotrophin signaling has since been implicated in the development and progression of a number of cancers, including neuroblastoma, melanoma, papillary thyroid carcinoma, prostate cancer and breast cancer [199].

Malignant melanomas, particularly those that advance to the brain, undergo progressive changes during their pathogenesis. Among the phenotypic changes that occur during metastatic melanoma progression, differences in the expression of receptors for paracrine growth factors and in the production of various autocrine growth factors are important. Although the significance of these factors in modulating the malignant properties exhibited by melanoma cells remains largely unknown, they are thought to be relevant in allowing malignant cells to survive in unusual compartments such as the brain. During malignant progression, melanoma cells show progression-associated increases in the expression of p75NTR, as witnessed by in situ examination of p75NTR levels in advanced stages of malignant melanoma [200]. The expression of p75NTR in melanoma has been associated with enhanced brain metastasis, independently of Trk. It was investigated the role of NTs and their receptors on melanoma invasiveness; using a migration assay, it was demonstrated that all NTs stimulate melanoma cell migration, and this is significantly higher in metastatic melanoma than in primary melanoma cell lines [201]. This seems to correlate to the work of Marchetti *et al.*, who have emphasized the role of NT in metastatic melanoma; effectively, blocking p75NTR inhibits NT-induced melanoma migration, high lighting the role of the low-affinity NT receptor [202].

p75NTR, similar to S100, is not a specific marker for melanocytic neoplasms and, therefore, in making an accurate diagnosis is necessary to rely on histologic features, immunohistochemistry and clinical characteristics. Data show that p75NTR is a useful ancillary marker to S100 in the diagnosis of spindle cell melanoma and desmoplastic

melanoma with a higher sensitivity. Although data of literature reveal p75NTR expression in melanoma to be superior to S100, these antibodies should be used together to increase their diagnostic sensitivity and to assure more reliable and accurate diagnosis [203].

The expression level of p75NTR was found to correlate with a number of properties:

- the ability of the cells to metastasize in nude mice;
- the extent of invasion of extracellular matrix stimulated by NGF;
- the ability of the cells to grow under stress conditions (normal melanocytes did not grow under these conditions).

Recent results show that melanoma cells proliferate through autocrine NT stimulation. K252a significantly reduces melanoma cell proliferation by inhibiting Trk phosphorylation. NT appeared to be important for melanoma cell migration *in vitro*, with special respect for metastatic cell lines. The migratory phenotype is necessarily dependent on the presence of both the high- and low-affinity NT receptors; cells treated with p75NTR small interfering RNA (p75NTR siRNA) fail to respond to NT stimulation, similarly, the administration of K252a blocks melanoma cell migration, confirming that NT stimulate melanoma cell migration and invasion cooperation of the low- and high-affinity receptors [204]. It has been hypothesized that the stubborn recurrence of cancer following a primary response to treatment is due to the survival of a subset of cancer cells that display an intrinsic resistance to treatment-induced cell death. The existence of cancer stem cells (CSCs), characterized by a less differentiated status, lower immunogenicity and resistance to immune rejection may be the source of cancer relapse and resistance to therapy. It is important to note that the term CSCs is more of a functional definition created to define a subgroup of cancer cells which can self-renew, initiate tumors, and differentiate into a heterogeneous progeny that partially maintains similarity to the original tissue from which they derived. The concept of CSCs and the hierarchical model of tumorigenesis have implications that may help advance the understanding of tumor biology and the development of more effective anti-cancer treatments.

A broad spectrum of melanomas that included primary cutaneous lesions as well as nodal, in-transit, and cutaneous metastasis were used to profile expression of CD271. CD271+ melanoma cells lack expression of TYR, MART and MAGE in 86%, 69% and 68% of melanoma patients respectively suggesting why T cell therapies directed at these antigens usually result in only temporary tumor shrinkage [205]. Boiko *et al*. demonstrated that p75NTR, which is considered a neural crest stem cell marker, allows the identification and isolation of melanoma cancer stem cells. Tumors derived from transplanted human CD271+ melanoma cells were capable of metastasizing *in vivo*, indeed [205]. Specifically, a CD271+ cell population isolated directly from aggressive melanoma samples and transplanted into T-, B and natural-killer cell deficient mice induce in melanoma formation at a markedly higher rate than CD271- cells [206]. Interestingly, CD271+ melanoma cells have a less differentiated phenotype. Melanoma stem cells may engage in several survival-promoting and virulence-conferring mechanisms, including chemoresistance and immune evasion and modulation. Moreover, melanoma stem cells may participate in “vasculogenic mimicry” melanoma progression, and may play a role in tumor metastasis. Although melanoma stem cells have not been definitively linked to epithelial-mesenchymal transition, the plasticity of the cells that participate in epithelial-mesenchymal transition and the stem cell model of cancer growth all suggest that primitive melanoma cells may play a role in these important processes [207].

Aims of the thesis

Human malignant melanoma is an highly aggressive and incurable cancer due to intrinsic resistance to apoptosis reprogramming proliferation and survival pathways during tumor progression. Several studies have shown that many natural dietary compounds can potently modulate various molecular targets, conducting prevention of cancer initiation, promotion and progression. Sulforaphane (SFN) is a compound derived from cruciferous plants.

Numerous studies have shown the antitumor effect of SFN in an adult tumor *in vitro* and/or *in vivo* but no reports on the different antitumor effects of SFN in human melanoma cells are available so far. However, in the literature, there is a single study that assesses the effect of SFN in human cell lines of melanoma (Me-18). The study analyzed the action of SFN together with the 2-oxoexyl isothiocyanate and showed that the two substances are able to induce growth arrest in dose-dependent manner, followed by apoptosis. Melanoma is a complex tumor and is especially important to understand the effect of several compounds in relation to the mutation present.

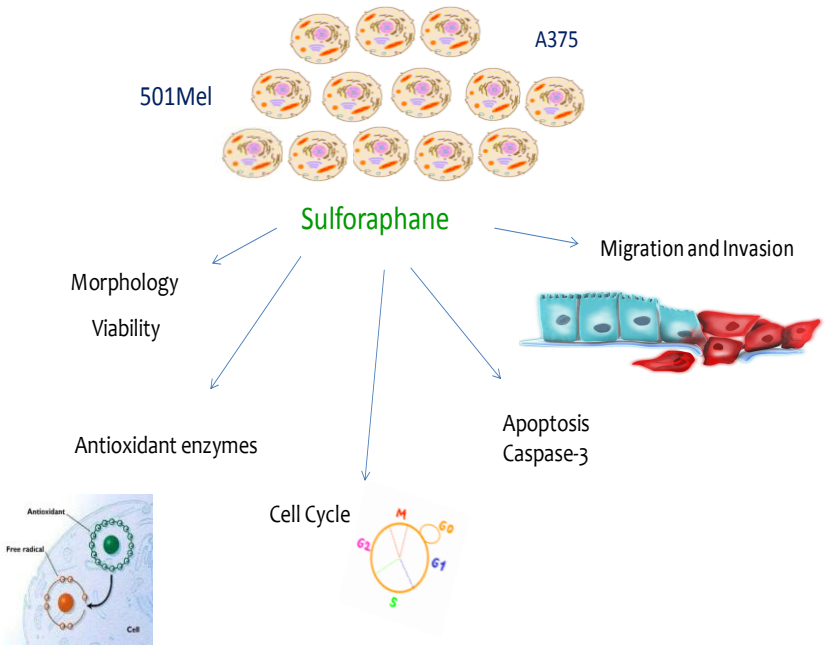
The current study aims to fill this gap. The goal of the proposed research was to investigate the antitumor potential of SFN, characterize the molecular basis of its activity and identify the mechanism by which it could interfere with the proliferation.

In particular I evaluated the effects of sulforaphane on human primary and metastatic melanoma cells in terms of:

i) Cell proliferation
ii) Cell cycle and apoptosis
iii) Antioxidant effect
iv) Migration and invasion
v) Pro-survival or apoptotic receptors involved
vi) Mechanisms of resistance

The results obtained allow to better understand the mechanisms of action of SFN with regard on the regulation of apoptosis and cell cycle and to explore the therapeutic potential in primary and metastatic melanoma cells. Moreover, all this could open newer avenues for the prevention of tumor progression and/or treatment of human malignancies.

Experimental designed:



Materials and methods

1. Reagents

Sulforaphane (SFN), dimethyl sulfoxide (DMSO), propidium iodide (PI), ponceau red and collagenase were purchased from Sigma Aldrich; trypan blue, Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, L-glutamine 100X, fetal bovine serum (FBS), penicillin-streptomycin, 0,25% tripsin-EDTA, medium 254, human melanocyte growth supplement-2 PMA-Free (HMGS-2), gentamicin, RNAse-A, DEPC-water, collagen I bovine, Hank's Balanced Salt Solution (HBSS), trypsin neutralizer solution and 0,025% trypsin/EDTA solution were obtained from Gibco Life Tecnologies.

Other chemical supplements and compounds were purchased from several companies as follows: CellTiter-Glo luminescent cell viability assay (Promega), human β-nerve transforming growth factor (β-NGF) (Cell Signaling), turbo DNA-free (Ambion, Life Technologies), FITC Annexin V apoptosis detection kit I (BD Pharmingen), TRIzol reagent (Ambion, Life Tecnologies), blue metilene (Merck), BCA protein assay kit and NP-40 (Pierce, Thermo scientific), ECL Prime western blotting detection reagents (Amersham), stripping buffer stripAblot and Tween 20 (EuroClone), protease inhibitor cocktail (Roche), PageRule prestained protein ladder (Fermentas Part of Thermo Fisher Scientific), Superscript III Reverse Transcriptase (Life Technologies) iQ™ SYBR® green supermix (Bio-Rad). Acetic acid, tris, chloroform, isopropanol, ethanol and methanol were obtained from VWR International. Moreover milk (non fat dried milk power), sodium chloride (NaCl) glycine, glycerol, ammonium perdulfat (APS), 1,4-Dithio-DL-threit(ol) (DTT), acrylamide (29:1), tetrametiletilendiammina (TEMED) were obtained from AppliChem (Delchimica).

2. Cell lines and treatments

A375 are human malignant melanoma cell lines, they were obtained from American Type Culture Collection (ATCC). The cells are maintained in a DMEM medium containing 10% FBS, 1% L-glutamine (200 mM) and 1% penicillin-streptomycin (10.000 units/ml). 501Mel cell lines were obtained from surgically removed metastases in melanoma patients at the National Cancer Institute, Milan, Italy. The cells are maintained in RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine (200 mM) and 1% penicillin-streptomycin (10.000 units/ml). HEMa-LP are human epidermal melanocytes isolated from lightly pigmented (LP) adult skin; they were obtained from Invitrogen (Life Technologies). HEMa-LP were propagated in Medium 254 supplemented with HMGS-2 with 10 µg/ml gentamicin.

Sulforaphane was dissolved in DMSO and stock solutions (40 mg/ml) were freshly prepared and added to the cell cultures to obtain the indicated final concentrations (0.1, 1, 2, 5 and 10 µg/ml) [49]. DMSO concentration was used at 0.001% and the same dose was used as a negative control.

Human β-NGF was dissolved in sterile PBS to 50 µg/ml stock concentration; to determine the effects of NGF on cultured melanoma cells, serial concentrations (10, 50 and 100 ng/ml) of purified human β-NGF has been tested. Cells were serially passaged as monolayer and cultured in T25 or T75 culture flasks and 24 or 6 well plates (Corning). They were cultured in a humidified atmosphere with 5% CO_2 at 37°C, until reaching approximately 70% confluence, and then treated witch different amounts of compounds as indicated.

3. Cell proliferation and morphology analysis

Cells (A375, 501Mel and HEMa) were seeded at a density of $3x10^5$ cells/well onto 6-well plates and after 24h a range of concentrations of SFN were added to each well at final concentrations (0.1, 1, 2, 5 and 10 μg/ml). As controls, one group received no treatment, another group was treated with the same concentration of DMSO (0.001%) as used in the corresponding experiment. Cell lines were examined after incubations and photographed under a phase contrast microscope at 10X magnification (Nikon Eclipse) to observe morphological changes.

For viability, cells (A375, 501Mel and HEMa) were plated at density of $5x10^3$ per well in a volume of 100 μL on 96-well white plates (Cellstar, Greiner) for tissue culture and were treated with different concentrations of SFN, hβ-NGF and both simultaneously for 24, 48 and 72 hours. Sulforaphane was added at final concentrations of 0.1, 1, 2, 5 and 10 μg/ml; human β-NGF was added at final concentration of 10, 50 and 100 ng/ml, while when used together, final concentrations were 2 μg/ml SFN and 10 ng/ml NGF. After times of incubation, the viability was determined, by CellTiter-Glo Luminescent Cell Viability Assay, based on quantification of the ATP indicating the presence of metabolically active cells, through Luminoskan Ascent (Thermo Labsystems). The results were expressed as percentage, based on the ratio of the absorbance of treated cells to that of controls (100%). Percent survival vs. control is reported as the mean (+/-) standard deviation.

4. Cell cycle analysis

Cells at $3x10^5$ cells/well were maintained on 6-well plates and cultured under the conditions described above. They were incubated with SFN for 24, 48 and 72 hours. After incubation, cells were trypsinized, washed with ice-cold phosphate-buffered saline (PBS), and fixed in 70% ethanol overnight. Then cells were re-suspended in PBS plus RNAse-A (30 mg/mL) and incubated at 4°C for 5 minutes. After adding FACS

buffer (PBS + 2% FBS) and PI (1 mg/mL), they were incubated at 4°C for 30 min and analysed immediately after incubation. The number of cells in each phase was measured using FACSCalibur flow cytometer with CellQuest software (Becton Dickinson). Each experiment per sample was determined by recording 20,000 events. The analyses were performed in triplicate for statistical evaluation.

5. Detection of apoptosis

5.1 Flow cytometry analysis for measurement of sub-G_1 phase

Cells were prepared as describes above for cell cycle analysis. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G_1 peak in the cell cycle pattern. Each experiment per sample was determined by recording 20,000 events. The analyses were performed in triplicate for statistical evaluation.

5.2 Apoptotic Cell Death Assay

A hallmark of apoptotic cells is the translocation of the membrane glycerophospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Annexin V is a 35-36 kDa Ca2+-dependent phospholipid-binding protein that has high affinity for PS. Annexin V is very sensitive and detects PS exposure occurring early in the onset of apoptotic cells. PS redistribution in a plasma membrane was measured by the binding of FITC-annexin V according to the manufacturer's protocol. Cells were plated at $3x10^5$ on 6-well plates and 24 hours later cells were treated with SFN for for times established.

Upon completion of the treatment with SFN, floating cells were collected combined with trypsinized adherent cells, then they were stained with Annexin V and PI according to the following protocol:

- cells were washed two times twice with PBS and resuspended cells in 1X Binding Buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$).
- cells were transferred 100 µl of the solution (1 x 10^5 cells) to a 5 ml culture tube.

- 5 µl of FITC Annexin V and 5 µl PI (100µg/ml) were addedin each tube.
- samples were vortexed and incubated for 15 min at RT (25°C) in the dark.
- 400 µl of 1X Binding Buffer to each tube were added.

Analyzed by flow cytometry within 1 hour.

The following controls are used to set up compensation and quadrants:

a) unstained cells;

b) cells stained with FITC Annexin V (no PI);

c) cells stained with PI (no FITC Annexin V).

Cells were subjected to FACS analysis, percentages of annexin V-FITC/PI+ and annexin V FITC/PI- cells were determined with CellQuest software. For this assay 10.000 events were counted. The analyses were performed in triplicate for statistical evaluation.

5.3 Western blotting analysis

Procedure:

A. Sample preparation

1. Lysis buffers
2. Protease and phosphatase inhibitors
3. Preparation of lysate from cell culture
4. Determination of protein concentration
5. Preparation of samples for loading into gels

B. Electrophoresis

1. Preparation of PAGE gels
2. Molecular weight markers
3. Loading samples and running the gel

C. Transfer of proteins and staining (Western blotting)

1. Visualization of proteins in gels
2. Transfer
3. Visualization of proteins in membranes: Ponceau S Stain
4. Blocking the membrane
5. Incubation with the primary antibody
6. Incubation with the secondary antibody
7. Development methods

5.3.1 Electrophoresis Solutions and Western Blotting Solution:

RIPA (Radio immunoprecipitation assay) Buffer:

- ✓ 50mM Tris-HCl pH 7.4
- ✓ 150mM NaCl
- ✓ 1% NP-40

Running gel (10% Acrylamide) (gel volume 20ml):

- ✓ 7.9ml H_2O
- ✓ 6.7ml Acrylamide:N,N'-methylene-bis-acrylamide (29:1) in H_2O deionized
- ✓ 5.0ml Tris-HCl 1.5M pH 8.8
- ✓ 0.2ml 10% SDS
- ✓ 0.2ml 10% APS (ammonium persulfate)
- ✓ 0.008ml TEMED (N,N,N',N'-tetramethylethylenediamine)

5% Stacking gel (gel volume 5 ml):

- ✓ 3.4ml H_2O
- ✓ 0.83ml Acrylamide: N,N'-methylene-bis-acrylamide (29:1) in H_2O deionized
- ✓ 0.63ml Tris-HCl 1M pH 6.8
- ✓ 0.05ml 10% SDS
- ✓ 0.05ml 10% APS (ammonium persulfate)
- ✓ 0.005ml TEMED

5X Loading buffer

- ✓ 0.25M Tris-HCl (pH 6.8)
- ✓ 10% SDS
- ✓ 0.5% Bromophenol blue
- ✓ 50% Glycerol
- ✓ 0.5 M Dithiothreitol (DTT) (add DTT from a 1M stock before the buffer is used)

Protein Markers

- ✓ Standard molecular-weight markers are commercially available PageRule Prestained Protein ladder (10 – 170 kDa).

1X Tris-glycine electrophoresis buffer

- ✓ 25mM Tris
- ✓ 250mM Glycine
- ✓ 0.1% SDS

Blotting buffer

- ✓ 25mM Tris
- ✓ 192mM Glycine

- ✓ 0.01% SDS
- ✓ 20% Methanol

Wash Buffer (2X TBS/T)

- ✓ 300 mM NaCl
- ✓ 20mM Tris
- ✓ 0.1% Tween 20

Ponceau S staining solution

- ✓ 0.2% Ponceau Red
- ✓ 3% Acetic Acid
- ✓ Adjust the volume with H_2O

Blocking Solution

- ✓ 5% w/v Milk Powder
- ✓ 1X TBS/T

Antibody diluition Buffer

- ✓ 1% w/v Milk Powder
- ✓ 1X TBS/T

Western blotting is an analytical technique for detection of specific proteins in a given sample. Cells were treated with 0, 2 and 5 µg/mL sulforaphane for 24, 48 and 72h, harvested by trypsinization, and washed with ice-cold PBS. Whole-cell extracts were isolated using a protein extraction buffer (RIPA) with added protease inhibitor cocktail. Cell lysates were quantified by a Bicinchoninic Acid (BCA) protein assay kit. All samples containing equal amounts of cell lysate proteins (70 µg), after the addition of the loading buffer full of 1M DTT, were denatured by boiling for 5 min and they were loaded on a 10% denaturing SDS-PAGE. The SDS-PAGE was finished when the bromophenol blue band has left the separating gel which occurred approximately after further 45 min applying a voltage of 150 V (~60 mA) to the chamber. Therefore, a 0.2 µm nitrocellulose membrane (PROTRAN, Whatman, Sigma) was cut to a rectangle with the similar side length as the separating gel. The stacking gel was removed from the separating gel. Finally, three layers of wet 3M

filter paper and one fiber sponge completed the transfer sandwich which was transferred to the gel holder cassette and put in the MiniProtean® 3 Cell (Biorad) in blotting buffer. The electrophoretic transfer was performed at a constant amperage 400mA for 2h at 4°C. The membrane was quickly rinsed with distilled H_2O and protein bands detected with a Ponceau-S stain. After washing twice with H_2O, the membrane was incubated with gentle agitation throughout all steps. After bathing in blocking buffer for 1h at RT to reduce unspecific binding, the membrane was incubated with primary antibody (anti-procaspase-3/9, table 4), diluted in antibody dilution buffer for 1h at room temperature. Then, the membranes were washed (5 washings with 5 min incubation) with TBS-Tween (0.1%) and incubated with appropriate secondary antibodies HRP-conjugate (Table 4) diluted in antibody dilution buffer for 1h at RT. Anti-β-actin antibodies as loading controls, it is good loading controls because its levels rarely change in cells. The detection of the HRP conjugated antibodies on the membrane was done after five washing steps using of the ECL Prime western blotting detection and visualized on Amersham hyperfilms ECL after automatic development in CawoMat 2000 IR. Densitometry analysis was performed with Image J software (JAVA); the results were normalized before with β-actin in each sample and subsequently with the single control.

Antibody	Ab diluition, source
β-actin	mouse 1:5000 SelleckBio
Caspase-3	rabbit 1:1000, Cell Signaling Technology
Caspase -9	rabbit 1:1000, Cell Signaling Technology
Anti-rabbit IgG-peroxidase	1:80000, Sigma Aldrich
Anti-mouse Polyvalent Immunoglobulins (IgG, IgA, IgM) Peroxidase	1:10000, Sigma Aldrich

Table 4. List antibodies.

5.4 Caspase-3 activation

Cells at $3x10^5$ cells/well were maintained on 6-well plates and cultured under the conditions described above. They were incubated with 2 μg/ml SFN for 24, 48 and 72 hours. After incubation, cell were harvested and fixed-permeabilized using the PE-conjugated monoclonal active caspase-3 antibody apoptosis kit 1 (BD), according to manufacturer's instructions.

Solutions:

- Cytofix/Cytoperm solution is used for the simultaneous fixation and permeabilization of cells prior to intracellular staining. The solution contains neutral pH-buffered saline, saponin and 4% (w/v) paraformaldehyde.
- Perm/Wash Buffer is used to permeabilize cells for intracellular staining; it also serves as an antibody diluent and cell wash buffer. The buffer contains fetal bovine serum (FBS), sodium azide and saponin. Dilute 1:10 in distilled H_20 prior to use.
- The PE conjugated monoclonal active caspase-3 antibody has been pre-titrated for use at 20 μl/$1x10^6$ cells in flow cytometry.

Procedure:

a) Wash cells twice with cold PBS, then resuspend cells in Cytofix/Cytoperm solution at a concentration of $1x10^6$ cells/0.5 ml.

b) Incubate cells for 20 min on ice.

c) Pellet cells, aspirate and discard Cytofix/Cytoperm solution; wash twice with Perm/Wash buffer at a volume of 0.5 ml buffer/$1x10^6$ cells at room temperature.

d) Resuspend cells in 100 µl Perm/Wash Buffer plus 20 µl PE-conjugated rabbit anti-active caspases-3 antibody and incubate for 30 min at room temperature.

f) Wash each sample in 1.0 ml Perm/Wash Buffer, then resuspend the sample in 0.5 ml Perm/Wash buffer and analyze by FACS with CellQuest software.

Each experiment per sample was determined by recording 10,000 events. The analyses were performed in triplicate for statistical evaluation.

6 Real-time RT-PCR analyses

6.1 RNA extraction

Cells at $3x10^5$ cells/well were maintained on 6-well plates and cultured under the conditions described above. They were incubated with SFN (2 μg/ml) for 24, 48 and 72 hours. Total RNA was isolated with monophasic acidic phenol guanidine isothiocyanate solution (TRIzol Reagent) in the following way. After treatment, at the cells was added 1 mL Trizol directly in the plate 6 well (1 ml in the culture dish per 10 cm^2 of culture dish surface area). The mixture was passed through a 21-22 G needle (Terumo, Italy). To separate the phases, 0.2 ml chloroform per ml of TRIzol was added. The samples were vortexed vigorously for 15 seconds and incubated them at room temperature for 2 to 3 min to permit the complete dissociation of nucleoprotein complexes. The samples were centrifuged at no more than 12,000 x g for 15 min at 4°C to separate the aqueous and inorganic phases. RNA remains exclusively in the aqueous phase. The aqueous, upper phase was recovered, mixed with 0.5 ml of isopropanol per ml of TRIzol Reagent, the samples were incubated at 15 to 30°C for 10 min and centrifuged at not more than 12,000 x g for 15 min at 4°C. The pellet was washed once with 75% ethanol, centrifuged again for 5 min at 4°C, air-dried and dissolved in 50 μl DEPC treated water. Total RNA was stored at -80°C.

To check purity and concentration the RNA were examined spectrophotometrically (Spectrostar Nano spectrophotometer, BMG Lab Tech) by measuring the A_{260}/A_{280} ratio. The ratio of the absorbance values at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA (pure RNA has an A_{260}/A_{280} ratio of 1.8 to 2.1).

Concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer, at A_{260} 1 unit corresponds to 40 μg of RNA per ml.

The concentration in μg/ml can be calculated from the absorbance value:

A_{260} x dilution factor x 40 μg/ml

6.2 DNAse treatment of total RNA sample

To ensure that contamination with genomic DNA did not contribute as templates at the PCR reactions, the RNA was treated with kit Turbo DNA-free as described by manufacturer. For this reaction were used 5 µg of RNA diluted in DECP water.

Procedure Overview:

- ✓ Add 0.1 volume 10X DNase Buffer and 1 µL TURBO DNase to the RNA, and mix gently.
- ✓ Incubate at 37°C for 20–30 min.
- ✓ Add resuspended DNase inactivation reagent (typically 0.1 volume).
- ✓ Incubate 5 min at room temperature, mixing occasionally.
- ✓ Centrifuge at 10,000 x g for 1.5 min and transfer the RNA to a fresh tube.

6.3 cDNA Synthesis and Quantitative Reverse-Transcription PCR (qRTPCR)

RNA was transcribed into cDNA using Superscript III Reverse Transcriptase; 1 µg total RNA of each sample was mixed with 1 µl Oligo dT20 (50 µgM) and 1 µl 10 mM dNTP mix. DEPC-treated water was added up to the final volume of 10 µl. Each sample was incubated at 65°C for 5 min for primer hybridisation and chilled on ice for at least 1 min. Samples were briefly centrifuged and 10 µl of the cDNA Synthesis mix were added (10X RT Buffer, 25 mM MgCl2, 0.1 M DTT, RNaseOUT (40 U/µL), Superscript III RT (200 U/µL)). Contents of the tubes were mixed and incubated at 50°C for 50 min to cDNA synthesis. The reaction was termined by incubating at 85°C for 5 min, in order to inhibit the enzymes. To each sample was added 1 µl (2 U/µL) of RNAse H to remove RNA followed by incubating the tubes at 37°C for 20 min (Figure 20). The cDNA was stored at -20°C. The cDNA synthesis was verified by amplification of the actin gene via PCR with control primers provided by the manufacturer.

Quantitative PCR reactions were performed on an I-cycler iQ Real-Time PCR (Bio-Rad), using iQ™ SYBR® Green Supermix, High-Profile 96-Well Semi-Skirted PCR Plates (Bio-Rad) and microseal (Bio-Rad) optical adhesive plate sealers. All reactions were performed in triplicate, with controls (contain all of the amplification reagents except template cDNA extracted from the samples; instead of template cDNA a

corresponding volume of nucleic acid free water is added to the reaction). Each 20 µl PCR reaction contained 12,5 ng cDNA, 2x qPCR mix, contains dNTPs, iTaq™ DNA polymerase, MgCl2, SYBR® Green I, enhancers, stabilizers, fluorescein, and finally primers diluted to a final concentration of 500 nM. Detection system was realized with reaction mode set at 95°C (3 min), followed by 40 cycles of 94°C (10 s), 61°C (10 s) and 72°C (20 s); then the melt curve has been made with 64 cycles starting at 68°C (10 s) with 0.5°C increments per cycle. The primers for β-actin, GAPDH, NQO1 and HO-1, TrkA and p75NTR were designed with Perl primer software with NCBI EntrezGene reference sequences as templates (Table 5) and synthesized by Sigma.

To assure specificity, primer pairs were designed to span across two neighboring exons and detection of a single peak in dissociation curve analysis. The number of times of changes for a given gene is derived from the equation $n = 2^{-\Delta\Delta Ct}$.

The ΔΔCt method (Livak & Schimittgen, 2001) was employed to quantify the amplification-fold difference between treatment and control groups, with Ct value of target genes being adjusted to individual housekeeping gene (GAPDH, β-actin) whichever expression was not affected by treatment protocols. Measurements were done in triplicate with variability < 0.5 Ct.

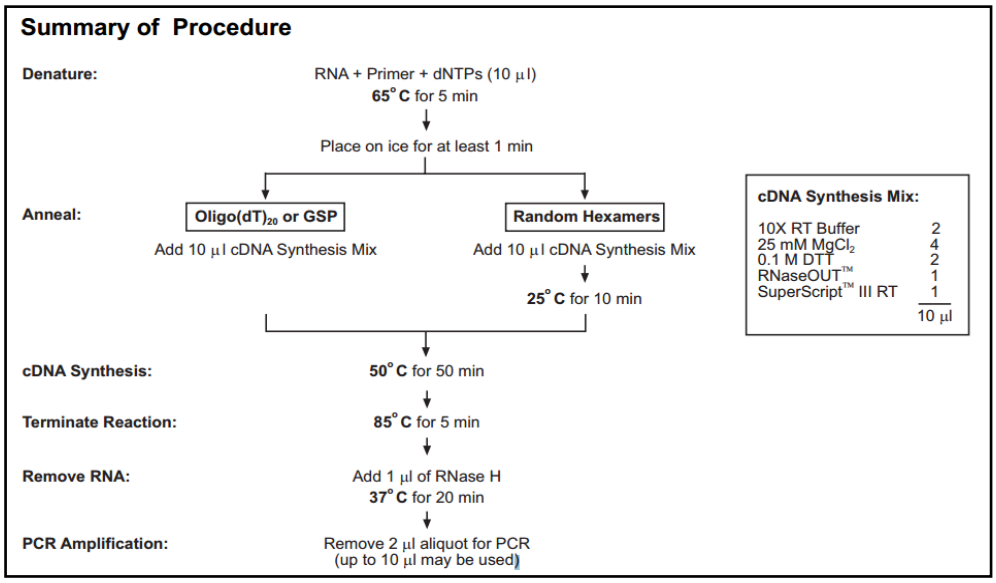


Figure 20. Aliquots of cDNA equivalent to 1 µg RNA were used for Real-time PCR iCycler iQ (Biorad).

Target gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
β-Actin	AAGATCAAGATCATTGCTCCTC	ACTCGTCATACTCCTGCT
GAPDH	GGAGTCAACGGATTTGGTC	GGCAACAATATCCACTTTACC
NQO1	TCTATGCCATGAACTTCAATCC	CTTCAGTTTACCTGTGATGTC
HMOX1	AAAGTGCAAGATTCTGCCC	GAGTGTAAGGACCCATCGG
TrkA	CATCCCTGACACTAACAGCA	CTCCGTCCACATTTGTTGAG
p75NTR	CCTCCAGAACAAGACCTCAT	GTTCCACCTCTTGAAGGCTA
GCLM	CCTTGAATGAATGGAGTTCCC	TCTGGAAACTCCCTGACC
GCLC	AAACCCAAACCATCCTACC	AATATAGAAGTAGCCTCCTTCC

Table 5. Lists of human primers used in Real Time PCR.

7. Determination of NGF receptors expression

The cells at $3x10^5$ cells/well were maintained on 6-well plates and cultured under the conditions described above. After 24h, 2 µg/ml SFN, 10 ng/ml NGF or at the same times, were added to the culture medium and cells were further incubated for times established.

Cells were then harvested, using 2 mM PBS/EDTA, pre-incubated with 0.5% BSA in PBS for 15 minutes at room temperature (RT) to minimize unspecific staining, followed by centrifugation, and 1h incubation at 4°C with either anti-human TrKA-PE (10 µl/$1x10^6$ cells from datasheet) (cat. FAB1751P, R&D Systems), and anti-human p75NTR (extracellular)-FITC (5 µl/$1x10^6$ cells, 1 mg/ml antibody concentration) (cat. ANT007-F Alomone Labs, Israel), in 0.5% BSA in PBS (PBS/BSA). Cells were then washed in PBS/BSA, and analyzed by FACSscan using 488 nm wavelength laser excitation for TrkA and p75NTR, with CellQuest software. Each experiment per sample was determined by recording 10,000 events. Moreover all experiments included negative controls and the analyses were performed in triplicate for statistical evaluation.

8. Scratch would healing assay

The wounding is test developed for the in *vitro* evaluation of cell migration and proliferation which includes the creation of a groove, said "scratch", on a confluent monolayer of cells. The monitoring of the scratch, through a camera connected to a microscope, to beginning and at regular intervals and the comparison and analysis of the images to quantify the degree of cell migration. In experiments cells were seeded in 6-well plates at a concentration of $5x10^4$ cells/well in 1 ml of complete medium and placed in incubator for 24 hours in order to allow adhesion of the cells. After 24 hours, the medium was removed and the scratch was created vertically to the centre

of the well using a sterile tip to 1000 µl. The cells were subsequently washed with PBS and treated with 2 µg/ml SFN, 10 ng/ml NGF, and with both at same times. The groove was monitored and photographed immediately and after 4, 24, 48 and 72 hours from the creation of the scratch using a phase-contrast microscope (Evos, Zeiss) with 4x magnification. The migration was evaluated as a residual area of the groove in the time frame under consideration through counts of three different fields for each condition. The wound area was calculated by tracing along the border of the wound using Image Software, image analysis software, and the percentage wound closure was calculated using the following equation:

[Wound Area (0h) - Wound Area (Xh)] x 100 /Wound Area (0h)= % Wound Closure

9. Invasion assay with collagen

Collagen matrix was generated from bovine type I collagen at a final concentration of 1mg/ml according to the manufacturer's protocol. Collagen has been plated immediately into the 24-well plates and then it has been incubated in a 37°C 95% humidity incubator for 30 minutes or until a gel is formed. Cells were seeded at a density of $5x10^4$ cells/well after collagen polymerization; then, they were incubated with 2µg/ml SFN for 24, 48 and 72 hours. After treatment, the collagen matrix, without adherent cells, was fixed with paraformaldehyde (4%), migrated cell nuclei were stained with blue methylene (1:10) and the membrane was analyzed using a microscope to count cell numbers (n=6 independent fields for each condition). All experiments included negatives controls.

10. Establishment of sulforaphane resistant cancer cell lines

Sulforaphane-resistant cells were established by exposure to increasing concentrations of SFN. A375 and 501Mel cells were initially cultured in its medium (see above) with starting from a concentration of 0.1 μg/ml SFN and then the cells were sub-cultured every two weeks with increased concentrations of SFN (50% increase each time). Finally, the resultant cell lines that grew exponentially in the presence of 2 μg/ml for A375 and 4 μg/ml 501Mel were designated as sulforaphane resistant melanoma cell lines, and named A375-rSFN and 501Mel-rSFN.

For the cell viability assay, 3×10^5 cells were plated onto 6 well culture plates and cultured at 37°C. Cells were collected 0,24,48 and 72 hours after the fresh medium replacement on day. The percentage of dead and living cells was then determined by staining with 0.1% trypan blue.

11. Statistical analysis

All the experiments were performed at least three times, independently. The data obtained were expressed as 'mean ± standard deviation'. Wherever appropriate, the data were analyzed by paired two tailed Student's t-test. The mean of the controls was compared with the mean of each individual treatment group by Dunnett's test. All statistical analyses were performed with Prism 5 Software. In all analysis the significance levels were specified at $p\leq0.05$ (*), $p\leq0.01$ (**) and $p\leq0.001$ (***).

RESULTS

1. SFN-induces morphological changes of melanoma cells

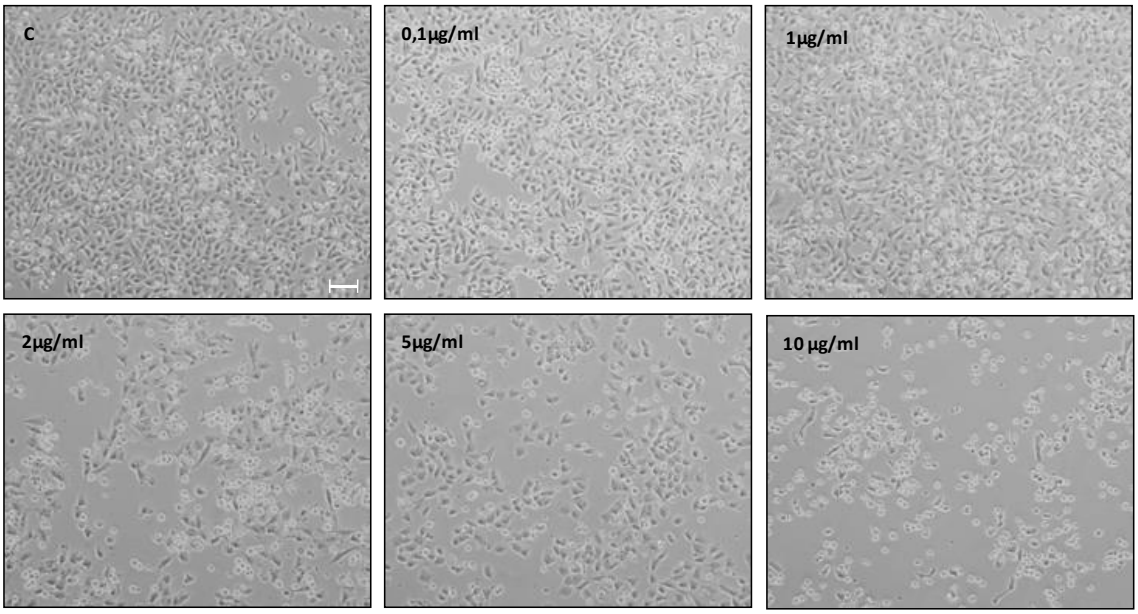
All the experiments described in the thesis are performed with two different human melanoma cells lines: i) A375 are human malignant melanoma cell lines obtained from American Type Culture Collection (ATCC), and ii) 501Mel obtained from surgically removed metastases in melanoma patients at the National Cancer Institute, Milan. As a control I used HEMa-LP human epidermal melanocytes isolated from lightly pigmented (LP) adult skin obtained from Invitrogen (Life Technologies). Melanoma cells in culture exhibited primarily a fibroblastic appearance during growth; however, many clumps of round cells were observed at confluence. The treatment of melanoma cultures with SFN causes dramatic changes in morphology which are dose and time-dependent.

1.1 A375 human melanoma cell lines

The A375 have been treated with different concentrations of SFN for 24, 48 and 72 h and with DMSO at the same concentration used for the dilution of SFN. Unlike the control and DMSO-treated cells (Figure 1.4), the addition of SFN to the A375 resulted an increase in cellular size (Figure 1.1). The treatment from 2 at 10 µg/ml of SFN, after 72h, induces considerable morphological changes; starting from the concentration of 2 µg/ml, A375 cells appeared large and elongated, moreover, at higher concentrations (5-10 µg/ml), cells were granular and much larger than those found at the lower concentration and in the control (Figure 1.1). The effect already appears at 48h post-

treatment, but it becomes much more marked after 72h. This qualitative analysis of cells indicated an altered morphology of the melanoma cells treated with SFN.

A



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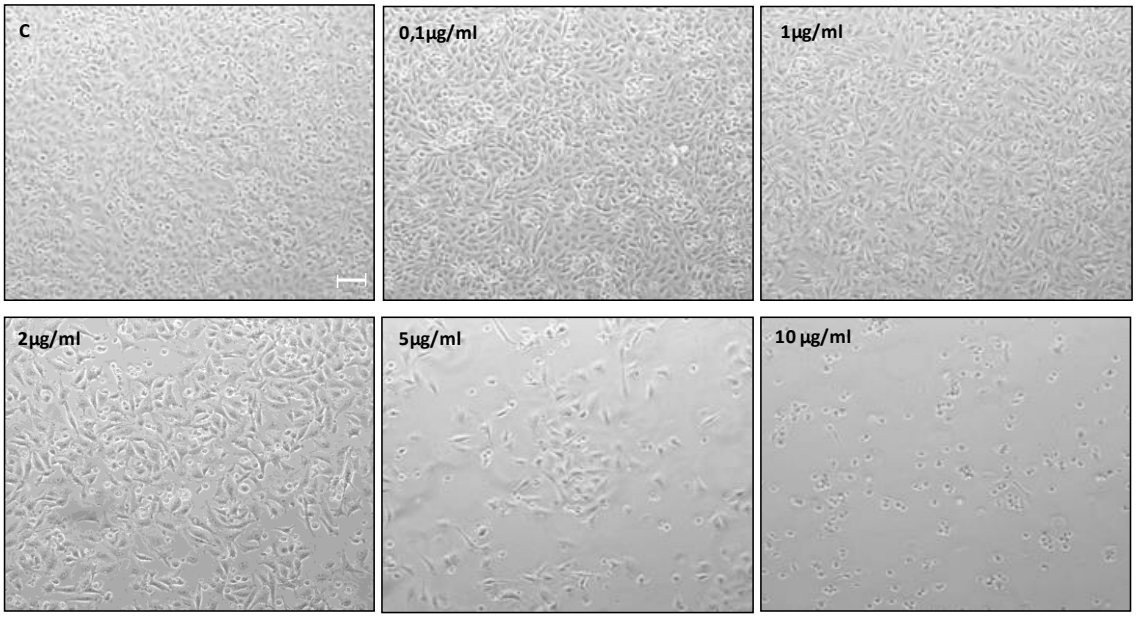

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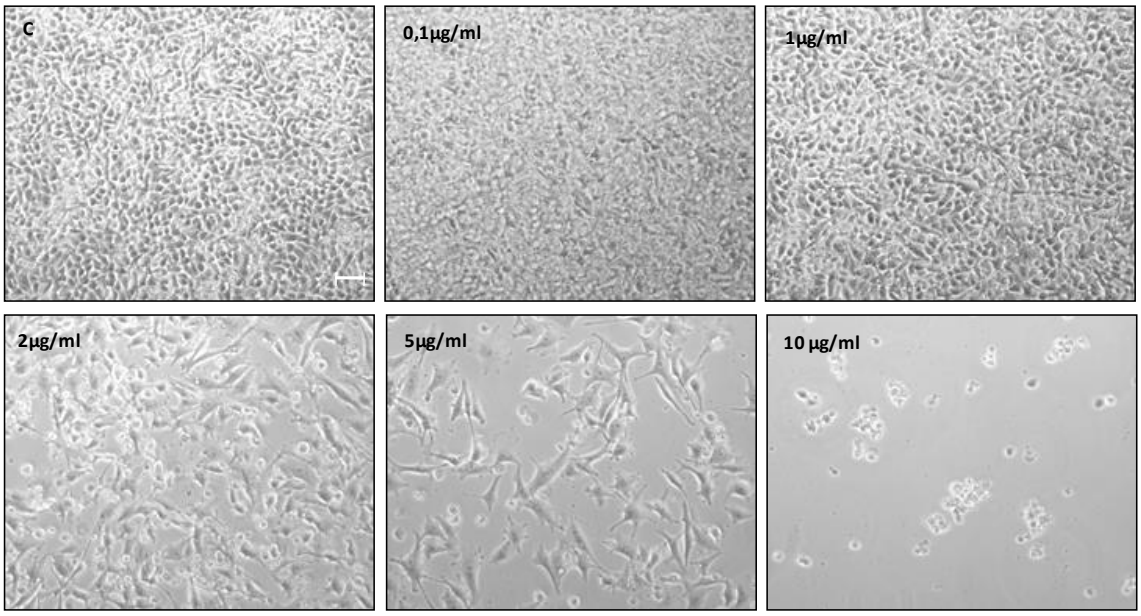


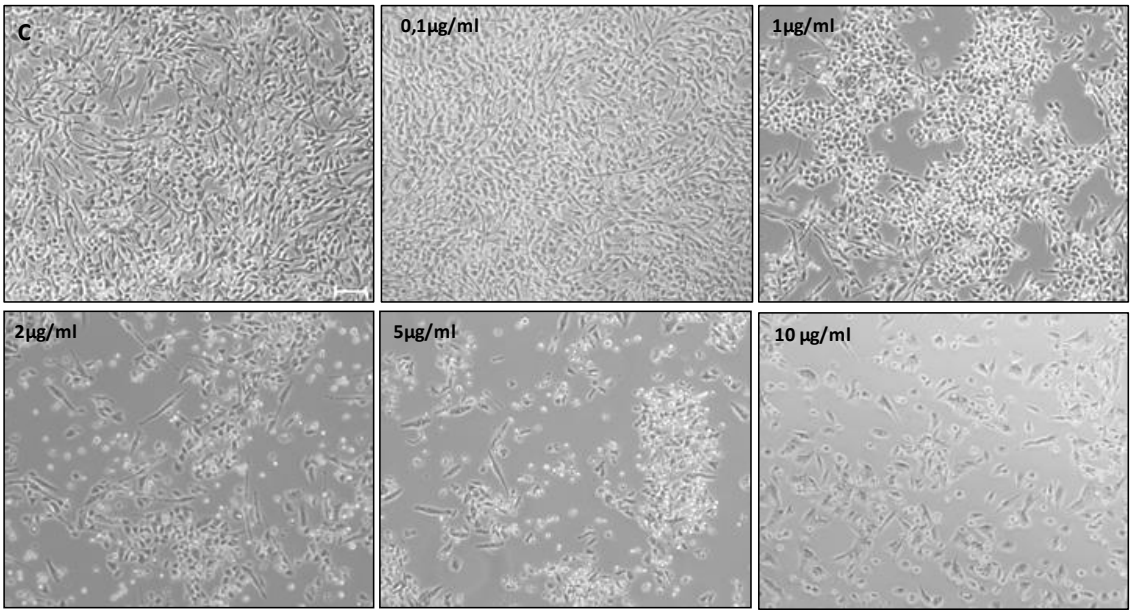
Figure 1.1 [A B C]. Recorded morphological changes over time in A375.

Pictures present the phase contrast microscopy figures (x10) of monolayer cultures of A375 untreated (control) or treated with 0.1, 1, 2, 5 and 10 µg/ml SFN at 24 [A], 48 [B] and 72 [C] hours. Scale bar = 100 µm.

1.2 501Mel human metastatic melanoma cell lines

Differences in cell morphology were observed between SFN-treated and control 501Mel cells by light microscopy. The treated cells exhibited an irregular morphology of the nuclei and typically had more vacuoles in the cytoplasm than control cells. As shown in figure 1.2, the most conspicuous changes were observed in treated cells with high concentration of SFN; it was observed that fewer attached cells remained after 72 hours of SFN (5-10 µg/ml) treatment. This qualitative analysis of cells indicated an altered morphology of the metastatic melanoma cells treated with sulforaphane. No differences were detected in the DMSO‑treated cells (Figure 1.4).

A


C
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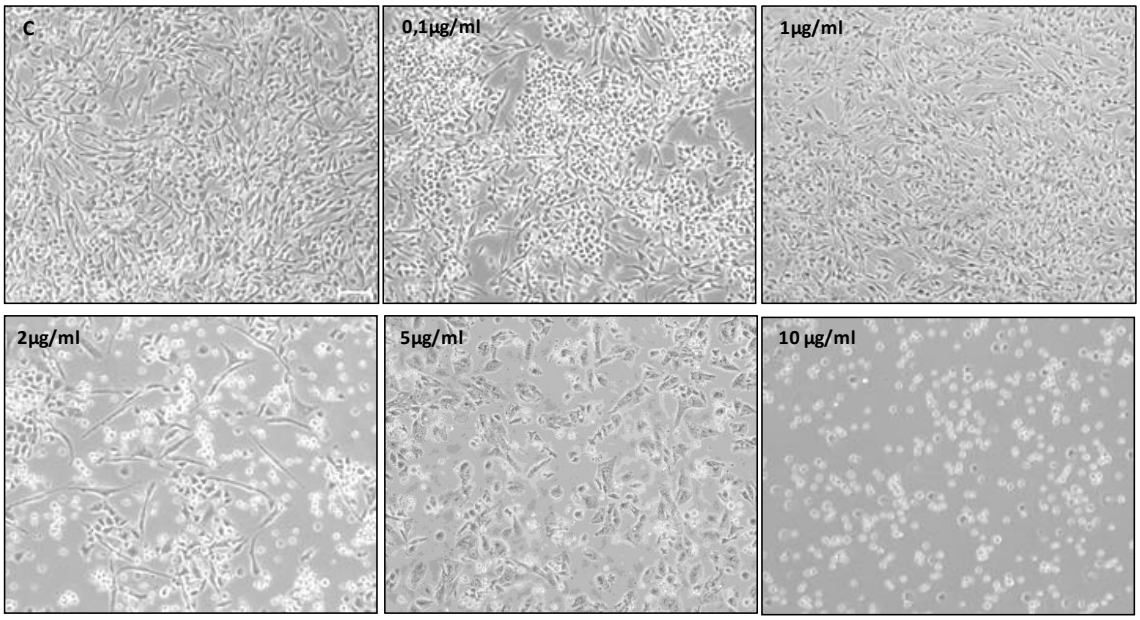
2µg/ml
5µg/ml
10 µg/ml

B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C

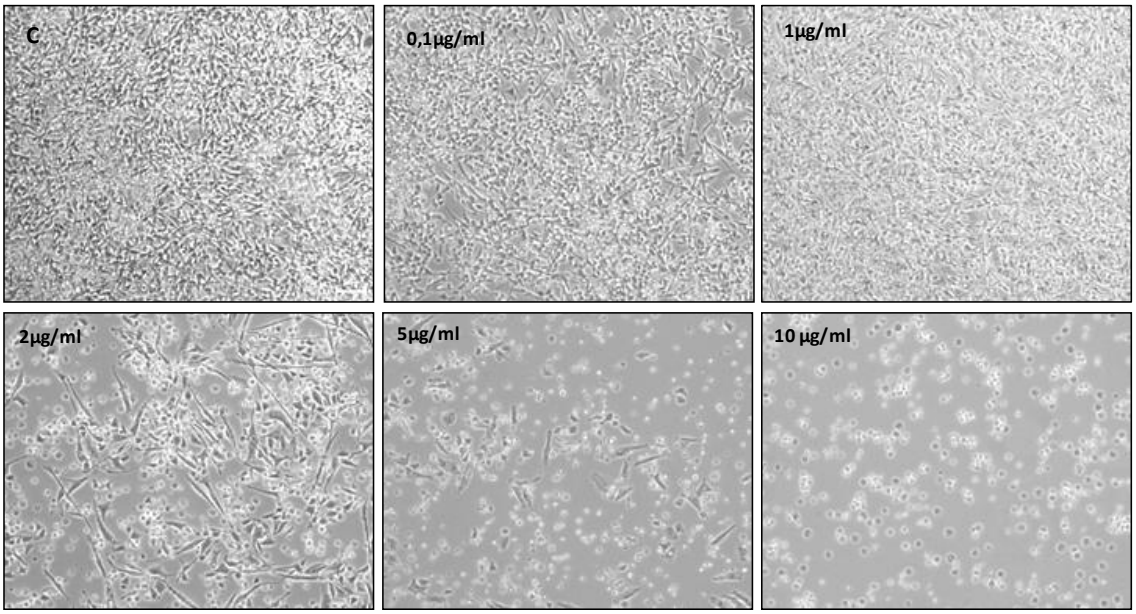
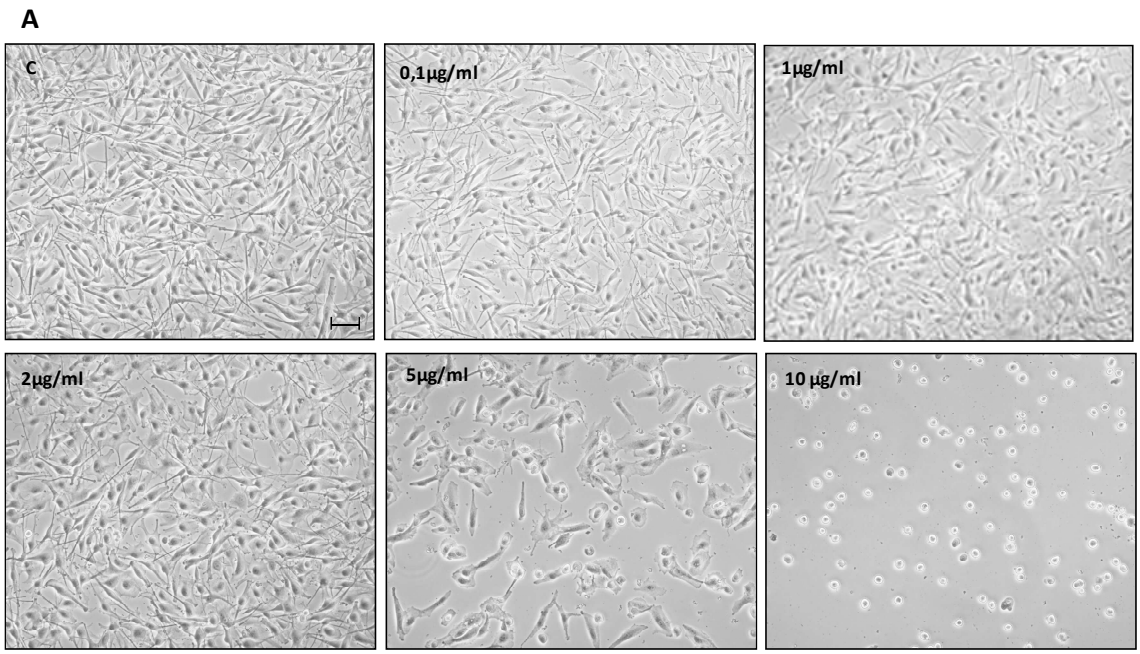


Figure 1.2 [A B C]. Recorded morphological changes over time in 501Mel.

Pictures present the phase contrast microscopy figures (x10) of monolayer cultures of 501Mel untreated (control) or treated after treatment with SFN at 24 [A], 48 [B] and 72 [C] hours. Scale bar = 100 µm.

1.3 Human epidermal melanocytes cells (HEMa)

In contrast to melanoma cells, HEMa cell lines did not exhibit any significant morphological alterations when treated at concentrations ranging from 0.1 to 2 µg/ml SFN at 24, 48 and 72 hours respectively (Figure 1.3). Only at high concentration (5-10 µg/ml) of SFN, most of the melanocytes became round and granulated and some cells are detached from plates. This was due to the toxicity of SFN treatment. No differences were detected in the DMSO‑treated cells (Figure 1.4).



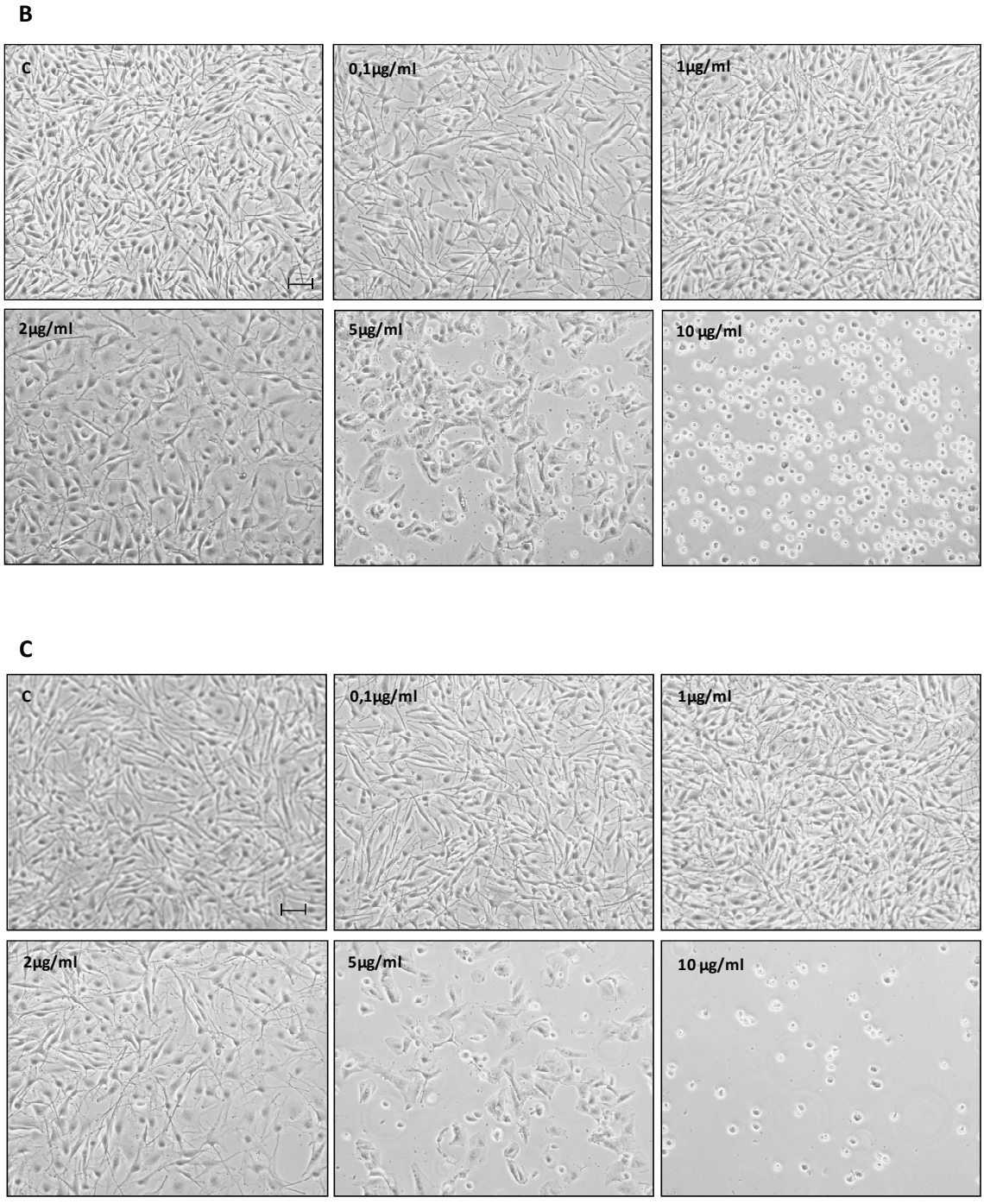


Figure 1.3 [A B C]. Morphology of HEMa after SFN treatment.

HEMa untreated (control) or treated with SFN at 24 [A], 48 [B] and 72 [C]. Scale bar = 100 µm.

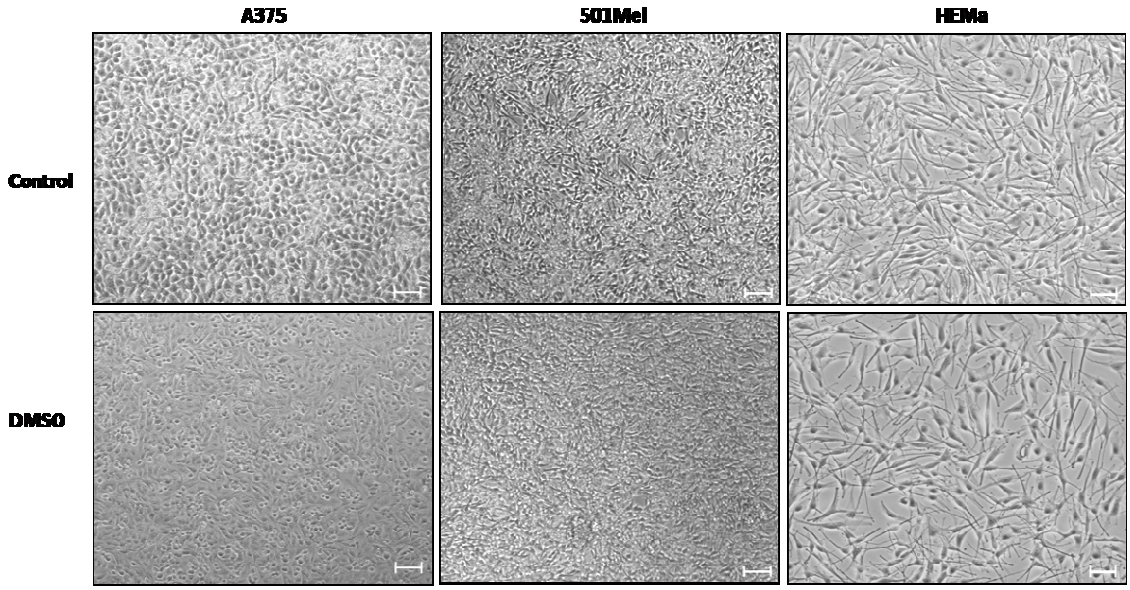


Figure 1.4 . Morphology of DMSO-treated cells.

Cells (A375, 501Mel and HEMa) untreated (control) or treated with DMSO (0.001%) at 72 hours and the same concentration was used to dissolve SFN. Scale bar = 100 µm.

4. Evidence of SFN induced apoptosis of melanoma cells

Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G_1 peak in the cell cycle pattern. Moreover the induction of apoptosis was identified by Annexin V-FITC binding assay and with propidium iodide (PI) staining. After staining cell population with annexin V and PI, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. All experiments were performed in three independent experiments and the results were similar.

4.1 A375 human melanoma cell lines

Cells with DNA content below G_1 phase, defined as a hypodiploid sub-G_1 peak, were regarded as apoptotic cells. The results showed that the percentage of sub-G_1 populations was: 2.3% and 14.2% ($p<0.01$), after a 24 hour treatment with 0 and 2 μg/ml sulforaphane (Figure 4.1). It is interesting to note that, after 48 and 72h of treatment the fraction of cells in sub-G_1 phase is equal to 29,5 and 21.3% respectably. There was no obvious increase of sub-G_1 populations after treatment with DMSO (data not shown). To confirm these results, it was evaluated the effects of SFN on apoptosis in A375 cells by using Annexin V-FITC and PI staining.

The apoptotic rate of the control group (apoptosis rate = early apoptosis + late apoptosis rates) and the SFN-treated group after 72 hours of treatment was respectably 5.7% and 34.55% ($p<0.05$). Only a very small percentage stained positive for PI, indicating that the cells were not necrotic (data not shown). The apoptotic rate of sulforaphane was significantly higher compared with control. No significant differences were detected in the DMSO-treated on control (data not shown).

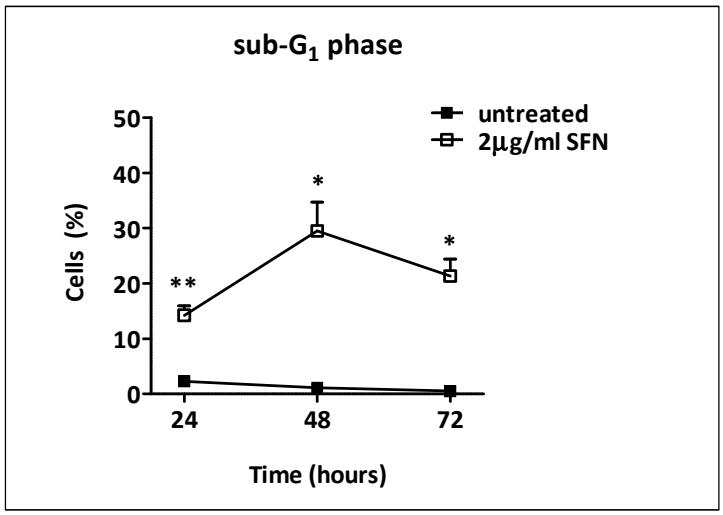


Figure 4.1. Determination of sub-G_1 cells in SFN-treated A375 cells by flow cytometry.

A375 cells were treated with 2 µg/ml SFN for 24, 48 and 72 h. The method of flow cytometry used is described under Materials and Methods. Data were shown by mean of percent PI stained cells ± SD (vertical bar) of triplicates experiments. p≤0.05 (*) and p≤0.01 (**).

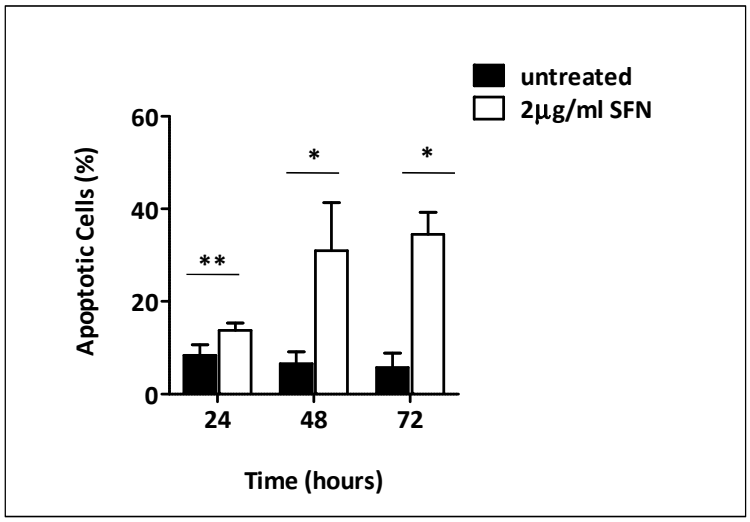


Figure 4.2. SFN induces apoptosis in A375 cells.

A375 cells were incubated with 2 µg/ml SFN for 24, 48 and 72 hours, followed by staining with Annexin-V/PI. The mean ± S.D. are shown from the sum of early and late apoptosis from three independent experiments. p≤0.05 (*) and p≤0.01 (**).

4.2 501Mel human metastatic melanoma cell lines

By measuring the fraction of cells with sub-G_1 DNA content, it was observed that dose of sulforaphane below 2 μg/ml induce a significant level of apoptosis in 501Mel cell lines (Figure 4.3). The sub-G_1 fractions of SFN-treated 501Mel cells were significantly increased in a time-dependent manner (9.7%, 20% and 24% respectively at 24, 48 and 72 hours). There was no obvious increase of sub-G_1 populations after treatment with DMSO (data not shown). It was next investigated whether this SFN-induced cell death was a result of 501Mel necrosis or apoptosis. Using Annexin-V-FITC /PI stained and flow cytometry, it was showed that treatment with 2 μg/ml SFN for 24, 48 and 72 hours resulted in increased annexin-V-FITC staining (Figure 4.4). The FACS analysis revealed that, after 24 hours, only a small fraction of cells, both in control and in treaties, has apoptotic characteristic. At 48 and 72 hours, however, the difference between control and treated becomes significant. For example, after 48 hours of treatment with 2 μg/ml SFN 18% are apoptotic, while only 10% in the control ($P < 0.001$).

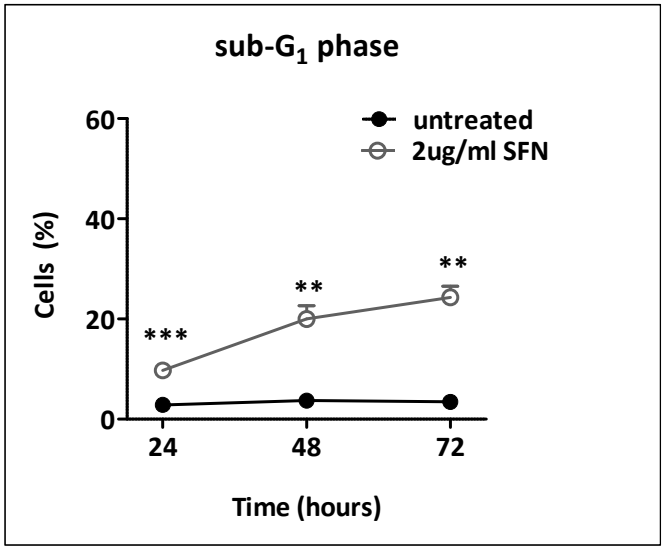


Figure 4.3. Determination of sub-G1 cells in SFN-treated 501Mel cells by flow cytometry.

501Mel cells were treated with 2 µg/ml SFN for 24, 48 and 72 hours. The method of flow cytometry used is described under Materials and Methods. Data were shown by mean of percent PI stained cells ± SD (vertical bar) of triplicates experiments. p≤0.01 (**) and p≤0.001 (***).

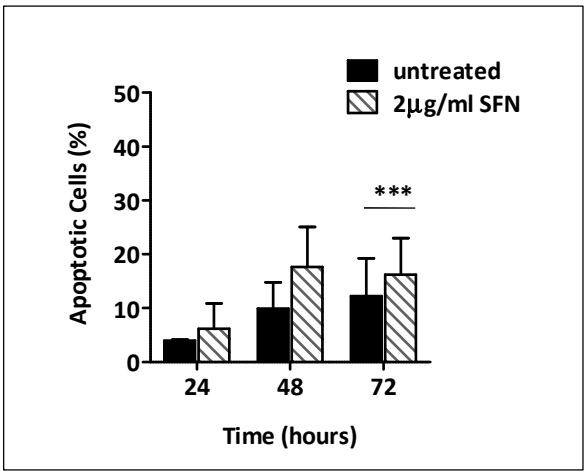


Figure 4.4. Sulforaphane induces apoptosis in 501Mel.

501Mel cells were treated for 24, 48 and 73 hours with 2 µg/ml SFN. Afterwards, all cells were collected and stained with Annexin V-FITC and PI. Staining intensity was determined for 10,000 cells using flow cytometry and subsequent quadrant analysis. The mean ± S.D. are shown from the sum of early and late apoptosis from three independent experiments. P < 0.001 (***) compared to untreated.

4.3 SFN did not induce apoptosis in HEMa cell lines

Sulforaphane does not seem to have any apoptotic activity on the melanocytes cell lines. The treatment has been shown to have no activity on the cells HEMA, furthermore after 72 hours with 2µg/ml SFN did not increase significantly the percentage of cells in sub-G_1 phase compared to control (6% vs 3.7%) (NS) (Figure 4.5).

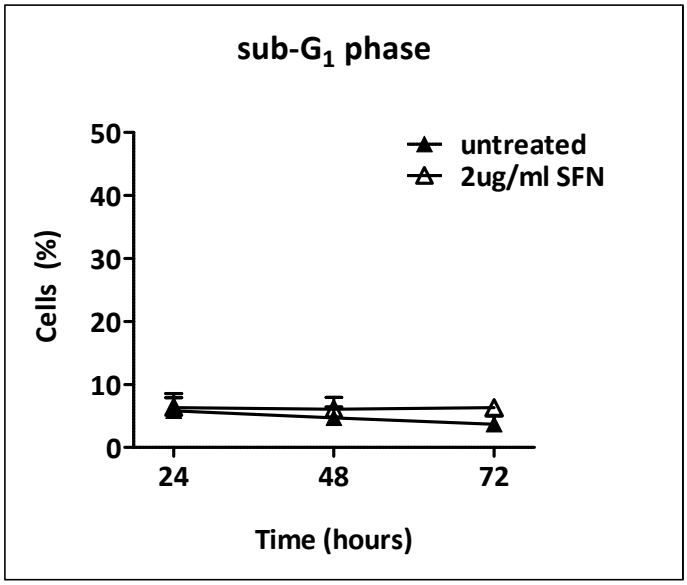


Figure 4.5. Determination of sub-G1 cells in SFN-treated HEMa cells by flow cytometry.

HEMa were treated with 2 µg/ml SFN for 24, 48 and 72 hours. The method of flow cytometry used is described under Materials and Methods. Data were shown by mean of percent PI stained cells ± SD (vertical bar) of triplicates experiments. p≤0.84 (24h NS) p≤0.29 (48h NS) and p≤0.11 (72h NS).

5. Molecular evidence of SFN induced apoptosis of melanoma cells

Since the pro-apoptotic effects of SFN are particularly marked after 48 hours of treatment the levels of caspases were analyzed in these conditions experimentally. Precisely, it was examined the effect of SFN on several key this process regulatory proteins, such as caspase-3 and caspase-9 using western blotting.

As shown in Figure 5.1(A), A375 cells upon SFN treatment (2 and 5 μg/ml), the level of procaspase-3 markedly decreased in a dose-dependent manner after 48 hours, while in figure 5.2 procaspase-9 was reduced mainly at 72 hours but the decrease started already at 48 hours (data represented two experiments). The level of procaspase-9 (47kD) was consistently reduced on treatment with 5μg/ml SFN. The changes in protein expression induced by SFN are consistent with the above-described induction of apoptosis. Furthermore, to confirm the data obtained, it was detected the active form of caspase-3 through FACS analysis. After treatment with 2 μg/ml sulforaphane for different times, A375 cells expressed increased caspase-3 activity (Figure 5.1(B)). The active form caspase-3 reached its maximum when the duration of treatment was 48h (20.60% versus 3.50% $p \leq 0.01$ (**)). These results indicate that sulforaphane-induced A375 cells apoptosis involves a caspase-3 and caspase-9 activation.

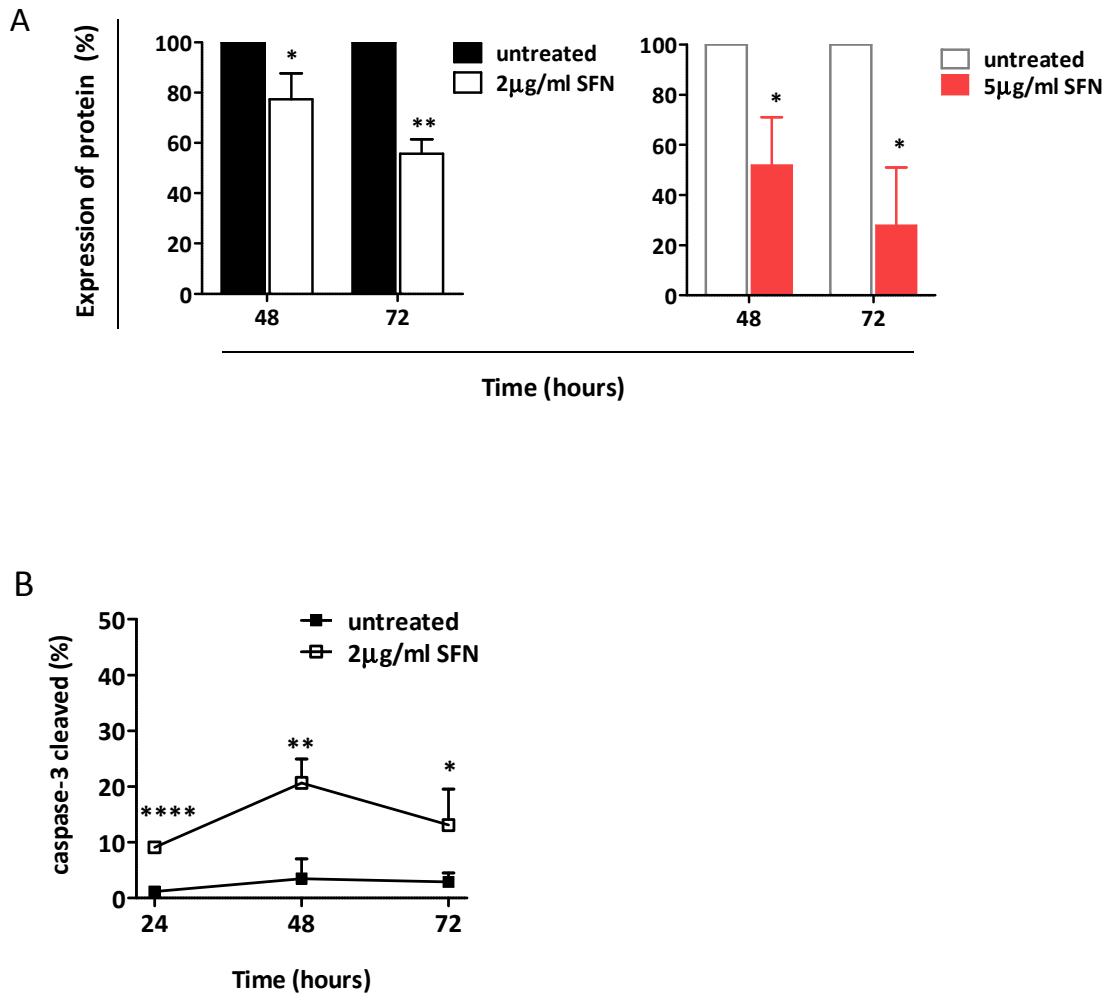


Figure 5.1 [A, B]. Activation of caspase-3 during SFN induced apoptosis in A375 cells.

[A] Cell lysates were prepared and subjected to immunoblotting with antibodies to caspase-3 (35kD) and β-actin (43kD). A375 cells were incubated with 2-5 µg/ml of sulforaphane for various time periods (48 and 72 h). The values represented below the figure represent the densitometric estimation of each band normalized by β-actin. The caspase-3 activity of control cells was set to 100% and the relative changes in the activity were shown. [B] Activation of caspase-3 by 2 µg/ml SFN is followed by FACS in A375 cells. For intracellular staining, cells were stained with PE-conjugated anti-active caspase-3 antibody as described in materials and methods. Data represent the mean ± SD of three independent experiments, data were analyzed by paired two tailed Student's test ($p \leq 0.05$ (*) and $p \leq 0.01$ (**)).

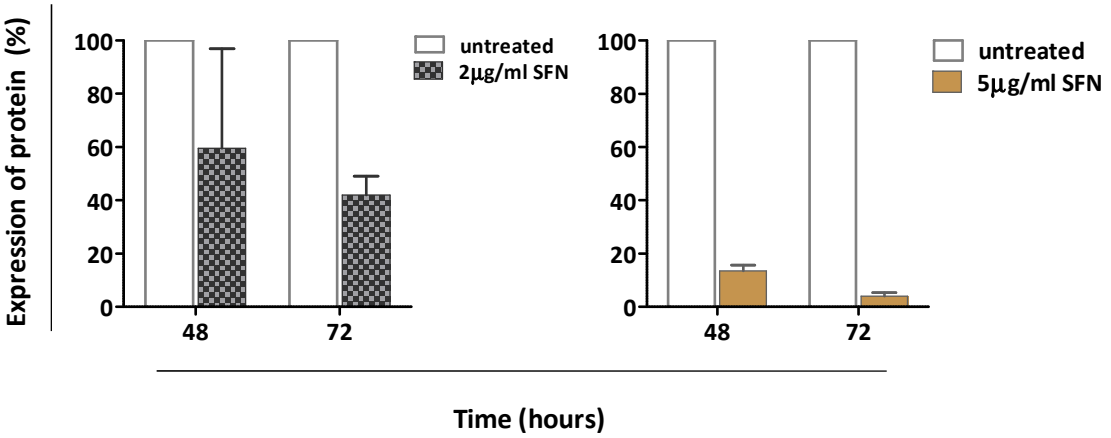


Figure 5.2. Activation of caspase-9 during SFN induced apoptosis in A375 cells.

Cell lysates were prepared and subjected to immunoblotting with antibodies to caspase-9 (47 kD) and β-actin (43kD). A375 cells were incubated with 2-5 µg/ml of sulforaphane for 48 and 72 hours. The values represented below the figure represent the densitometric estimation of each band normalized by β-actin. The caspase-9 activity of control cells was set to 100% and the relative changes in the activity were shown. Data represent the mean ± SD (vertical bar) of two independent experiments.

6. Effect of sulforaphane on the expression and activity of phase 2 enzymes

Numerous studies have shown the ability of SFN to induce phase 2 enzymes detoxification in different cell models. Since, to date no studies have been reported on the effect of SFN in human melanoma cells, objective of this study was to evaluate the effect of treatment with 2 μg/ml SFN, for different times, on the expression of phase 2 enzymes detoxifying/antioxidant (HMOX-1, NQO1, GCLM and GCLC).

6.1 A375 human melanoma cell lines

As shown in Figure 6.1, HMOX-1, NQO1, GCLC and GCLM mRNA expression in A375 induced by SFN. It was reported levels of mRNA, obtained by RT-PCR, of phase 2 enzymes, normalized against GAPDH, following supplementation of the cultures with 2 μg/ml SFN for several times. It can be seen a significant increase in the levels of expression gene, for each of the genes considered. After treatment, HMOX-1 mRNA was dramatically increased compared to the control at 6 (81.00-fold) and 12 hours (48.40-fold), while it was reduced at 24h (5.00-fold). Regarding NQO1 mRNA levels, after treatment, at 24, 48, and 72h were induced 1.40, 2.70 and 2.85-fold, respectively. Thus SFN produces a sustained NQO1 transcriptional response. In addition, SFN induced up-regulation of GCLM and GCLC gene expression compare to control. After treatment, GCLM mRNA levels at 6, and 24 h were induced 2.42, and 4.50-fold, respectively, while GCLC mRNA levels were little higher compared in treated cells as compared to the control cells (1.4-fold at 24h).

6.2 501Mel human metastatic melanoma cell lines

The mRNAs of four phase II genes, including HMOX-1, NQO1, GCLC and GCLM, were measured by real-time PCR in 501Mel cells after treatment. As shown in Figure 6.2, except for GCLC, the mRNAs of these genes were significantly increased by SFN treatment. At 24 hours post treatment increased the mRNA of HMOX-1, NQO1, GCLC and GCLM, by 2.40-, 2.67-, 1.40- and 5.71-fold, respectively. However, at 48 and 72

hours increased the mRNA only of HMOX-1 and NQO1 by 3.30-, 4.23-fold and 5.80-, 4.70-fold, respectively.

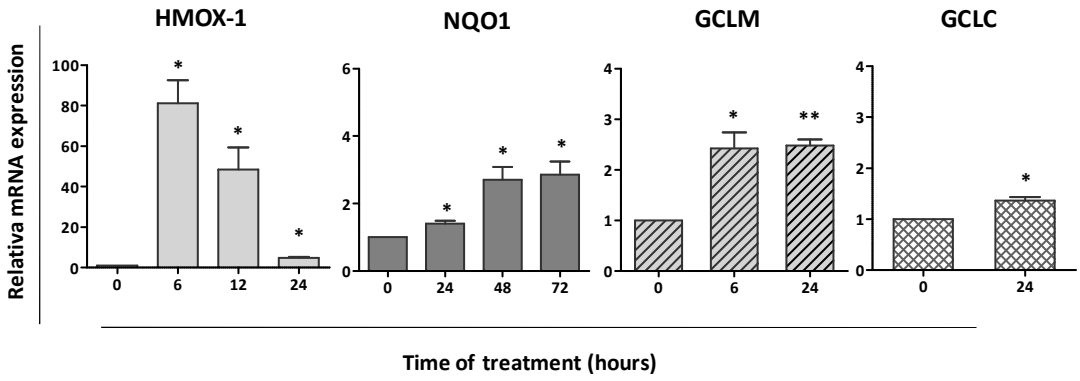


Figure 6.1. HO-1, NQO-1, GCLM and GCLC mRNA levels after treatment with sulforaphane in A375.
Fold-induction of phase 2 enzymes HMOX-1, NQO1, GCLM and GCLC in A375 treated with SFN. The levels of mRNA were assessed by RT-PCR analysis as described in the materials and methods. Levels of mRNA were normalized against GAPDH. Cells were untreated (control) or 2 μg/ml SFN for several times. Data in graphs represent mean ± SD (n=3). The significance level compared to control was specified at $p<0.05$ (*) and $p\leq0.01$ (**) using paired two tailed Student's t-test.

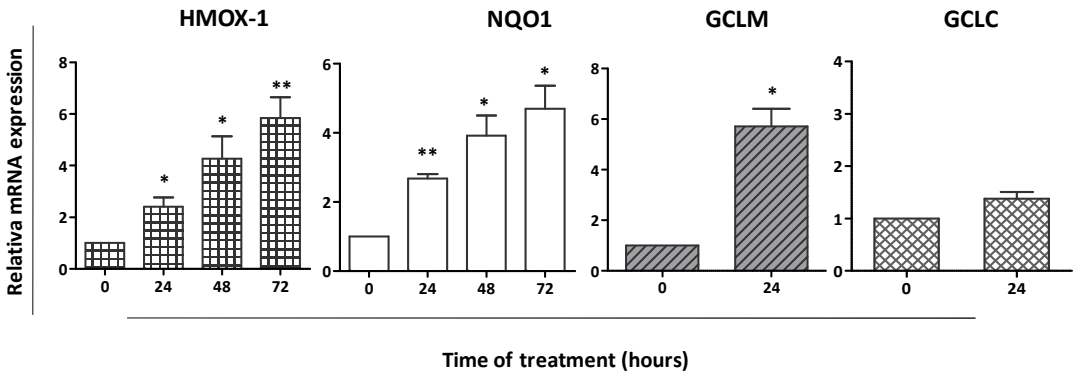


Figure 6.2 . HO-1, NQO-1, GCLM and GCLC mRNA levels after treatment in 501Mel.

Temporal changes in the expression of HO-1 ,NQO1 GCLC and GCLM mRNA after SFN (2 µg/ml) exposure in 501Mel. The levels of mRNA were assessed by RT-PCR analysis as described in the materials and methods. Levels of mRNA were normalized against GAPDH. Data in graphs represent mean ± SD (n=3). The significance level compared to control was specified at $p<0.05$ (*) and $p<0.001$ (**) using paired two tailed Student's t-test.

7. SFN upregulates TrKA and p75NTR expression

To better characterize the molecular events induced by SFN on melanoma, it was investigated the expression of NGF receptors TrkA and p75NTR, known to be involved in melanoma progression. The mRNA expression levels of the two receptors in A375 cells are depicted in Figure 7.1. The mRNA transcripts encoding both the high-affinity NGF receptor TrkA and the common low-affinity receptor p75NTR revealed significantly higher expression levels in SFN-treated A375 as compared with the untreated control. The expression level of p75NTR at 48h of treatment was 3.87-fold significantly higher as compared to untreated cells. In contrast, mRNA expression of TrkA was significant increased 2.44-fold, only at 72h ($p<0.02$).

To test whether the expression of p75NTR and TrkA mRNA correlates with the proteins, A375 was stained with anti-TrkA and anti-p75NTR antibodies and cells were analyzed by flow cytometry. Cells were incubated with β-NGF, SFN or β-NGF+SFN (Figure 7.2). SFN induced a significant increase TrKA positive (TrKA(+)) cells in a time-dependent manner than control (44.70, 58.80 and 68.40% vs. 31.70, 21.00, 29.20%). Similar effect was obtained with the combination of β-NGF and SFN (65.30, 59.00 and 72.30%). β-NGF treatment did not modified TrKA(+) cells (Figure 7.2A). Furthermore, analysis of FACS analysis demonstrated the significantly increase in the percentage of p75NTR positive (p75NTR(+)) cells after SFN alone or in combination with β-NGF in a time-dependent manner than control. In particular, SFN treatment showed fivefold increase compared to control at 48 and 72h (27.00 and 30.00% vs. 5.60 and 6.60% respectively). The double treatment determined a significantly increase of p75NTR(+) cells similar to SFN alone (22.00 and 27% at 48 and 72 h respectively). β-NGF treatment did not induce any significant modification of $p75NTR^{+}$ cells in all examined conditions (Figure 7.2B). FACS analysis confirmed the TrKA/p75NTR prosurvival ratio of proliferating control and β-NGF treatment cells. In contrast, exposure to SFN and β-NGF plus SFN, upregulates p75NTR, this 'switch' led to a marked reduction in the TrKA/p75NTR prosurvival ratio at 48 and 72 h versus control (2.60 and 2.60 vs. 7.00 and 5.00 respectively) (Figure 7.2C). Sulforaphane induces an increase of mRNA

expression and of positive cells regarding NGF receptors. Sulforaphane up-regulates p75NTR expression, converting the TrKA(+) A375 cells into TrKA(+)/p75NTR(+), and inducing cell death.

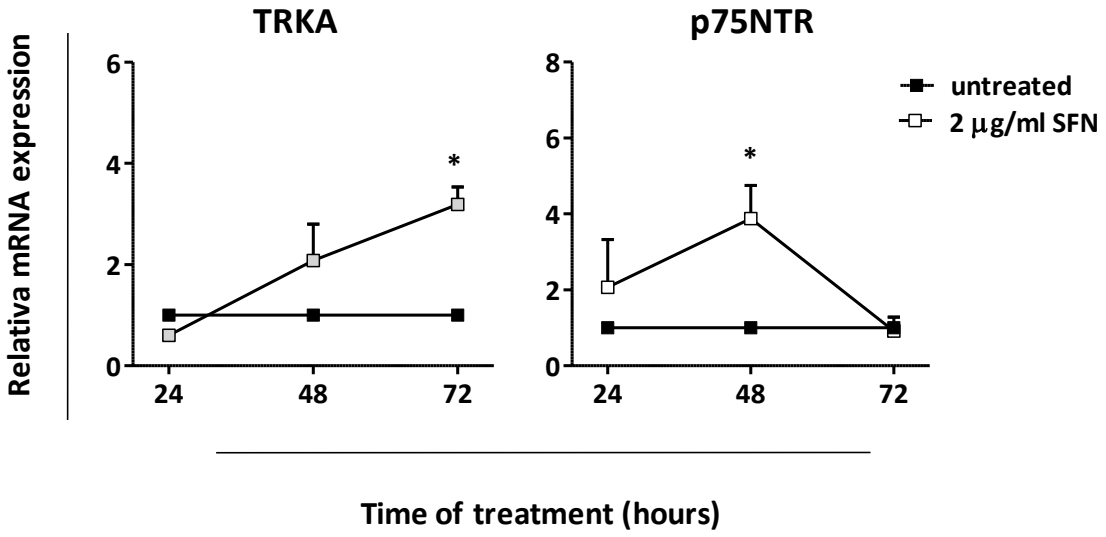


Figura 7.1. TrkA and p75NTR mRNA levels by sulforaphane.

Changes in mRNA expression of TrkA and p75NTR in A375 cells after SFN (2 μg/ml) exposure.

Data in graphs represent mean ± SD (n=3). The significance level compared to control was specified at $p<0.05$ (*) using a paired two-tailed Student's t-test.

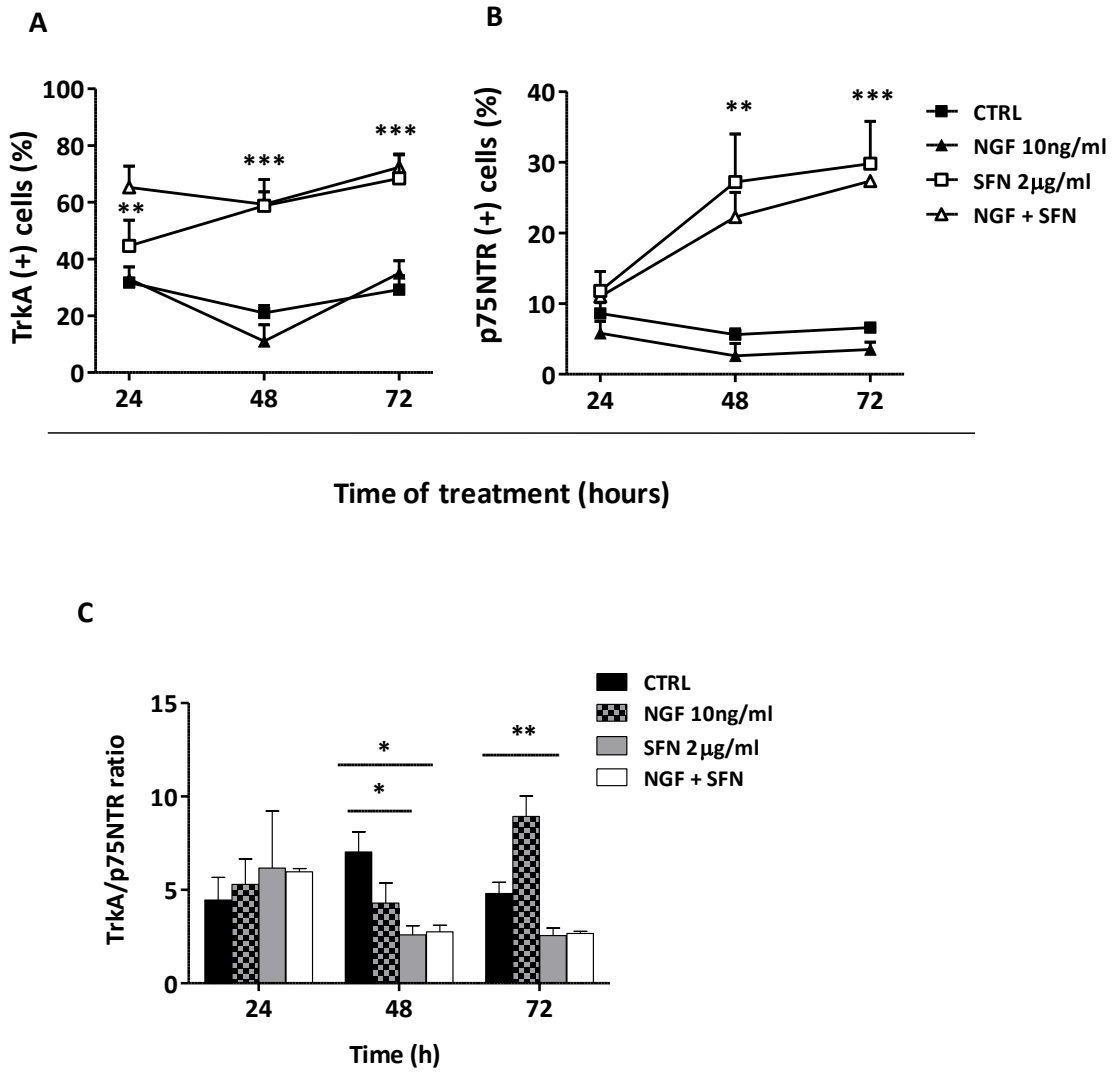


Figura 7.2 [A, B, C]. TrkA(+) and p75NTR(+) cells as analyzed by flow cytometry after treatments.

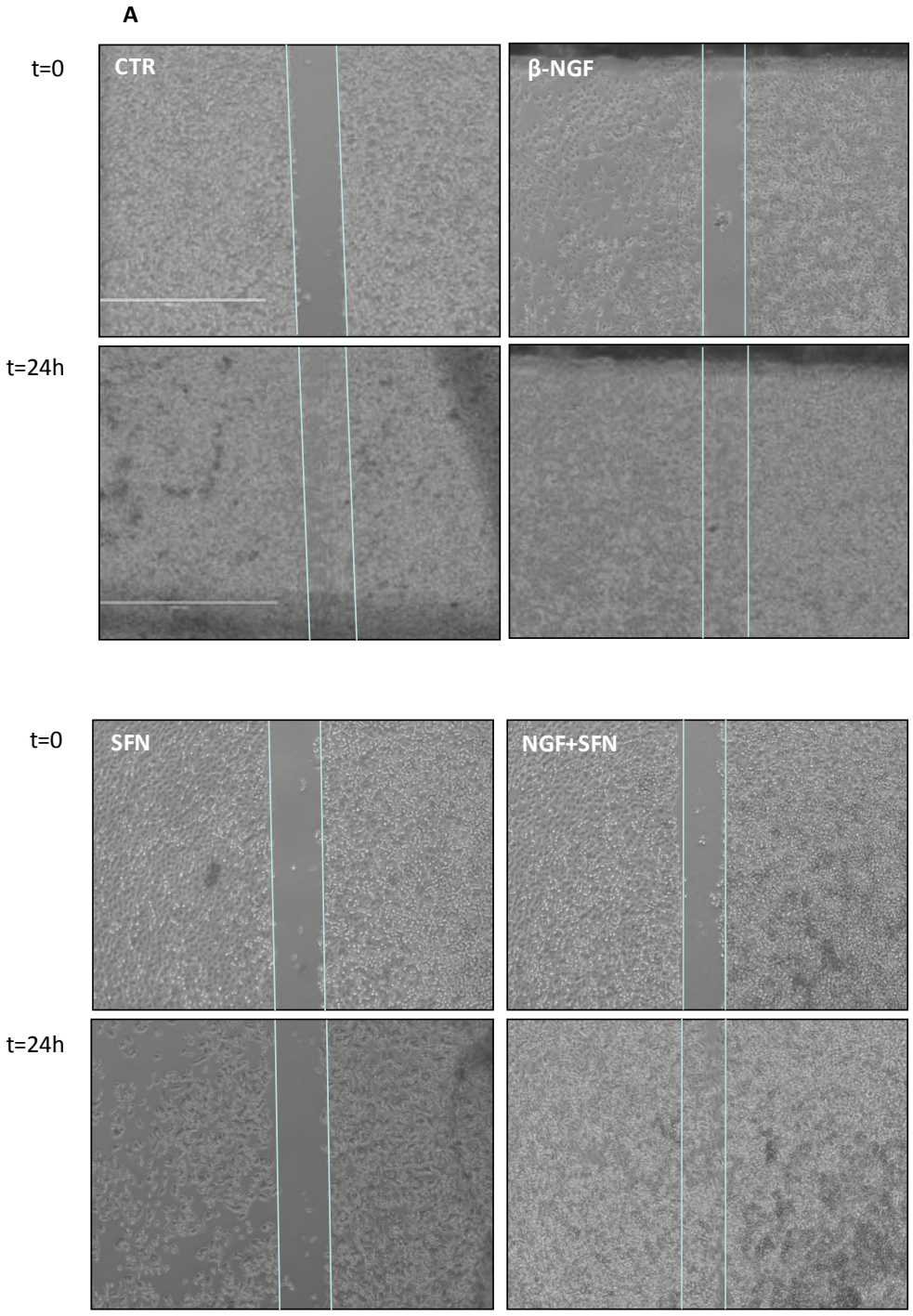
Positive cells for TrkA [A] and p75NTR [B] were followed by FACS in A375 cells after different incubation (β-NGF, SFN and β-NGF+SFN) at 24, 48 and 72 hours. Data in graphs represent mean ± SD (n=3). The p75NTR/TrKA expression ratio [C] was calculated using data from [A] and [B]. The significance level of SFN cell treated compared to control was specified at $p<0.05$ (*) and $p<0.001$ (***) using a paired two-tailed Student's t-test. [A] β-NGF+SFN versus CTRL 24h (*), NGF+SFN versus CTRL 72h (**). [B] β-NGF+SFN versus CTRL 48h (*), NGF+SFN versus CTRL 72h (**). [C] β-NGF+SFN versus CTRL 48h (*).

8. Sulforaphane reduces migration and invasion in A375 melanoma cells

To investigate the effect of SFN on migration of melanoma cells, *in vitro*, mechanically wounded A375 cell monolayers were incubated with medium alone, β-NGF, SFN and β-NGF+SFN and the wound area was measured at 0, 4, 24, 48 and 72 hours. After incubation, phase-contrast microscopy was used to capture images at various time points (Figure 8.1[A]). The wound area was calculated by tracing along the border of the wound using Image-J software. Sulforaphane significantly reduced A375 cells migration in scratch-wound assays. The difference between SFN-treated and control cells was statistically significant ($p<0.0003$). It was observed to decrease from approximately 42% to 11.5% wound closure after 24 hours (Figure 8.1[B]). In β-NFG- and (β-NGF+SFN)-treated monolayers, 85 and 59% of the wound area was closed after 24h, 100 and 87% after 72h respectively (Figure 8.1[B]). A375 cells with β-NGF had higher invasive ability than controls, moreover the addition of SFN led to a reduction of migration effect induced by NGF ($p<0.05$). These data suggest that SFN plays an important role in regulating the migration of A375 melanoma cells.

The ability of cells to penetrate through a basement membrane and invade adjacent tissues is also critical for the formation of metastases by cancer cells. As SFN has been shown to be involved in cell invasion, it was examined its potential invasive activity in A375 human melanoma cell lines (Figure 8.2[A]). Following treatment with 2µg/ml SFN, the invasion of cells was decreased when compared with untreated cells. The number of cells in collagen gel, in un/treated cells was 91 versus 41 (number of cells obtained from count of 6 independent fields for each condition) at 24h and became 145 versus 39 at 48h. In SFN-treated A375, the number of cells that invaded remained constant at the times analyzed (Figure 8.2[B]). Wound closure and invasion assay results showed that SFN plays an important role in the regulation of melanoma cells migration and invasion. As sulforaphane increases the expression of TrkA and p75NTR, it is interesting to understand if this is confirmed by invasion. They have been analyzed supernatant cells, adherent cells and collagen matrix cells. At 48h, SFN induced

increases of percentage TrkA(+) cells in collagen gel (42%). In treated cells, it is interesting to note that p75NTR is highly expressed in supernatant (47%) and adherent cells (33%), while the percentage of p75NTR(+) cells was very low in migrated cells (6%), at 48h. This remained constant for the times studied. The percentage of p75NTR(+) cells strongly decreased, when these were in collagen gel.


A
t=0
CTR
β-NGF
t=24h
t=0
SFN
NGF+SFN
t=24h

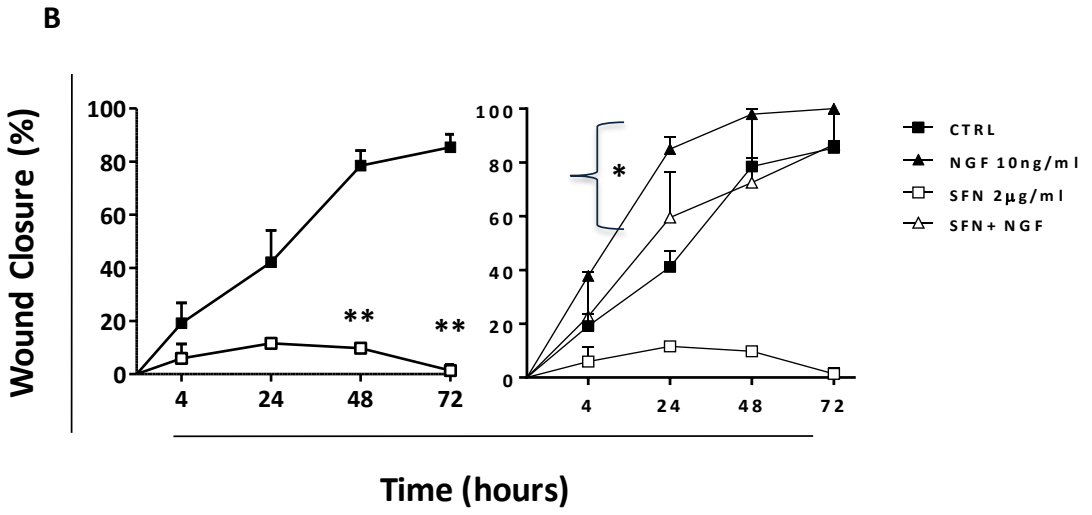
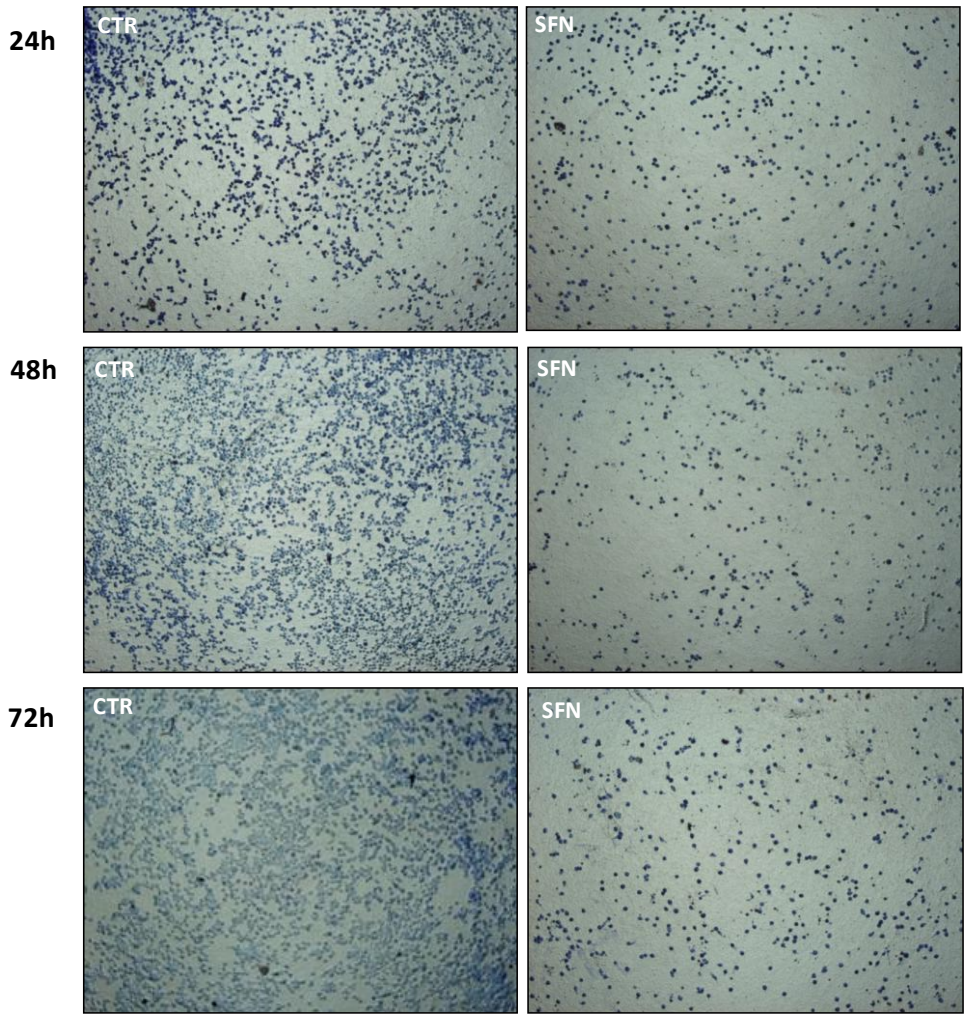


Figure 8.1 [A, B]. Inhibition of A375 migration by SFN.

A375 grown to confluence on 6-well culture plates were scratched off with a 1000µL pipette tip. After a change of complete medium, the recovery of the vacant space was observed at 0 4, 24, 48 and 72h with medium alone, 2µg/ml SFN, 10ng/ml NGF, and with both at same time. Data represent mean ±SD of three separate experiments, each performed in triplicate. [A] Cell migration assay shows the movement of A375 captured at 0 and 24h after incubation using phase-contrast microscope. Scale bar, 100 µm. The time-dependent wound closure with different incubation (untreated, NGF, SFN and NGF+SFN). The percentage of closed wound area compared with T= 0h was measured after 4, 24, 48 and 72h. The statistical analysis was done by comparing NGF+SFN against NGF alone using a paired two-tailed Student's test ($p<0.05$ (*)). The significance level compared to control was specified using a paired two tailed Student's test $p<0.01$ (**).

A



B

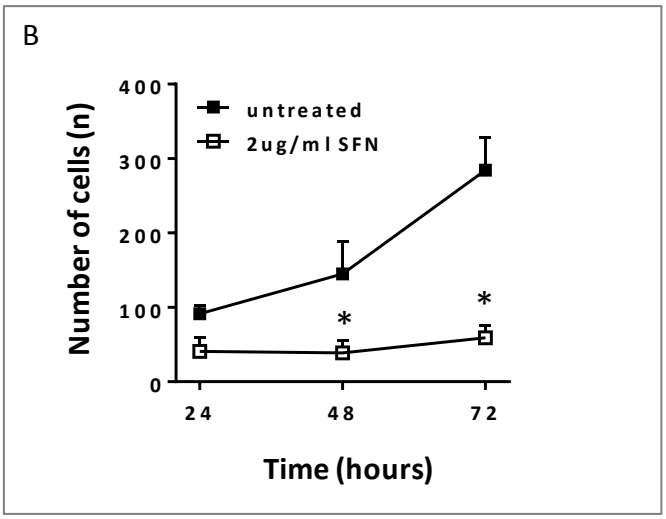
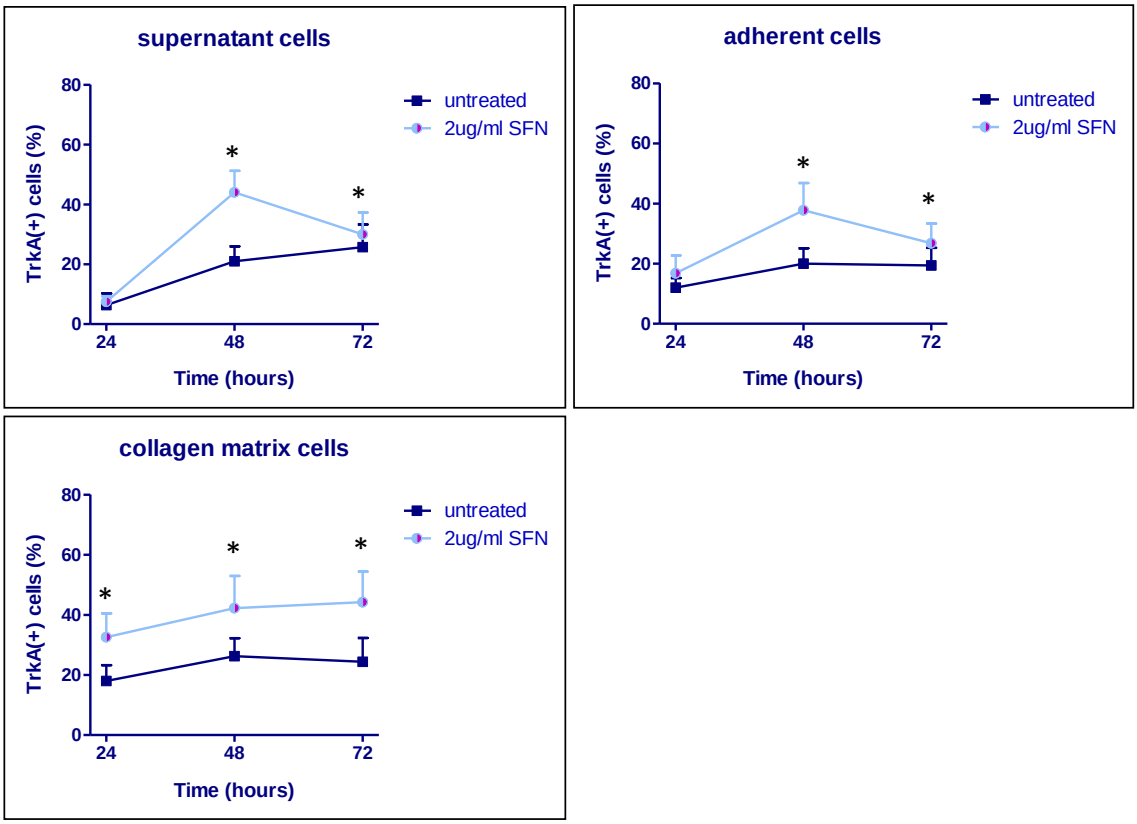


Figure 8.2 [A, B]. Inhibition of A375 invasion by SFN in collagen.

[A] Invasion assay of A375 cells with 2µg/ml SFN. The invaded cell number was determined at 24, 48 and 72h after treatment. Migrated cell nuclei were stained with blue methylene and images were captured by phase contrast microscopy (4x). Scale bar = 100 µm. [B] Number of cells to collagen gel at different time points. Data represent the mean and standard deviation of the evaluated clusters of randomly selected field (6 fields for each condition). The significance level compared to control was specified at $p<0.05$ (*) using a paired two-tailed Student's t-test.

A



B

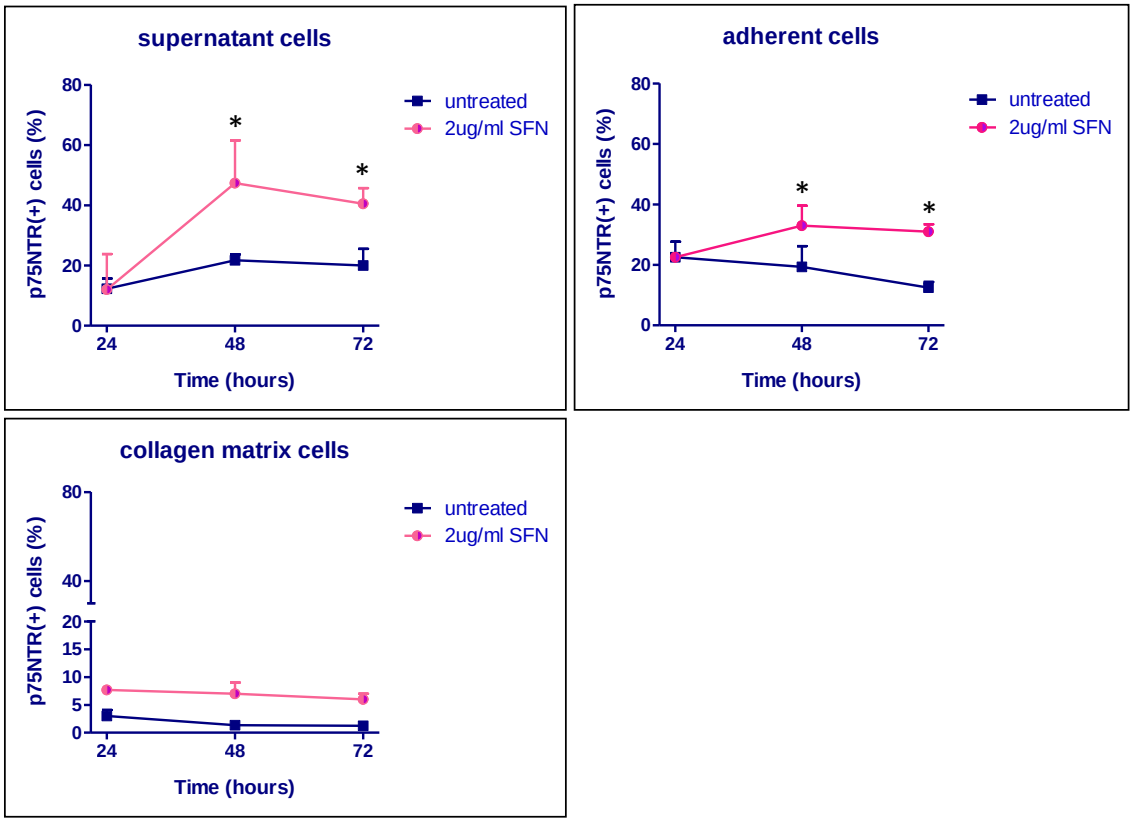


Figure 8.3 [A, B]. TrkA(+) and p75NTR(+) cells in invasion assay after SFN treatment.

[A] TrkA(+) in supernatant cells, adherent cells and collagen matrix cells, post treatment with 2µg/ml SFN. [B] p75NTR(+) in supernatant cells, adherent cells and collagen matrix cells, post treatment with 2µg/ml SFN. Cells were analyzed by FACSscan using 488 nm wavelength laser excitation for TrkA and p75NTR, with CellQuest software. Each experiment per sample was determined by recording 10,000 events. Moreover all experiments included negative controls and the analyses were performed in triplicate for statistical evaluation. The significance level compared to control was specified at $p<0.05$ (*) using a paired two-tailed Student's t-test.

9. Establishment of sulforaphane-resistant human melanoma cell lines.

Sulforaphane resistant cells were established by exposure to increasing concentrations of SFN.

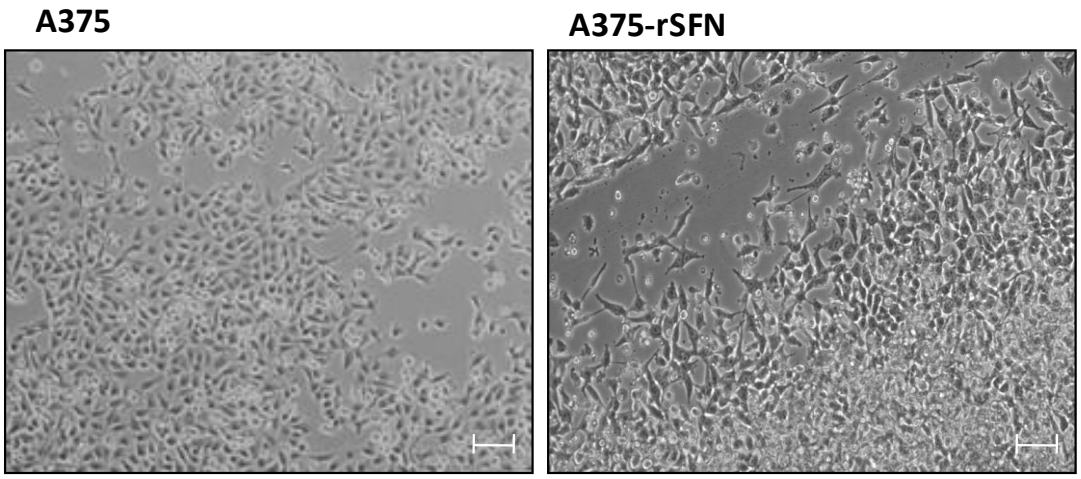
9.1 A375-rSFN

No much morphologic difference was found in sulforaphane resistant A375-rSFN cell lines (Figure 9.1A). The growth of cells was obtained by trypan-blue exclusion assay. Data showed that A375-rSFN cells grew slower than A375, the number of resistant cells following the addition of SFN (2 μg/ml) was 3.5, 4.8 and 5.5 x 10^5, in contrast the number of cell of A375 was 7.5, 11, 18.5 x 10^5 at 24, 48 and 72 hours, respectively (Figure 9.1B).

9.2 501Mel-rSFN

Differences in cell morphology were observed between SFN-resistant 501Mel cell line (501Mel-rSFN) and control by light microscopy (Figure 9.2A). 501Mel-rSFN cells exhibited an irregular morphology of the nuclei and different cell sizes than control cells. The 501mel are still ongoing selection, below has been reported the growth compared to the control under 4 μg/ml of SFN. The growth of the cells and the SFN-resistant cells (Figure 9.2B) show that the 501Mel-rSFN cells grew more slowly. The number of resistant cells following the addition of SFN (4 μg/ml) was 4.2, 7.5 and 11.0 x 10^5, in contrast the number of cell of 501Mel was 5.4, 12.5 and 24.5 x 10^5 at 24, 48 and 72 hours, respectively.

A



B

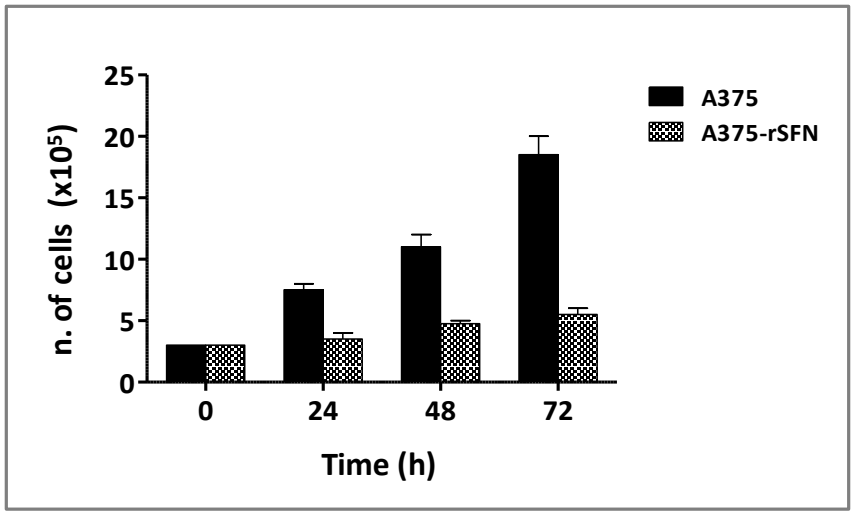


Figure 9.1 [A,B]. Recorded morphological changes in A375-rSFN.

[A] Pictures present the phase contrast microscopy figures (x10) of monolayer cultures of A375 untreated (control) and resistant at 2µg/ml SFN (A375-rSFN). Scale bar = 100 µm.

[B] Growth of A375 and A375-rSFN after 24, 48 and 72 h by trypan-blue exclusion assay. Cell counts were expressed as mean ± standard deviation (SD).

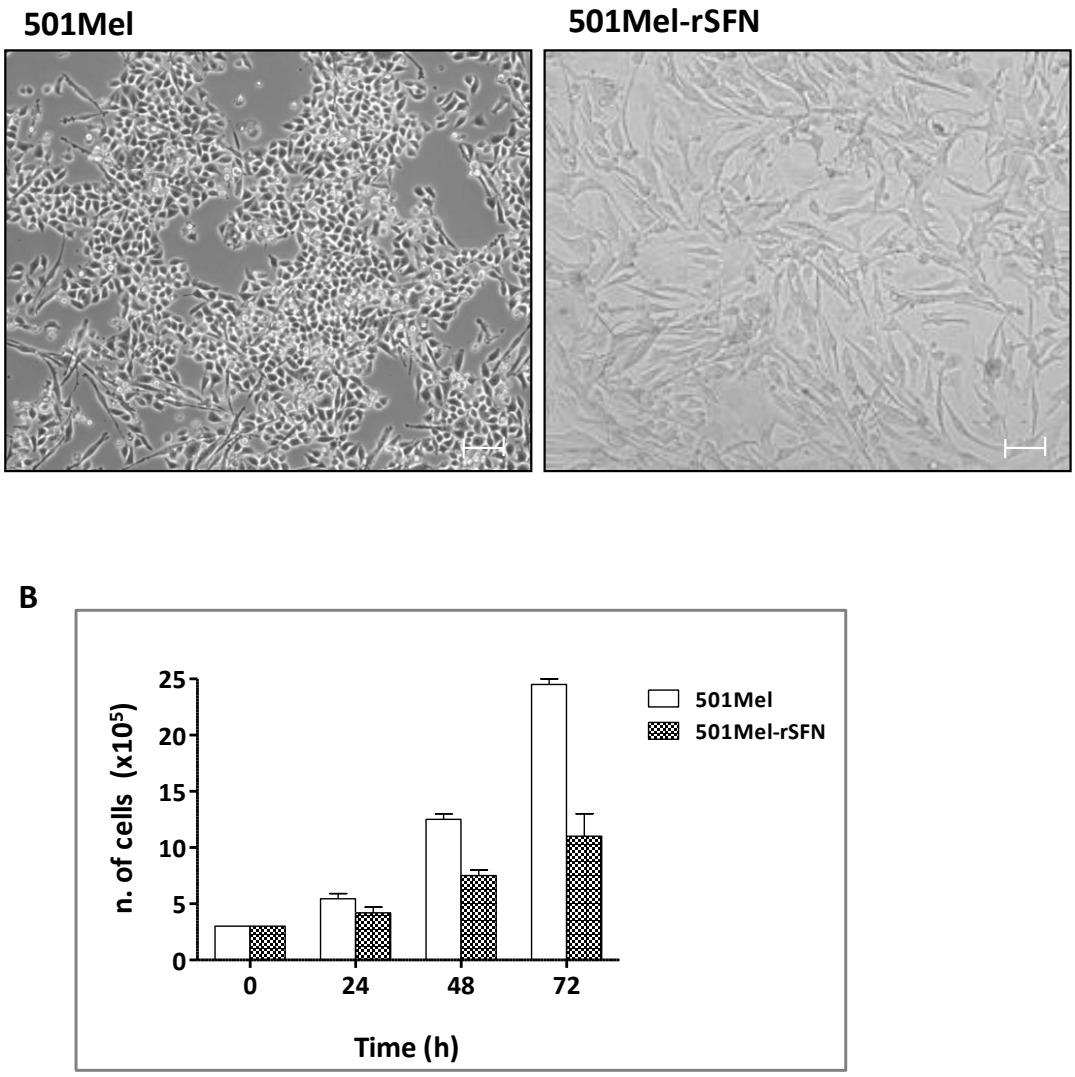


Figure 9.2[A, B]. Recorded morphological changes in 501Mel-rSFN.

[A] Pictures present the phase contrast microscopy figures (x10) of monolayer cultures of 501Mel untreated (control) and resistant at 4 µg/ml SFN (501Mel-rSFN). Scale bar = 100 µm. [B] Growth of 501Mel and 501Mel-rSFN after 24, 48 and 72 h by trypan-blue exclusion assay. Cell counts were expressed as mean ± standard deviation (SD).

Discussion and conclusion

Human malignant melanoma is an highly aggressive and incurable cancer due to intrinsic resistance to apoptosis reprogrammed proliferation and survival pathways during tumor progression. Use of medicinal natural products is widespread in patients with various types of cancer. Evidence from epidemiological and pathologic studies suggests that cancer could be prevented or progression could be blocked. Plant foods, including citrus fruit, cruciferous vegetables, and soybeans, show promising potential in reducing the risk of various types of cancers [96]. One possible candidate is sulforaphane, derived from glucoraphanin found in crucifers, which has been shown to have effects as a chemopreventive and anticancer agent in multiple preclinical systems through phase 2 enzyme induction, cell cycle arrest, and apoptosis in several cancer cell types. In B16F-10 murine melanoma cell line SFN reduces invasion, inhibits activation of matrix metalloproteinases and, consequently, the development of lung metastases, and prevents melanin synthesis and tyrosinase expression by affecting phosphorylation of MAP kinase family. However, the molecular mechanisms that underlie the antitumor effects of sulforaphane in melanoma models have not been fully clarified. In the past couple of decades the efficacy of isothiocyanates, particularly the one of SFN, in cancer chemoprevention has been recognized and these compounds to be extensively studied for their pharmacological effects. Moreover, to date SFN is under numerous clinical trials including a phase II trial for prostate cancer and a pilot study for the evaluation of the role of sulforaphane in atypical nevi--precursor lesions. Starting from these experimental evidences, it was conducted research of investigating the action of SFN in human melanoma cells.

In this study, it was observed that SFN inhibits the proliferation of human primary (A375) and metastatic (501Mel) melanoma cell lines by inducing cell cycle arrest and apoptosis. Sulforaphane reduced the viability of A375 and 501Mel cells in a dose – and time –dependent manner. High concentrations of SFN are anyway toxic for cells as demonstrated with experiments in human epidermal melanocytes adult cell line

(HEMa), but the viability of HEMa was not minimally affected at 2 µg/ml SFN treatment suggesting that this dose not exhibited minimal toxicity, if any, to normal cells. Therefore concentrations of 2 µg/ml or lower result to be useful for further investigations. In addition, each cell line was shown to undergo accumulation of G_2/M-phase cells in a time-dependent manner, which was accompanied by a decrease in G_0/G_1 phase cells. The results suggested that the growth inhibitory effect of SFN might be initiated by arresting the cells at G_2/M phase before undergoing apoptosis. Cells posses mechanism to maintain genomic stability through cell arrest. At least two cell cycle checkpoints play a role in the cellular response, allowing the DNA to be repaired prior to DNA duplication (G_1/S checkpoint) or mitosis (G_2/M checkpoint). Cell cycle deregulation is a hallmark of tumor cells, and targeting the proteins that mediate critical cell cycle processes is an emerging strategy for the treatment of cancer. This was the first time that a G_2/M cell cycle arrest was detected in human melanoma cells after sulforaphane treatment. The G_2/M arrest triggered by sulforaphane might be also explained by changes in the expression and/or activity of G_2/M cell cycle regulators. Future studies may also involve elucidation on expression of cyclin A , cyclin B1 and p53 and p21 proteins.

The results of the present study indicate that SFN treatment induced apoptosis in both A375 and 501Mel cell lines (but not in melanocytes) associated with an increase of sub-G_1 phase cell number and an annexin V positivity. In this study, many of the typical structural modifications that happen during the apoptotic pathway were observed which include cell shrinkage, translocation of PS to the outer layer of the plasma membrane and DNA fragmentation.

As highlighted in figure 4.1 and 4.3, the fraction of cells in sub-G_1 phase was visible during the analysis of cell cycle, 24, 48 and 72 hours after treatment with 2µg/ml SFN suggesting clearly an induction of apoptosis due to sulforaphane treatment. The inhibition of cell growth by SFN appears to be cytostatic and cytotoxic; all the results in this study that showed cell growth inhibition and cell-cycle arrest caused by SFN were obtained with cells that are going into apoptosis. It is interesting to note that the primary melanoma cells was more sensitive to SFN-induced apoptosis than the

melanotic and metastatic cell line 501Mel; probably, as regards 501Mel, the decrease of viability was supported not only by apoptosis but also by necrosis process.

It was evaluated the activation of caspases, an aspartate-directed cysteine proteases, that play a key role in the initiation and execution of apoptosis. The pathways of apoptosis are extremely complicated, the intrinsic and extrinsic pathways (or death receptor) have been well described that involve a number of proteins. Another particular pathway of apoptosis has also been recognized and involves perforin/ granzyme-A or B. Since caspase-3 is an executioner caspase that can be activated through both caspase-9 and caspase-8-dependent pathways, it was next determined the extent of activation of these initiator caspases following treatment of SFN. Western blot results demonstrated that SFN-induced apoptosis was associated with activation of both caspases-3 and -9 and with reduction of procaspase-3 and procaspase-9, in a dose- dependent fashion. These data suggest that the effect that SFN exert on these cells involves the mitochondrial-mediated apoptotic pathway.

Sulforaphane, besides, inhibits cancer initiation and progression through the reduction of formation and metabolic activation of carcinogen, and induction of its detoxification capacity. The results reveal that treatment of A375 and 501Mel cells with SFN of leads to a several fold increase in the mRNA expression of NQO1, HMOX-1, GCLM and GCLC genes (Figure 6.1 and 6.2). Significant increase of these phase 2 enzyme expression in human melanoma cell lines occurred with maximal enzyme induction observed generally at 6 and 24h for A375 and at 72 hours for 501Mel. This study demonstrates the potential of antioxidant phase 2 enzymes induction in the human melanoma as a strategy to reduce inflammatory effects and to promote apoptosis. Induction of the HMOX-1 protein has been reported to protect against a variety of stress conditions such as hydrogen peroxide, cisplatin, UV irradiation, and inflammatory cytokine-mediated cell damage. Moreover, HMOX-1 has been shown to inhibit proliferation and induce apoptosis in several cancer cells such as breast carcinoma cells. NQO1 is an activating enzyme for some anticancer drugs that plays an important role in regulating the activity of these agents, and it is a target for enzyme-directed tumor targeting. NQO1 participates in cellular defense against oxidative stress and regulates apoptosis

via p53- and NFκB-mediated pathways, in fact, some studies suggest that it may help to regulate the stability of p53 and apoptosis in human and mouse cells. The heterodimer GCLM and GCLC subunits have an essential role in GSH homeostasis by catalyzing the first-step, ratelimiting enzymatic reaction in GSH biosynthesis; the induction of GCLC and GCLM regulates cell proliferation and detoxification.

To better characterize the molecular events induced by SFN on melanoma, it was investigated the expression of nerve growth factor (NGF) receptors, known to be involved in melanoma progression. Sulforaphane treatment in A375 cells increased TrkA and p75NTR positive cells this was correlated with fold increase of mRNA expression. Therefore, whereas TrkA is generally a prosurvival, the role of p75NTR is more complex and enigmatic, as it can modulate divergent signaling pathways by forming complexes with different co-receptors to induce specific responses. Depending on the cell type, p75NTR can act synergistically, antagonistically, or independently from TrKs. In presence of mature NTs, it can function as a coreceptor for the TrKs, increasing their selectivity and prosurvival signaling. The more relevant novelty of this study is the cytotoxic mechanism of sulforaphane in these cells, which appeared to be associated with the upregulation of p75NTR, leading to p75NTR-dependent apoptosis. Moreover it was investigated the ratio of NGF receptors, FACS analysis confirmed that the TrKA/p75NTR prosurvival ratio of proliferating control and β-NGF treatment cells. In contrast the exposure, to SFN and β-NGF plus SFN, upregulated $p75NTR^{+}$cells; this 'switch' led to a marked reduction in the TrKA/p75NTR prosurvival ratio at 48 and 72 h versus control, suggesting that coexpression of p75NTR interferes with TrKA prosurvival signaling, inducing cell death. Changes induced by SFN in p75NTR expression was associated with an increase in multi-caspase activity. Indeed, as expected, addition of SFN to NGF-treated A375 cells reduced p75NTR(+) cells. These observations led to the hypothesis that SFN induces apoptosis through p75NTR and that p75NTR-dependent apoptosis could represent a homeostatic mechanism to eliminate damaged cells – similar to Fas-dependent apoptosis associated with inflammation – and possibly a 'class effect' of death receptors. This response might be associated with the inhibition of the

PI3K/Akt/NF-kappa B signaling pathway, future studies may also involve elucidation of the mechanisms by which SFN affects the p75NTR and TrkA pathway.

Certain chemotherapeutic agents are known to induce apoptosis through induction of death receptors. In this study, it was also demonstrated that SFN significantly suppresses the migration of A375 performing a scratch assay. Cell migration is a crucial step in cancer development. Melanoma is very aggressive, and it is thus very important to clarify the migration mechanism of melanoma cells, it is a cancer that is difficult to cure because the potential for malignancy is so high. The ability of cells to penetrate through a basement membrane and invade adjacent tissues is also critical for the formation of metastases by cancer cells. As SFN has been shown to be involved in cell invasion, it was examined its potential invasive activity in A375 human melanoma cell lines. Sulforaphane inhibition of invasion in collagen gel was found to be time dependent, with a relatively short treatment during the 24h assay only, which was sufficient to significantly decrease invasion. Moreover, cells treated with β-NGF had higher invasive ability than control cells, and the addition of SFN led to a reduction of migration effect induced by NGF. This suggests a direct effect of SFN on β-NFG, and the potential of this compound in moderating the effects of NGF in particular on migration. Collagen gel invasion and scratch assays indicated that SFN significantly inhibits A375 cell migration and invasion *in vitro*.

Furthermore, another important aim of this study was to establish SFN-resistant cell lines *in vitro* and to elucidate molecular mechanisms leading to drug resistance in melanoma. In this study, SFN-resistant melanoma cell lines were respectively established from A375 and 501Mel, by stepwise and continuous exposure to SFN. Each resistant cell lines grow in presence of SFN ranging from 2 to 4 µg/ml in A375-rSFN and 501Mel-rSFN respectively. Future studies aim to sequence the trascriptome using HiSeq 1500 Illumina platform to better understand signaling networks within of normal, treated and SFN-resistant melanoma cells. The success of chemotherapeutic agents would be attributed to its potentialities in inducing apoptosis in malignant cell populations. Considering that some chemoresistant melanoma cells might acquire sensitivity to chemotherapeutic agents, present results can suggest a possible role of

sulforaphane as, also, modulator of classical chemotherapeutical compounds to treat melanoma. For example in metastatic melanoma, clinical treatment with low SFN (2 μg/ml) dose might arrest the cells at G_2/M phase, enhancing the efficacy of the chemotherapeutic agent.

The question arises as to whether treatment with 2μg/ml (11.2 μM) SFN is relevant to humans. From literature it is known that SFN was detectable in the plasma of rats 1h after the administration of an oral dose of 50μM SFN and that the peak plasma level of sulforaphane was 20μM at 4h, and the level of SFN declined with a half-life of 2.2h. Although further pharmacokinetic studies in humans are necessary, the SFN concentration used in the present *in vitro* study is highly achievable *in vivo.* Finally, it is important to mention that there are other glucosinolates and metabolites found in cruciferous vegetables which possess similar chemistry, metabolism, and anticancer effects as SFN (eg. glucobrassicin- I3C, gluconasturtiin- phenethylisothiocyanate, glucoerucin-erucin (sulfide analog of SFN), glucoiberin-iberin). Also, there is a large body of research that has examined SFN effects on many other cancers such as breast, hepatic, bladder, osteosarcoma, glioblastoma and leukemia. Although larger scale clinical trials are in progress, already dietary SFN shows promise as a safe and effective anti-cancer strategy that includes incorporating easily accessible foods into an individual's regular diet. Considering that sulforaphane is a natural constituent of diet and demonstrates safe pharmacology, its therapeutic applications could become a promising tool. Therefore, it is important to promote the development of therapies that involve SFN in the treatment of cancer, including melanoma. Plants continue to be important for development of new anti-cancer drugs.

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