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"Neuroendocrine aspects of cutaneous melanoma: focus on somatostatin receptors (SSTRs) expression and role of the pan-SSTR-agonist Pasireotide on cell proliferation"

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. Fanelli F, Gambineri A, Belluomo I, Repaci A, Di Lallo VD, Di Dalmazi G, Mezzullo M, Prontera O, **Cuomo G**, Zanotti L, Paccapelo A, Morselli-Labate AM,Pagotto U, Pasquali R.**Androgen profiling by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in healthy normal-weight ovulatory and anovulatory late adolescent and young women.** J Clin Endocrinol Metab. 2013 Jul;98(7):3058-67. doi: 10.1210/jc.2013-1381. Epub 2013 Jun 18.

2. Fanelli F1, Belluomo I, Di Lallo VD, **Cuomo G**, De Iasio R, Baccini M, Casadio E, Casetta B, Vicennati V, Gambineri A, Grossi G, Pasquali R, Pagotto U. **Serum steroid profiling by isotopic dilution-liquid chromatography-mass spectrometry: comparison with current immunoassays and reference intervals in healthy adults.** Steroids. 2011 Feb;76(3):244-53. doi: 10.1016/j.steroids.2010.11.005. Epub 2010 Nov 26.
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Abbreviations

ALM: Acral Lentiginous Melanoma

bFGF : fibroblast growth factor

CDKN2A**:** Cyclin-Dependent Kinase inhibitor 2A

CMM: Cutaneous Malignant Melanoma

CTLA-4: cytotoxic T-lymphocyte-associated antigen 4

DITC: Dacarbazine

EGFR: epidermal growth factor receptor

FDA: Food and Drug Administration

GPCRs: g-protein coupled receptors

GPCRs: G-protein coupled receptors

ICC: Immonocytochemistry

IGF-IR : insulin-like growth factor IR

IL-2: Interleukin

INF-α: Interferon-α

LDH : lactate dehydrogenase

LMM: Lentigo Maligna Melanoma

MAPKs: Mitogen-activated protein kinases

MDM2: Murine Double Minute

NCC: neural crest cell

NET: Neuroendocrine tumor

NM *:* Nodular Melanoma

NST: nervous system tumours

OS: Overall survival

PDGFRb : platelet-derived growth factor receptor b

PI: propidium iodide

RGP : Radial-growth-phase

ROS: Reactive oxygen species

SSM*:* Superficial Spreading Melanoma

SST: somatostatin

SSTRs: somatostatin receptors

TMZ: temozolamide

TNM: tumor-node-metastasis

UV: ultraviolet

VGP: vertical-growth-phase

Abstract

Cutaneous malignant melanoma (CMM) is an aggressive malignancy whose incidence and mortality has increased worldwide. CMM is the most common cause of death from skin cancer. Wide surgical excision of early stage melanoma remains the main curative treatment. Unresectable advanced melanoma presents an aggressive behavior, tendency to rapidly metastasize and an intrinsic resistance to chemotherapy. The only targeted therapy approved for melanoma is vemurafenib, a small molecule targeting *BRAF* particularly when affected by common mutations in the nucleotides encoding for the aminoacid V600. These evidences suggest that novel therapeutic options for advanced CMM are still required. Melanocytes derive from neural crest cells and melanoma cells can express somatostatin receptors (SSTRs) suggesting that at least a subgroup of melanomas could have a neuroendocrine differentiation. The role of somatostatin (SST) pathway in CMM has been scantly investigated. The aim of this project was to evaluate SSTRs expression and to define the effects of SST analogs in relation to SSTR protein expression in *in vitro* models of CMM, exploring the role of SST pathway as a potential therapeutic target in human CMM. With this propose four cutaneous melanoma cell lines: A375, HMCB, COLO38 and M14 were used as *in vitro* models of CMM. The expression of SSTRs was evaluated by retro transcriptase quantitative polymerase chain reaction (RT-qPCR) and immunocitochemisty (ICC) in all four cell lines. The *in vitro* effects of daily administration of SST analogs pasireotide and octreotide and the *BRAF* inhibitor vemurafenib on cell viability, proliferation and cell cycle were investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay, DNA assay and fluorescence-activated cell sorting (FACS), respectively. Additionally the *in vitro* effects of daily administration of pasireotide in combination with vemurafenib, on cell viability, proliferation and cell cycle were investigated in two of the four cell lines (A375 and M14) that resulted sensitive to the effects of pasireotide. All tested melanoma cell lines express SSTR mRNA and proteins. At mRNA level, SSTR2 was the most expressed receptor followed by SSTR1, SSTR3 and SSTR5. The protein expression of SSTR1 was strong in A375, COLO38, M14 and moderate for HMCB; protein expression of SSTR2 was mild for A375 and COLO38, moderate for COLO38 and strong for M14; protein expression of SSTR5 was strong for A375 and M14, moderate for HMCB and mild for COLO38. The expression of SSTRs by ICC showed a predominant cytoplasmic localization in all melanoma cell lines used. Moreover, a perinucear staining for SSTR2 in COLO38 cells and for SSTR5 in HMCB and COLO38 cells was observed. Pasireotide significantly inhibited in a dose dependent-manner viability in A375 and M14 melanoma cell lines (maximal effects observed at dose of 10^{-7}M: 41% $p<0.01$ and 44% $p<0.001$ *vs* control, respectively). Octreotide significantly inhibited cell viability only in A375 cells (maximal effects observed at dose of 10^{-6}M; 54.15% $p<0.001$ *vs* control). Vemurafenib significantly inhibited A375 cell viability in a dose and time-dependent manner (maximal effects observed at dose of 10^{-6}M: 82.89%, $p< 0.001$ *vs* control). Pasireotide, and vemurafenib but not octreotide significantly inhibited cell proliferation in A375 (maximal effects observed at dose of 10^{-6}M: 20.57% and 21% $p<0.05$ *vs* control, with pasireotide and

vemurafenib respectively; IC_{50} $3*10^{-10}$M and $2.6*10^{-8}$M, respectively) and M14 cells (maximal effects observed at dose of 10^{-6}M: 20.57% and 21% $p<0.05$ *vs* control, with pasireotide and vemurafenib respectively; IC_{50} $3.8*10^{-8}$M and $1.228*10^{-7}$M, respectively). Combined treatment with vemurafenib and pasireotide had additive inhibitory effects in A375 only on cell viability (maximal effects observed at dose of vemurafenib 10^{-8}M + pasireotide 10^{-9}M: 41.79% $p>0.001$ *vs* control). The antiproliferative effects of pasireotide were observed only in cell lines presenting a strong SSTR5 protein expression (A375 and M14), suggesting that this pattern of SSTR protein expression could be predictive of response to this drug in CMM. Preliminary results of the FACS analysis suggest that the antiploferative effects of pasireotide in A375 and M14 could at least in part depend by an inhibition of cell cycle. Preliminary results of western blotting experiments, exploring the subcellular localization of SSTR2 and 5 in basal condition and after pasireotide or octreotide, suggest that the different trafficking of SSTR2 and 5 might explain the stronger antiproliferative effects observed with pasireotide compared to octreotide in these two melanoma cell lines. In conclusion this study firstly described the protein expression of SSTRs and suggested that the antiproliferative effects of pasireotide in human cutaneous melanoma cell lines could be related to a particular pattern of SSTR protein expression. This study has a potential translational value since the expression of SSTRs might indicate the potential use of SST analogs, radio-labeled SST analogs, SST analogs conjugate with chemotherapic agents and SSTR scintigraphy in the management of a subset of patients with CMM. This study encourages further studies to better define the role of SST pathway in diagnosis, prognosis and as potential target for treatment in human CMM.

Introduction

Cutaneous Malignant Melanoma (CMM) is a potentially lethal form of skin cancer, which results from the malignant transformation of melanocytes, which are the pigment-producing cells responsible for the colour of skin. Although the key triggers leading to malignant transformation of melanocytes have yet to be elucidated, they are multifactorial and include UV radiation damage and genetic susceptibility. CMM, accounting for 3 to 5 percent of all skin cancers, is responsible for approximately 75 percent of all deaths from skin cancers (Sladden MJ. *et al*. 2009, American Cancer Society. 2011). Worldwide, the incidence of melanoma continues to rise. The median survival time for melanoma patients with metastatic disease is 8-9 months, and the 3-year-survival rate is less than 15 percent (Balch CM. *et al*. 2009). Treatment of CMM is still a challenge. Conventional chemotherapy with dacarbazine (DITC) alone is associated with an objective response rate of, at most, 15 percent; moreover, nearly all of these responses are partial. Immune-based therapies, such as Interferon-α and Inerteleukin-2, have yielded comparable response rates, but they are associated with more intense toxicities and no clear impact on overall survival (OS) for metastatic melanoma patients (Lui P. *et al*. 2007, Eggermont AM. *et al*. 2009). Over the last decade, significant advances have been made in the understanding of genetic changes that drive melanoma development and progression, leading to the authorization of ipilimumab, a monoclonal antibody targeting citotoxic T-lymphocytes-associated antigen 4, and vemurafenib, a *BRAF* inhibitor used in patients whose tumors contain a V600 mutation in *BRAF* gene. Results of the BRIM-3 phase III clinical trial showed a response rate of 48% and 5% with vemurafenib and DITC, respectively, in previously untreated patients with metastatic melanoma harbouring V600E mutation. At 6 months, overall survival was 84% in the vemurafenib group and 64% in the DITC group. A phase III clinical trial reported a median overall survival of 10.0 months among patients receiving ipilimumab as compared with 6.4 months among patients receiving placebo (Hodi FS. *et al*.2010). Despite these encouraging data, severe toxicity and resistance occur after treatment with these compounds, therefore, new treatments for CMM are still required. An alternative targeted approach in the management of malignancies is the use of analogs of hormones, whose receptors are expressed on tumors and might influence tumor cell proliferation (Schally AV and Nagy A. 1999, 2004, Schally AV. 2008). Among them, an attractive target for treatment of melanoma and other types of cancers are somatostatin receptors (SSTRs). Somatostatin (SST) shows a pleiotropic inhibitory effect on cell proliferation and angiogenesis (Ferjoux G. *et al*. 2000, Dasgupta P. 2004, Hejna M. *et al*. 2002). Consequently, SST synthetic long-acting analogs have been developed and are currently used in management of neuroendocrine tumors (Woltering EA. 1997, Pollak MN and Schally AV. 1998). The expression of SSTRs on CMM and other melanomas has been hypothesized since the 1995, taking into account the neural crest origin of such tumors (Williams S. *et al*. 1997). Therefore in chemoresistant melanoma acquiring neuroendocrine phenotype SST analogs might represent an alternative treatment. However, the role of SST analogs in CMM is still

controversial. SST and its analogs effects are mediated by different G-protein coupled receptors (GPCRs) that are differently expressed on normal and pathologic tissues. Activation of SSTRs leads to different signalling processes through intracellular pathways including Ras/Raf/MAPK (mitogen-activated protein kinase) pathway that is classically associated to cell survival and proliferation. This pathway seems to be critical to oncogenic signalling in melanoma as supported by the existence of driver mutations in genes coding for proteins belonging to this pathway.

1.1 Cutaneous Melanoma

Melanoma is a group of biologically distinct malignancies with heterogeneous features including cell of origin, age of onset, ethnic distribution, clinical and histologic characteristics, pattern of metastasis and aetiology. Among risk factors a causative role of UV radiation, predisposing germ-line alterations, mutational processes and patterns of somatic mutations have been identified. CMM arises from neoplastic transformation of skin melanocytes. Neoplasms are initiated by gain-of-function mutations in oncogenes, which lead to benign melanocytic nevi which in turn, due to additional genetic aberrations, progress to malignant melanoma.

1.2 Epidemiology

The incidence of melanoma is continuing to increase worldwide. Once a rare cancer, the incidence of malignant melanoma skin cancer in most developed countries has risen faster than any other cancer type since the mid-1950s (Hall H *et al*. 1999). CMM is the sixth most commonly diagnosed cancer in the USA in both genders (Jemal A. *et al*. 2006). It is estimated that the annual increase in the incidence rate of melanoma has been approximately 3–7 percent per year worldwide for Caucasians (Parkin DM. *et al*. 2001). The estimated lifetime risk of an American developing invasive melanoma is 1 in 59 and is projected to rise to 1 in 50 by the year 2015 (Fig.1). About the European melanoma incidence and mortality, they present differences between European countries, possibly related to missed opportunities for early diagnosis and incomplete reporting of melanoma in Eastern Europe. The estimated age-standardized incidence of melanoma (measured per 100 000 person-years) varies widely from 19.2 in Switzerland to 2.2 in Greece calculated by GLOBOCAN (the standard set of worldwide estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer for 2008). Melanoma mortality rates of 1.5 are similar in CEE (Central and Eastern Europe) and Western Europe, although rates vary with a high of 3.2 in Norway and a low of 0.9 in Greece (Forsea AM. *et al* . 2012)

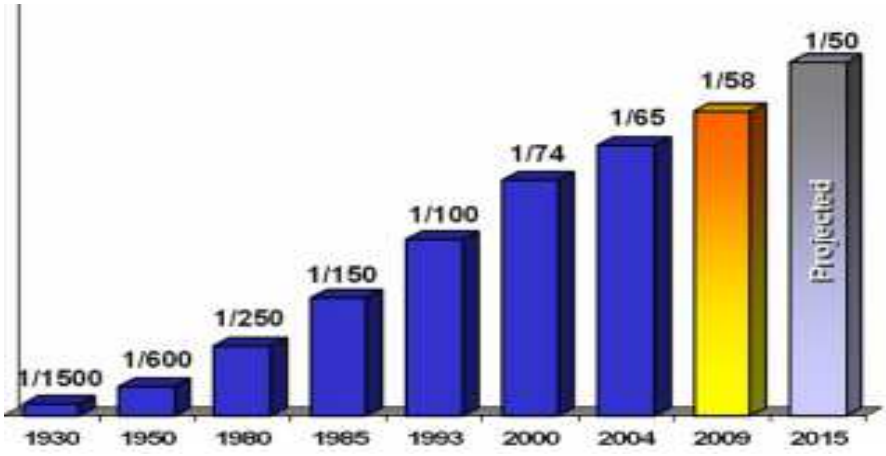


Figure 1. US invasive melanoma lifetime risk

This increased incidence may be attributable to better and earlier detection of melanomas and enhanced public awareness. Patients with deep primary tumors or tumors that metastasize to regional lymph nodes frequently develop distant metastases. Median survival after the onset of distant metastases is only 6–9 months, and the 5-year survival rate is less than 5 percent (Houghton AN and Polsky D. 2002). An analysis of melanoma trends stratified by thickness, based on the Surveillance, Epidemiology and End Results (SEER) registry data from 1988 to 2006, showed that all four thickness categories (≤1, 1.01–2, 2.01–4 and >4 mm) increased in incidence over the 19-year study period (Criscione VD and Weinstock MA. 2010). Furthermore, Jemal *et al*. reported that this increase was different by gender; females had a greater increase in thin lesions (4.1 percent per year) than men, where the increase was greatest in thick melanomas (6.1 percent per year) (Jemal A. *et al*. 2001). Another analysis of nine SEER registry databases (1975–2006) showed that age-specific melanoma incidence rates were greater among women than men prior to age 40 years at diagnosis. In addition, melanomas on the trunk were more frequently diagnosed in US men than women, although changes in behavior and life style lead to an increase of the incidence of melanomas in the trunk in women over the years (Bradford PT. *et al*. 2010).

1.3 Melanoma risk factors

Risk factors for any malignancy can be subdivided into genetic and environmental with interaction between the two (Fig.2). The principal established and also postulated risk factors for CMM are:

- Invasive cutaneous melanoma in one or more first-degree relatives;
- Previous personal primary invasive melanoma;
- Multiple banal melanocytic naevi (>100);
- Three or more clinically atypical (dysplastic) naevi;
- High solar exposure in early childhood (before age 10);
- Pale Caucasian skin;
- Red or blond hair;
- Past history of one or more severe blistering sunburns;
- Higher socioeconomic group;
- Past sunbed use, especially before age 30;
- Occupation (airline crew);
- Past pesticide exposure.

Approximately 5 percent of all invasive CMM occur in a familial setting with two or more close relatives affected. This observation indicates that, in a small minority of melanoma patients, low prevalence/high penetrance genes are involved. In addition, the typical phenotype of the melanoma patient, with pale Caucasian skin, red or blond hair and blue eyes indicates that high prevalence/low penetrance genes may interact with environmental factors, particularly with sun exposure.

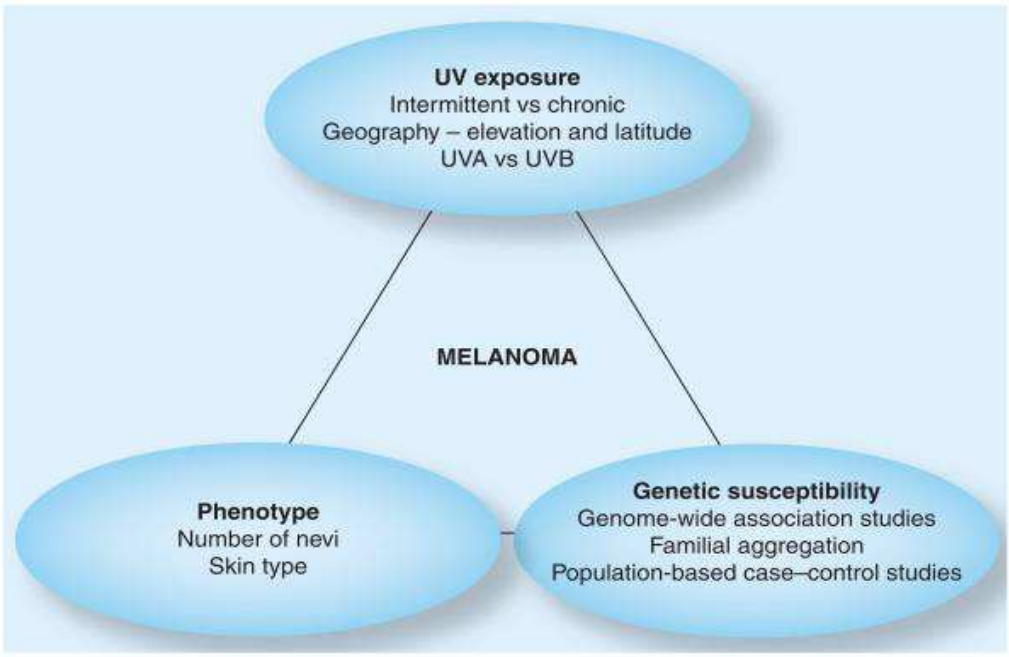


Figure 2. The complex interaction of genetic and environmental factors that concur to melanoma development and progression.

It has been demonstrated that around one-third of patients in melanoma families worldwide have an identifiable germline mutation in CDKN2A, a gene important in controlling entry into the cell cycle. A wide range of mutations has been reported in these families, with concentration of specific mutations in certain geographic areas, such as the Mediterranean, Sweden and Scotland, indicating the likely source of the founder mutation (Pho L. *et al*. 2006). Functional studies on some of these mutations have indicated that they are likely to be a significant causative factor in melanoma development. Nevertheless, in more than 50 percent of all families with pathologically confirmed invasive CMM no putative responsible gene has yet been identified. A number of research groups are currently actively investigating these families for new melanoma susceptibility genes. The principal phenotypic risk factor for melanoma is Caucasian pale-skinned patient. Furthermore, several studies conducted in different countries like Australia (Whiteman DC and Green AC. 2005), North America (Cho E, Rosner BA and Colditz GA. 2005) and Europe (Swerdlow AJ *et al*. 1986) have all shown that a high count of banal melanocytic naevi is a major risk factor for sporadic melanoma. Other independent risk factor for sporadic melanoma is the presence of large, atypical naevi (dysplastic naevi). Sun exposure plays a primary and supporting role in most melanoma tumors. There is evidence that for the four main types of CMM, the pattern of excess sunlight exposure which is most damaging varies (Habif TP *et al*. 1996, Ivry GB *et al*. , MacKie RM. 2006). In the environment the ultraviolet (UV) irradiation present in sunlight is the most important carcinogen for human skin (Matsumu Y and Ananthaswamy HN. 2004). The molecular mechanisms responsible for the carcinogenic effects of UV in human skin are various and not fully understood (Situm M *et al*. 2007). Currently, it is thought that the DNA damaging trough the formation of dimeric photoproducts and gene mutations, inflammatory, and immunosuppressive properties of UVR all contribute to initiation, progression, and metastasis of primary melanoma (Garibyan L and Fisher DE. 2010). In particular, UVB carcinogenicity is ascribed to the ability of this waveband to induce promutagenic DNA lesions, primarily cis-syn cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4) pyrimidone photoproducts ((6-4)PPs) (Pfeifer *et al*. 2005). Reactive oxygen species (ROS) overproduction may stimulate malignant transformation to melanoma. Photodimeric CPDs and (6-4)PPs can induce single C-T or tandem CC-TT transition mutations (Brash *et al*. 1987, Otoshi *et al*. 2000, Pascucci *et al*. 1997, Wang *et al*. 1993), whereas oxidative DNA damage can mainly produce various base substitutions and single-strand breaks (Moriya. 1993, Shibutani *et al*. 1991). Changes in ROS signaling pathways play also important role in the damaging action of UVA and UVB irradiation on the skin. Several studies have also demonstrated that the sunburns are strongly related to the development of melanoma (J. M. Elwood *et al*.1985).

1.4 Staging of cutaneous melanoma

Staging systems for melanoma continue to evolve as our understanding of the complex biology of this disease improves. The official guidelines for staging melanoma were updated in 2009 by the American Joint Committee on Cancer (AJCC) (Balch CM. 2009). This staging system called TNM (tumor-node-metastasis) is based on the following: the thickness of the tumor (the thickness is described using the Breslow scale), whether the tumor is ulcerated (has broken the skin), whether the tumor has spread to the lymph nodes and if the lymph nodes are joined together, whether the tumor has spread to other parts of the body, mitoses within the primary tumor, the site of distance metastasis, level of serum of lactate dehydrogenase (LDH) (Fig.3). The TNM System is the most widely used system for cancer staging in the world. The system defines cancer stage by describing:
T: the features of the primary tumor. The three distinguishing features are tumor thickness, mitoses, and ulceration. Tumor thickness (also known as Breslow depth) is measured in millimeters (mm).

1 mm = .04 inch, or less than 1/16 inch
2 mm = between 1/16 and 1/8 inch
4 mm = between 1/8 and 1/4 inch
N: the presence or absence of tumor spread to nearby lymph nodes
M: the presence or absence of metastasis to distant sites

T Classification	***Thickness***	***Ulceration Status***
Tis	N/A	N/A
T1	≤ 1.0mm	a: w/o ulceration and mitosis < 1 / mm^2 b: with ulceration and mitosis ≥ 1 / mm^2
T2	1.01-2.0mm	a: w/o ulceration b: with ulceration
T3	2.01-4.0mm	a: w/o ulceration b: with ulceration
T4	> 4.0mm	a: w/o ulceration b: with ulceration
N Classification	***# of Metastatic Nodes***	***Nodal Metastatic Mass***
N0	No evidence of lymph node metastasis	
N1	1 node	a: micrometastasis b: macrometastasis
N2	2-3 nodes	a: micrometastasis b: macrometastasis c: In transit metastases/satellites without metastatic nodes
N3	4 or more metastatic nodes, or matted nodes, or in-transit metastases/satellites and metastatic nodes	
M Classification	***Site***	***Serum LDH***
M0	No evidence of metastasis to distant tissues or organs	
M1a	Distant skin, subcutaneous or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases Or any distant metastases	Normal Elevated

Figure 3. TNM staging system of melanoma updated by AJCC in 2009.

1.5 Molecular biology of melanocytes and their transformation into melanoma cells

Melanocytes are pigment producing cells of the skin in humans and other vertebrates. They constitute a heterogeneous group of cells originating from neural crest cells (NCC), capable to produce melanin. Melanocytes are predominately localized in the basal layer of the epidermis, however their presence in other sides of the body are documented. For this reason other functions of melanocytes, a part the production of the pigment melanin, are suggested. Melanocytes seem to have neuroendocrine functions, they play a role in detoxification in the brain, anti-inflammatory activities by reduction and binding of ROS in heart and adipose tissue, balance and hearing in inner hear and cochlea, hair and eyes pigmentation and protection against UV (Plonka PM *et al*. 2009). They are classically considered the cells of the basal layer of epidermis but their presence have also been found in hair, iris, inner ear, nervous system, heart , mucosal membrane and central nervous system (Tachibana M. 1999, Brito FC *et al*. 2008). (Fig.4).

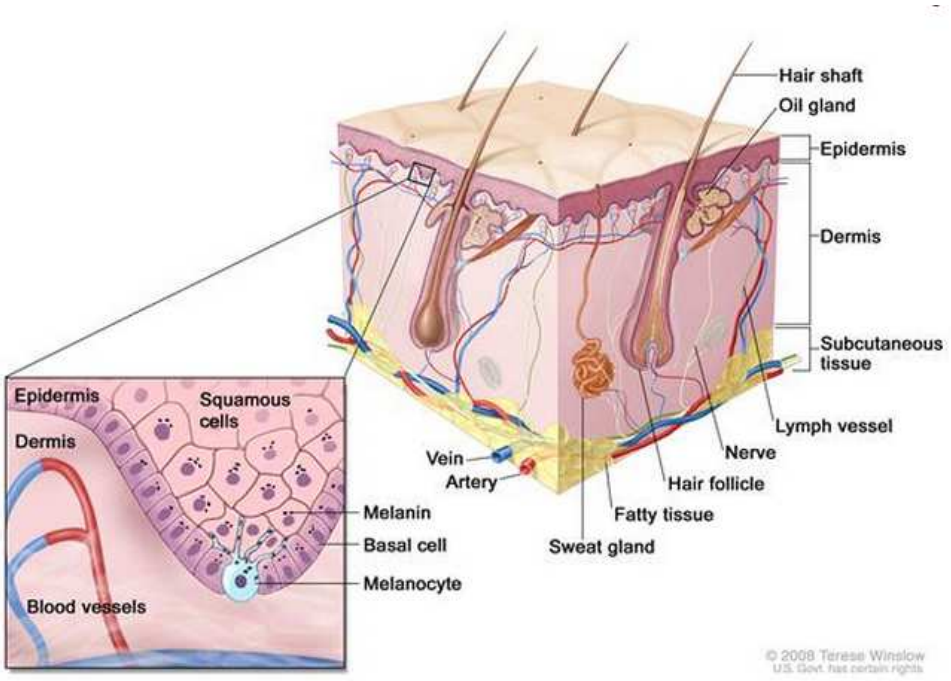


Figure 4. Anatomy of the skin, showing the epidermis, dermis, and subcutaneous tissue. Melanocytes are in the layer of basal cells at the deepest part of the epidermis.

The life cycle of melanocytes consists of several steps including lineage specification from embryonic neural crest cells (melanoblasts), migration and proliferation of melanoblasts, differentiation of melanoblasts into melanocytes, maturation of melanocytes (melanin production in special organelles – melanosomes) transport of mature melanosomes to keratinocytes and eventual cell death. Proliferation and differentiation of melanocytes during development is regulated by numerous genetic and epigenetic factors. Moreover, epigenetic factors from the surrounding tissue environment, such as keratinocytes and

fibroblasts, the pituitary gland, other organs and the blood supply, as well as environmental factors such as ultraviolet (UV) radiation and ionizing radiation are also important for the regulation of melanocytes proliferation and differentiation. In particular seems that keratinocytes are involved in regulating the proliferation and differentiation of melanocytes. *In vitro* experiments have demonstrated that in a culture of proliferating keratinocytes, melanoblasts and melanocytes start to proliferate around the keratinocyte colony, suggesting that keratinocytes produce and release melanocyte mitogens and melanogen in cooperation with basic fibroblast growth factor (bFGF) (Hirobe. 1994). The mechanisms leading to malignant transformation of melanocytes are poorly understood. In developing malignant melanoma, there is a complex interaction of environmental and endogenous (genetic) factors, including: dysregulation of cell proliferation, programmed cell death (apoptosis) and cell to cell interactions. It has been suggested that several genes involved in melanocytes development may also be associated with melanoma cell development (Audrey Uong *et al*. 2010). Even though progress have been made in understanding the molecular biology of malignant melanoma, it is still unclear how a normal melanocyte becomes a melanoma cell. Many evidences clearly indicate the existence of complex molecular machinery that provides checks and balances in normal melanocytes. Progression from normal melanocytes to melanoma cells is the result of a combination of down- or up-regulation of various effectors involved in different molecular pathways. A hypothetical model of melanoma development is represented by the melanoma derived from a pre-existing nevus, which represent about 25% of all melanoma cases. These malignancies develop through a multistep process regulated by a key set of genes. Melanocytes must acquire successive genetic abnormalities before they get a malignant behavior leading to melanoma formation. The figure 2 shows the various stages of melanocytic lesion. In each of these stages a new clone of cells acquire growth advantages over the surrounding tissues (Fig.5). In normal skin there is an homogeneous distribution of dendritic melanocytes within the basal layer of the epidermis. In the early stages, benign melanocytic naevi occur with increased numbers of dendritic melanocytes. According to their localization, naevi are termed junctional, dermal or compound. Some naevi are dysplastic, with morphologically atypical melanocytes. Subsequently, melanoma cells begin to growth in a radial mode, this step is called Radial-growth-phase (RGP) melanoma. This is considered to be the primary malignant stage. The final step of progression is the Vertical-growth-phase (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, by infiltration of the vascular and lymphatic systems. Pagetoid spread describes the upward migration or vertical stacking of melanocytes that is a histological characteristic of melanoma.

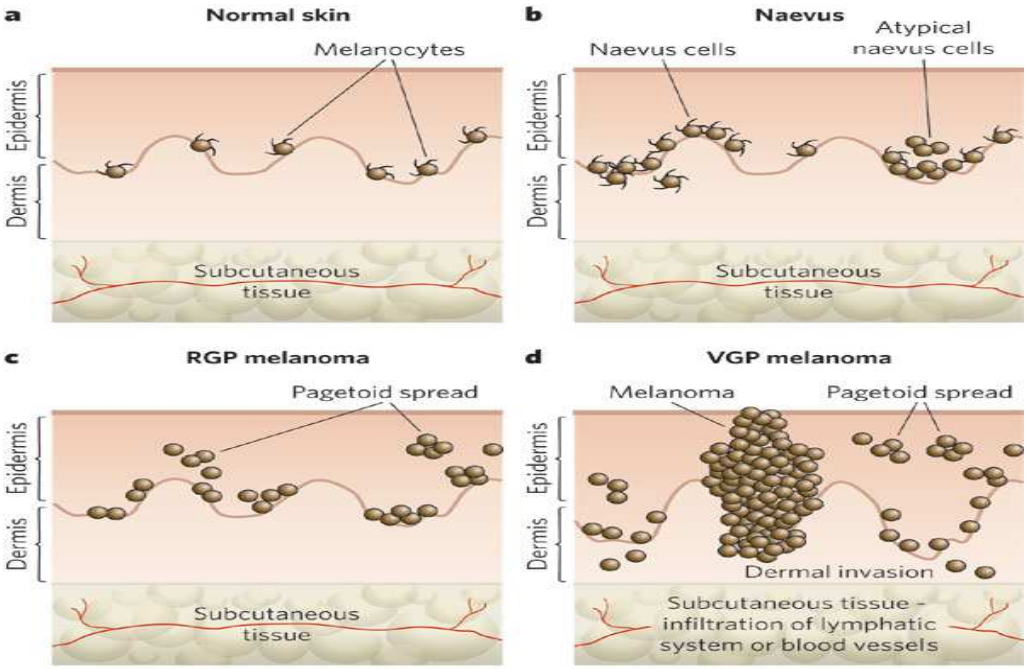


Figure 5. a, Normal skin. **b**, Naevus. **c**, Radial-growth-phase (RGP) melanoma. This is considered to be the primary malignant stage. **d**, Vertical-growth-phase (VGP) melanoma. (figure from the Article "Melanoma biology and new targeted therapy" of Vanessa Gray-Schopfer *et al*. 2007. Nature)

1.6 Molecular bases of CMM

CMM is a complex genetic disease in which several altered genes and molecular pathways are involved. The clinical heterogeneity of melanoma can probably be explained by the existence of distinct types of melanoma with different susceptibility to ultraviolet light.
Cutaneous melanomas, indeed, have four distinct subtypes:

- *Superficial Spreading Melanoma* (SSM), on intermittently exposed skin;
- *Lentigo Maligna Melanoma* (LMM), on chronically exposed skin;
- *Acral Lentiginous Melanoma* (ALM), on the hairless skin of the palms and soles;
- *Nodular Melanoma* (NM), with tumorigenic vertical growth, not associated with macular component.

Many studies conducted over several decades on benign and malignant melanocytic lesions as well as melanoma cell lines have implicated numerous genes in melanoma development and progression. This emerging pattern of molecular complexity in melanoma tumors mirrors the clinical diversity of the disease and highlights the notion that melanoma, like other cancers, is not a single disease but a heterogeneous group of disorders that arise from complex molecular changes. Understanding of molecular aberrations involving important cellular processes, such as cellular signaling networks, cell cycle regulation, and cell death, will be essential for better diagnosis, accurate assessment of prognosis, and rational design of effective therapeutics. The characterization of molecular signature of individual patient's lesions could provide new insights for selection of a personalized therapy and prediction of response to treatment. The principal molecular aberrations affecting functionally relevant cellular processes in the oncogenesis of melanoma, such as cell cycle control and cell-signaling mechanisms are the following:

1.6.1 *CDKN2A (Cyclin-Dependent Kinase inhibitor 2A)*

The best-characterized high-penetrance susceptibility gene predisposing to CMM is *CDKN2A* (N. Ibrahim and F. G. Haluska. 2009, Calder and M. B. Morgan. 2010, A. Sekulic *et al*. 2008). This tumor suppressor gene is located on chromosome 9p21 and encodes two distinct tumor-suppressor proteins (p14/ARF and p16/INK4a) implicated in the pathogenesis of 25-40 per cent of familial CMM. Over 60 different germline mutations in *CDKN2A* have been detected in more than 190 families wordwilde. The majority of these mutations are missense mutations in $p16^{CDKN2A}$ (Goldstein AM . *et al* 2006). To date, germline large deletions have been characterised at the 9p21 locus in only six families worldwide. A deletion involving *CDKN2A* exon1a, 2, and 3 and a deletion removing exon 1α and half of exon 2 were described in two melanoma-prone kindreds, originated from UK and from Norway, respectively (Mistry *et al*. 2005; Knappskog *et al*. 2006). Large deletions have also been found in families with combined proneness to melanoma and nervous system tumours (NST): a gross deletion ablating the whole *CDKN2A* and *CDKN2B*

genes has been reported in a French family (Bahuau *et al*. 1998; Pasmant *et al*. 2007), and a deletion of p14ARF-specific exon 1b of the CDKN2A gene has been found in one US family and in two UK families (Bahuau *et al*. 1998; Randerson-Moor *et al*. 2001; Mistry *et al*. 2005; Laud *et al*. 2006).

The $p16^{CDKN2A}$ protein inhibits the activity of the cyclin D1-cyclin-dependent kinase 4 (CDK4) complex, that drives cell cycle progression by phosphorylating the retinoblastoma (RB) protein. Thus, $p16^{CDKN2A}$ induces cell cycle arrest at G1 phase, blocking the RB protein phosphorylation. RB phosphorylation causes the release of the E2F transcription factor, which binds the promoters of target genes, stimulating the synthesis of proteins necessary for cell division. Normally the RB protein prevents the cell division. When the RB protein is absent or inactivated by phosphorilation, there is a promotion of the cell cycle progression (Pacifico A. and Leone G. 2007).

$p14^{CDKN2A}$ stabilizes p53, interacting with the Murine Double Minute (MDM2) protein, whose principal function is to promote the ubiquitin-mediated degradation of the p53 tumor suppressor gene product (Stott FJ. *et al*. 1998, Tsao H. *et al*. 2000, Piepkorn M. 2000). The p53 protein arrests cell division at G1 phase to allow DNA repair or to induce apoptosis of potentially transformed cells. In normal conditions, the expression levels of p53 in cells are low. In response to DNA damage, p53 accumulates and prevents cell division. Therefore, inactivation of the *TP53* gene results in an accumulation of genetic damage in cells which promotes tumor formation. In melanoma, the frequency of *TP53* mutations is low (Box NF and Terzian T. 2008). Different signals regulate p53 levels by controlling its binding with MDM2. Several kinases play this role, catalyzing stress-induced phosphorylation of serine in the trans-activation domain of p53. Moreover, several proteins, including E2F, stabilize p53 through the $p14^{CDKN2A}$-mediated pathway.
Data obtained from genetic and molecular studies over the past few years have indicated that the *CDKN2A* locus as the principal and rate-limiting target of UV radiation in melanoma formation (Goldstein AM. *et al*. 2005).

1.6.2 *CDK4* *(Cyclin-Dependent Kinase-4)*

It represents another high-penetrance melanoma susceptibility gene. Only three melanoma families worldwide are carriers of mutations in *CDK4* (Arg24Cys and Arg24His) (K.D. Meyle and P. Guldberg. 2009). Located on chromosome 12q14, *CDK4* encodes cyclin-dependent kinase 4 protein, a constituent of the complex *CDK4/6*. The Arg24Cys makes the $p16^{CDK2NA}$ protein unable to inhibit the D1-ciclyn-*CDK4* complex, resulting in a sort of oncogenic activation of *CDK4*.

1.6.3 RAS/RAF/MEK/ERK Signaling Pathway: the role of BRAF in melanoma

Among the signaling pathways that are constitutively activated in melanoma, mitogen-activated protein kinase (MAPK) pathway has been considered one of the most attractive targets for treatment (Satyamoorthy *et al*. 2003, Sharma *et al*. 2006, Smalley *et al*. 2006, Solit *et al*. 2006). This pathway represents the major signaling cascade involved in the control of cell growth, survival, proliferation and migration and it seems to be implicated in rapid melanoma growth, enhanced cell survival and resistance to apoptosis playing a major role in both development and progression of melanoma and seems (Davies H. *et al*. 2002, MMMP). When active in its GTP-bound state, *RAS* activates a number of downstream effectors, one of which is the *RAF* family of serine/threonine kinases. There are three isoforms of *RAF*, namely, *A-Raf*, *BRAF*, and *CRAF* (also called *Raf-1*). Once activated, *RAF* stimulates the MAPK cascade, resulting in the sequential activation of MEK1 and MEK2, which in turn activates ERK1 and ERK2 (Crews *et al*. 1992; Kyriakis *et al*. 1992). Once activated, the ERKs either activate cytoplasmic targets or migrate to the nucleus, where they phosphorylate transcription factors.

In melanocytes, the MAPK pathway is activated by growth factors released from the local microenvironment and through receptor tyrosine kinases activation. Under physiological conditions, these growth factors only induce a weak stimulation of the MAPK pathway that is insufficient to induce melanocyte proliferation. In most melanoma cells, the situation is very different and it has been shown that >90 per cent of clinical melanoma specimens have continuous hyperactivity in the MAPK pathway (Cohen *et al*. 2002). Although MAPK activity in melanoma cells can arise through autocrine growth factor stimulation (Nesbit *et al*. 1999), N-cadherin-based homotypic cell–cell adhesion (Li *et al*. 2001), and melanoma cell–matrix adhesion, it is more commonly activated after the acquisition of an activating oncogenic mutation.The first such MAPK-activating mutation to be reported in melanoma was in *NRAS* (Padua *et al*. 1984). Mutations in *NRAS* have since been identified in 15–20 per cent of all melanomas, and are most commonly the result of the substitution from leucine to glutamine at position 61.

The most common mutation to be reported in melanoma thus far is in *BRAF*, the serine–threonine kinase located downstream of *NRAS*. In fact, approximately 50 per cent of melanomas harbor activating *BRAF* mutations. Among the *BRAF* mutations observed in melanoma, over 90 per cent are at the codon 600, and among these, over 90 per cent are a single nucleotide mutation resulting in a substitution of a glutamic acid to a valine (*BRAF* V600E: nucleotide 1799 T>A; codon GTG>AAG). The second most common mutation is *BRAF* V600K substituting lysine for valine, that represents 5-6 per cent (GTG>AAG), followed by *BRAF* V600R (GTG>AGG), an infrequent two-nucleotide variation of the predominant mutation, *BRAF* V600 'E2' (GTG>GAA), and *BRAF* V600D (GTG>GAT) (Catalogue of Somatic Mutation in Cancer, COSMIC). The *BRAF* V600E mutation activates *BRAF* and induces constitutive MEK-ERK signaling in cells (Davies H. *et al*. 2002, Wan PT. *et al*. 2004) (Fig.6). The presence of *BRAF* mutations in nevi strongly suggests that *BRAF* activation is necessary but not sufficient for the development of melanoma. Acquisition of *BRAF* V600E mutation seems to be

an early event in melanoma development, moreover *BRAF* mutations occur at high frequency in melanomas that are strongly linked to intermittent sun exposure. *BRAF* V600E has been implicated in different mechanisms of melanoma progression, and principally, in addition to the MAPK pathway activation, evasion of senescence and apoptosis, unchecked replicative potential, angiogenesis. No clear differences in prognosis were noted between *BRAF*-mutated versus wild-type melanomas. Features of the antecedent primary melanoma significantly associated with *BRAF* mutation were the superficial spreading and nodular hisopatological subtypes, the presence of mitoses, the presence of occult primary melanoma, a truncal location and the age at the diagnosis of the primary tumor (<50 years).

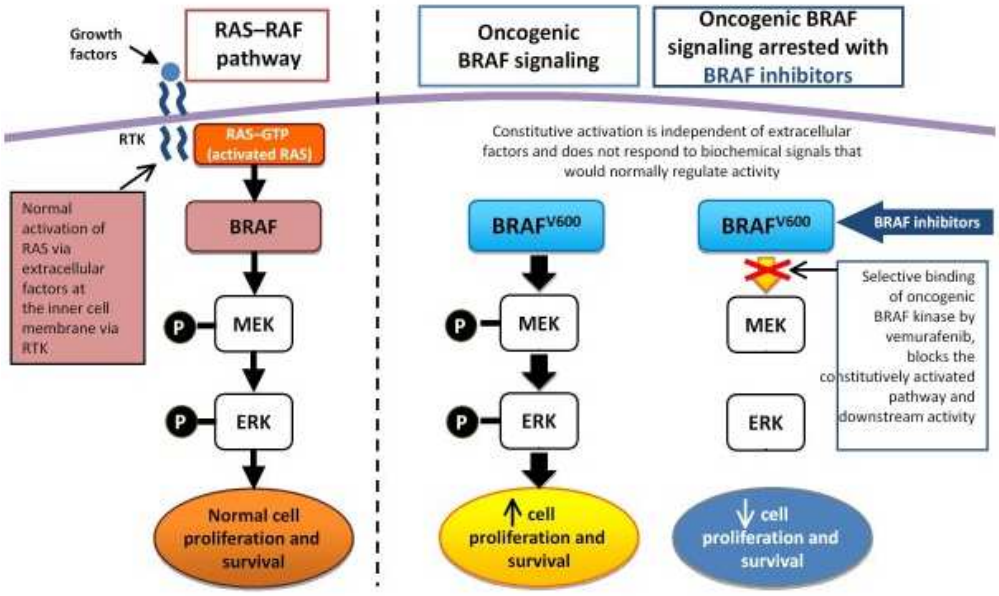


Figure 6. Oncogenic *BRAF* signaling pathway

1.6.4 PTEN (phosphatase and tensin homolg) and PI3K/AKT Pathway

Among the other mutated genes and pathways that may be equally important for melanoma development and progression there are the PTEN gene and PI3/AKT pathway. Activation of PI3/AKT pathway in melanoma occurs through either paracrine/autocrine growth factors or less of expression and/or mutation of negative pathway regulators (PTEN). In particular, the insuine-like growth factor-I is known to aid the growth of early stage melanoma cells, at least in part, through the activation of PI3/AKT pathway (Satyamoorthy *et al*. 2002). Activation of AKT pathway stimulates cell cycle progression, survival, metabolism and migration through phosphorylation of many physiological substrates (Stokoe D. 2001, Dania PL. 2000, Kandel ES and Hay N. 1999, Downward J. 2004). It has been proposed that a common mechanism of activation of AKT is DNA copy gain involving the Akt3 locus, which is found in 40-60 per cent of melanomas (it leads to a selective constitutive activation in AKT3). AKT expression strongly correlates with melanoma progression, and depletion of AKT3 induces apoptosis in melanoma cells and reduces the growth of xenografts (Staal SP. 1984, Stahl JM. *et al*. 2004). One of the most critical regulator of AKT is PTEN, that degrades the products of PI3K, thereby preventing the activation of AKT. However, the mechanism by which PI3K pathway is activated in melanoma remains not fully elucidated, but may involve the loss of expression or functional inactivation of PTEN (Fig.7).

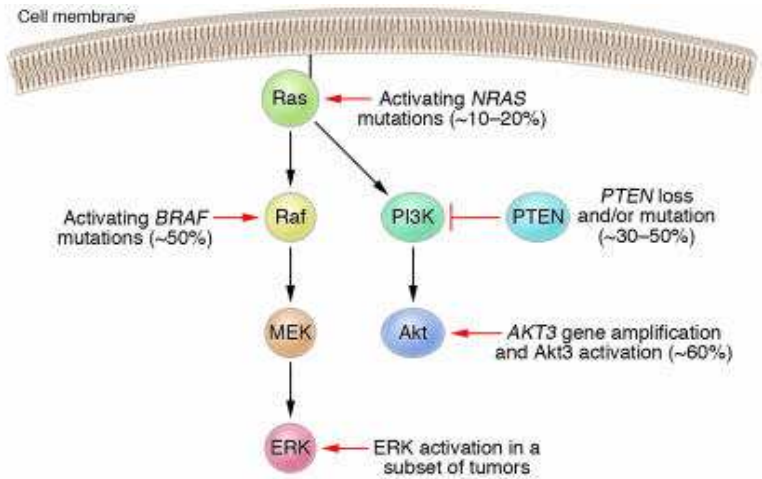


Figure 7. Schematic of the canonical *Ras* effector pathways Raf-MEK-ERK and PI3K-Akt in melanoma.

1.6.5 mTOR (mammalian target of rapamycin) pathway

Another signaling cascade intimately linked to the PI3K/AKT pathway and whose hyperactivation is involved in melanoma pathogenesis, is the mTOR pathway. mTOR signaling involves the activity of two signaling complexes, mTORC1 and mTORC2. Increased activity in these two downstream pathway components leads to increased protein translation and cell proliferation (Fig.8). The two different mTORC complexes have opposite effects on AKT signaling, with mTORC1 suppressing AKT signaling and mTORC2 directly activating AKT through a phosphorylation event at Ser473 (Sarbassov *et al*. 2004). There is also evidence that mTORC1 inhibition may lead to increased PI3K/AKT signaling through the upregulated expression of the insulin-like growth factor-I adaptor protein IRS2 (Tamburini *et al*. 2008). mTOR signaling is known to be active in melanoma, with immunohistochemical studies showing the constitutive phosphorylation of p70 S6 kinase in a panel of metastatic melanoma samples (Karbowniczek *et al*. 2008). Furthermore, results from other groups also indicate that the activation of mTOR pathway is related with MAPK pathway activation in melanoma. Some evidences suggest that the mTOR pathway activation seems to be associated with worse prognosis, especially in conjunctival melanomas. Overall, the alterations in major components of the MAPK, such as *BRAF* and *NRAS* mutations, and mTOR pathways, *PTEN* loss and AKT overexpression, seem to have substantial influence in melanoma progression, being both pathways linked to survival and chemoresistence in melanoma (Guertin DA. *et al*. 2007).

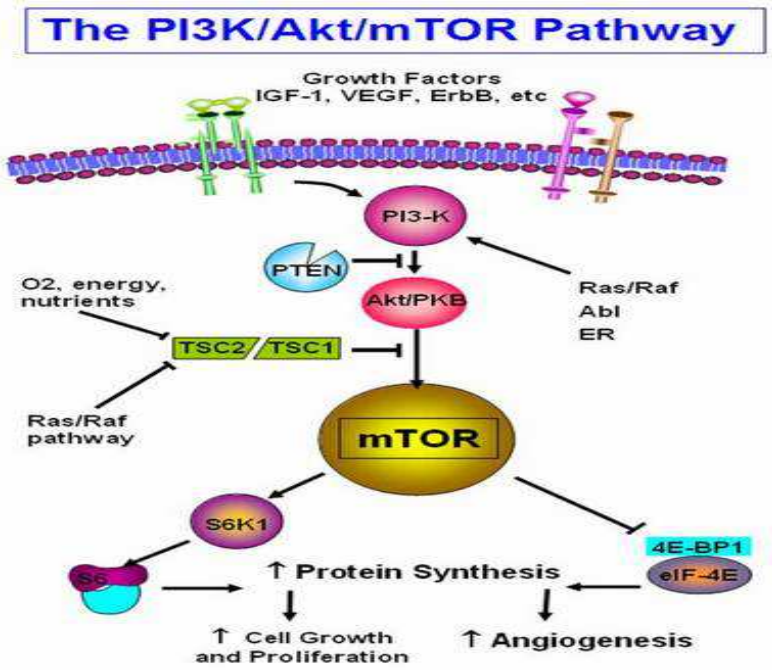


Figure 8. Schematic representation of mTOR pathway. The two best-characterized mTORC1 substrates, elongation factor 4e-binding protein 1 (4e-BP1) and ribosomal protein S6 kinase-1 (S6K1), are components of the translational control machinery and mediate cap-dependent translation and ribosome biogenesis, respectively.

1.6.6 MITF (microphthalmia-associated transcription factor)

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. MITF, in addition to its involvement into the differentiation pathways such as pigmentation, may play an important role in the proliferation and/or survival of developing melanocytes, contributing to melanocyte differentiation by triggering cell cycle exit. 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 (Wellbrock C and Marais R . 2005). In this regard, low levels of MITF protein were found in invasive melanoma cells (Hoek KS. *et al*. 2008) and have been associated with poor prognosis and clinical disease progression (Salti GI- *et al*. 2000).

1.7 Current therapies for CMM

Melanoma is an extremely aggressive disease with high metastatic potential and a notoriously high resistance to citotoxic agents. This is thought to be because melanocytes originate from highly motile cells that have high enhanced survival properties. Melanoma cells have low levels of spontaneous apoptosis *in vivo* compared with other tumor cell types, and relatively resistant to drug-induced apoptosis *in vitro* (Soengas M. and Lowe S. 2003). There are several approved postoperative adjuvant therapies for malignant melanoma like chemotherapy (dacarbazine, DTIC) and immunotherapy (Interferon-a, interleukin-2, ipilimumab).

1.7.1 Chemotherapy

DTIC: Chemotherapy continues to be an important tool in the treatment of melanoma. While not having demonstrated an overall survival benefit, chemotherapy has a clear role for palliation of patients with melanoma (Lee SM. 1995). Multiple chemotherapeutics have been evaluated in the treatment of advanced melanoma however only DTIC has been approved for use by the Food and Drug Administration (FDA). DTIC, and the analog drug temozolomide, are alkylating agents that damage DNA by introducing alkyl groups to guanine bases, eventually cell death via apoptosis and other cell death mechanisms. DTIC has become the “standard of care” benchmark for the treatment of metastatic melanoma. However, the drug has never been shown in a randomized phase III trial to improve overall survival. Generally, DTIC is associated with a response rate of approximately 10–20 per cent and a progression-free survival of approximately three to six months (Crosby T. *et al*. 2000). The side effect profile of DTIC is predominately dictated by nausea, vomiting and bone marrow suppression in the form of leucopenia and anemia. In a phase III trial of 305 patients, temozolomide (TMZ) was not found to be significantly more efficacious as compared to DTIC in terms of overall survival (OS), 7.7 versus 6.4 months, respectively, and progression-free survival (PFS), 1.9 months versus 1.5 months, respectively (Middleton MR. *et al*. 2000). A second phase III trial of TMZ versus DTIC in 859 patients confirmed this, showing no difference in OS, 9.1 versus 9.4 months or PFS, 2.3 versus 2.2 months, respectively) (Patel PM *et al*.2001). Despite various attempts to improve the efficacy of TMZ, it remains approximately that of DTIC. The major advantage of TMZ is the ease of dosing given the oral formulation of the drug. Despite this advantage, TMZ has not achieved FDA approval for the treatment of metastatic melanoma.Others classes of alkylating and cytotoxic agents with documented activity in melanoma are nitrosoureas, microtubule disrupting agent, taxanes and platinum. However, none of these agents has been approved by the FDA for treatment of advanced melanoma, except fotemustine that has been approved by some European regulators.Most chemotherapeutic drugs function by inducing apoptosis in malignant cells, so resistant 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. On the basis of these

principles, several new targeted agents are currently being evaluated and tested alone or in combination with conventional chemotherapy.

1.7.2 Targeted therapies in melanoma

The identification of activating mutations in melanoma, combined with a growing appreciation of the different pattern of genetic changes in the anatomically defined melanoma subtypes, has become the focus of a concerted effort to translate these discoveries into personalized therapeutic approaches for melanoma. Novel ways to modulate the immune system by monoclonal antibodies as well as various signalling pathway inhibitors are responsible for creating a whole new therapeutic landscape. Several novel targets are currently being investigated in melanoma. The increasing of knowledge of the molecular alterations associated with melanoma progression provides rational druggable targets for development of novel therapeutic strategies, including alterations in key intracellular signalling pathways and growth factor receptors.

BRAF inhibitors: A number of *BRAF* inhibitors are currently under clinical development and evaluation. Sorafenib (BAY43-9006, Bayer) is a bi-aryl urea small molecule broad-spectrum kinase inhibitor. It is able to inhibits the vascular endothelial growth factor receptor (VEGFR) and *RAF* kinase, which also has activity against *C-Kit* and platelet derived growth factor receptor beta (PDGFR-β). Activity against melanoma was demonstrated in phase I studies, and so it was further developed for this indication in combination with the usual combination of carboplatin and paclitaxel. The response rate in phase I trial was over 30 per cent, and so it was evaluated also in phase II and III trials that moreover failed. The addition of sorafenib to carboplatin and paclitaxel did not improve any of the relevant end points over placebo in advanced melanoma patients. Studies of sorafenib indicate that it lacks of selectivity and potency for *RAF*, and it is highly potent inhibitor of VEGFR2, VEGFR3, and several other kinases (Wilhelm SM. *et al*. 2004).

Vemurafenib (PLX3042): Vemurafenib is a potent and specific inhibitor of *BRAF* with the V600E mutation (Fig.9). It has marked antitumor effects against melanoma cell lines with the *BRAF* V600E mutation only. It is inactive in the cell lines with wild type *BRAF* (Flaherty KT, *et al*. 2010). Flaherty *et al*. conducted a Phase I and II trials for vemurafenib study in patients with unresectable, previously untreated stage IIIC or stage IV melanoma that tested positive for the *BRAF* V600E mutation. A phase I trial established the maximum tolerated dose to be 960 mg twice daily which showed responses against the tumor. A phase 2 trial involving patients who had received previous treatment for melanoma with the *BRAF* V600E mutation displayed a confirmed response rate of 53 per cent, with a median duration of response of 6.7 months. The levels of phosphorylated extracellular signal- regulated kinase (ERK), cyclin D1, and Ki-67 were markedly reduced at day 15 as compared with baseline in all specimens examined. This study proposed that vemurafenib inhibited the MAP kinase pathway, resulting in decreased cyclin D1 levels and decreased proliferation. Subsequently, Phase III trial was conducted in 680 patients with previously untreated, unresectable stage IIIC or stage IV

melanoma with *BRAF* V600E mutations. The patients were randomized to vemurafenib or DTIC (Guo J, *et al*. 2011). There was an increase in median survival from 8 months for DTIC to 12.3 months for vemurafenib (Chapman PB. *et al*. 2011). A total of 672 patients were evaluated for OS. At 6 months, OS was 84 per cent in the vemurafenib group compared to 64 per cent in the DTIC group. Estimated median progression-free survival (PFS) in the vemurafenib group and in the DTIC group was 5.3 months and 1.6 months respectively. The most common adverse events in the vemurafenib group were cutaneous events, arthralgias, and fatigue; photosensitivity skin reactions of grade 2 or 3 were seen in 12 per cent of the patients. Among patients treated with vemurafenib, 18 per cent were reported to have at least one squamous-cell carcinoma of the skin or keratoacanthoma. Vemurafenib displayed a relative reduction of 63 per cent in the risk of death and of 74 per cent in the risk of tumor progression in untreated, unresectable stage IIIC or stage IV melanoma with the *BRAF* V600E mutation, in comparison with treatment with DTIC. Vemurafenib 960 mg, orally administered twice daily was approved by FDA in 2011 to treat patients with metastatic or unresectable melanoma.

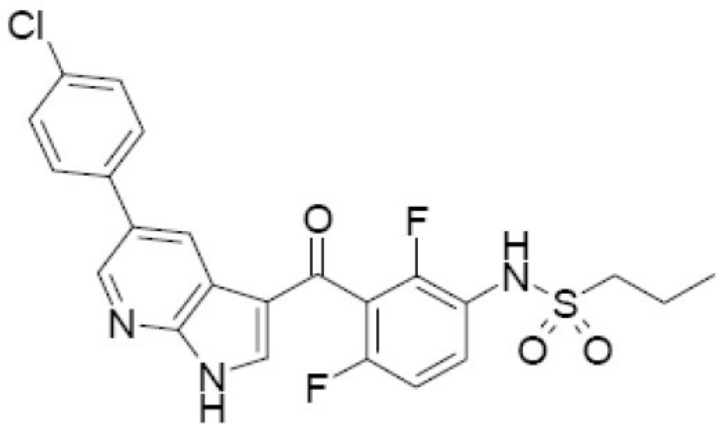


Figure 9. The structural formula of vemurafenib

Currently, several other studies are ongoing for the evaluation of different classes of *BRAF* inhibitors: dobrafenib that is a selective kinase inhibitor that is active against several mutated forms of *BRAF* kinase including *BRAF* V600E, *BRAF* V600K, and *BRAF* V600D or Trametinib that reversibly and selectively inhibits the activation of mitogen-activated extracellular signal regulated kinase (MEK) 1 and MEK2 and inhibits their kinase activity.

1.7.3 Immunotherapy

Ipilimumab: Melanoma is characterized as one of the most immunogenic tumors due to the presence of tumor infiltrating lymphocytes in resected melanoma, occasional spontaneous regressions, and clinical responses to immune stimulation. The immunogenicity of melanoma has led investigators to study novel immune strategies to overcome tumor immune evasion. One

mechanism by which T cells self-regulate their activation is through expression of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). CTLA-4 functions as a negative co-stimulatory molecule for the T cell, and therapies that antagonize CTLA-4 remove the brakes from the T cell leading to a net effect of T cell hyper-responsiveness. In march 2011 FDA approved the cancer immunotherapy drug ipilimumab (yervoy) for metastatic melanoma patients. Ipilimumab is human IgG1 monoclonal antibody that blocks CTLA-4, thereby increasing T-cell activity and promoting antitumor activity. Two phase 3 randomized clinical trials have evaluated ipilimumab in metastatic melanoma. In the first trial of patients with previously treated unresectable stage III or IV melanoma, ipilimumab demonstrated an improved overall survival versus glycoprotein 100 peptide vaccine (gp100) (10.1 *vs* 6.4 months) (Robert C. *et al*. 2011). In the second phase 3 trial in previously untreated patients with metastatic melanoma, ipilimumab plus DTIC demonstrated improvement in OS versus single agent DTIC (11.2 vs 9.2 months. In both phase 3 studies, the response rate, complete response (CR) and partial response (PR) was only 10 per cent to 15 per cent and the disease control rate (CR, PR, and stable disease (SD) was approximately 30 per cent. In addition, the improvement in percent of patients alive at one and two years is consistently 10 per cent better than the non-ipilimumab containing arms. While the response rate and improvement in OS in ipilimumab is relatively modest, the toxicities of the therapy, including immune-related enterocolitis, hepatitis, and dermatitis, are highly manageable.

1.8 Neuroendocrine differentiation of melanoma cells

Neuroendocrine tumors (NETs) are a heterogeneous group of neoplasms which take origin from the neuroendocrine cell system and are characterized by embryological, biological and histopathological differences. Traditionally considered as a rare and "niche" pathology, over the last decades they have gained significant attention from the scientific community, even because of their increasing incidence and prevalence probably imputable to the availability of more sensitive diagnostic tools and to the development of higher awareness among clinicians. However, commonly, neuroendocrine tumors seem to be characterized by the secretion of specific markers (for example Chromogranin, synapsin…) and a distinctive pattern of receptors expression. The term "neuroendocrine" has been used to define the phenotype of cells that secrete their products in a regulated manner, in response to a specific stimulus. Neuroendocrine features have been used as evidence of a common embryological origin for normal and neoplastic cells. However, it is now recognized that neuroendocrine characteristics can be observed in various cell types that do not have a common embryological origin with neurons and endocrine cells. Although melanoma is not commonly classified as neuroendocrine tumor, some subtypes of melanoma can exhibit a neuroendocrine phenotype. Moreover, another feature of melanoma as neuroendocrine tumor is the expression of SST receptors (SSTRs), G protein-coupled receptors with inhibitory capacity growth, proliferation and secretion in some NETs. Although the evaluation of the distribution of SSTRs by imaging with ^{111}In-pentetreotide scintigraphy (OctreoScan) has limited sensitivity for localizing melanomas, tumors that can be imaged by OctreoScan may be amenable to adjuvant therapy with octreotide or targeted therapy with high-energy radioisotope-labeled octreotide (Sharon S. Lum. 2001). In 2005, Eyden B. *et al*., demonstrated, by immunohistochemistry experiments(IHC) , that malignant melanoma showed a neuroendocrine differentiation. In addition to expression of typical melanoma markers such as S100 protein, HMB-45 and melan-A, melanoma cells may be shown to express a range of neuroendocrine markers, including synaptophysin, chromogranin, neurofilament protein, CD56, VIP and GFAP. Ultrastructure may reveal typical membrane-bound neurosecretory granules (Eyden B. 2005, Banerjee SS. And Eyden B. 2007). However, the knowledge about the “neuroendocrine” aspects of melanoma cells are sill poor and further studies are required. More than 15 years ago, it has been proposed for the first time that melanocytes are the sensory and regulatory cells with computing and amplifying capabilities, which detect and transform external and/or internal signals/energy into organized regulatory network(s) for the maintenance of the cutaneous homeostasis. This concept is in agreement with a hypothesis that melanocytes are ‘neurons of the skin’ formulated by Aaron B. Lerner. Melanocytes produce classical stress neurotransmitters, neuropeptides and hormones, and that this production is stimulated by ultraviolet radiation, biological factors and other agents that act within the skin neuroendocrine system. Furthermore, their production is not random, but hierarchical and follows the structures of classical neuroendocrine organizations such as hypothalamic-pituitary-adrenal axis, serotoninergic, melatoninergic and catecholaminergic systems. An example of an intrinsic but overlooked neuroendocrine activity is production and secretion of

melanogenesis intermediates including L-DOPA or its derivatives that could enter circulation and act on distant sites. Such capabilities have defined melanocytes as neuroendocrine cells that not only coordinate cutaneous but also can affect a global homeostasis.

1.8.1 Somatostatin and cancer

Somatostatin, SST (also known as growth hormone-inhibiting hormone (GHIH) or somatotropin release-inhibiting factor (SRIF) is a peptide hormone that regulates the endocrine system and affects neurotransmission, cell proliferation and numerous secondary hormones release inhibition. Two active biologically forms derive from the C-terminus portion of a single pro-peptide: SST-14 and SST-28. SST acts on its multiple cell targets via a family of six receptors that originate from five genes: SSTR1, SSTR2a, SSTR2b, SSTR3, SSTR4, SSTR5. SSTR2 is alternatively spliced at its C-terminus producing the SSTR2a and the SSTR2b variants that have a somewhat different tissue distribution. Besides their expression in normal tissues, SSTRs have been identified in tumor cell of different aetiology including pituitary, pancreatic, breast and hematopoietic tissues. Moreover, the majority of human tumors do express SSTRs, often more than one receptor subtype (Hofland, L.J. and S.W. Lamberts. 2001). As mentioned above, the SSTRs are members of the G-proteins coupled receptors (GPCRs) superfamily and so modulate cellular function through multiple pathways coupled to G-protein dependent signalling pathways. The different signaling pathways activated by the various SSTRs subtypes vary according to the receptor subtype and tissue localization. However, all SSTRs subtypes inhibit Adenylate Cyclase and cAMP production upon ligand binding (Patel, Y.C. 1999). All of the pleiotropic effects of SST in the different target tissues can be explained by two basic biological mechanisms: inhibition of secretion and inhibition of proliferation. As already mentioned, the SST peptides inhibit secretion (of neurotransmitters or hormones) from cells in different tissues such as the pituitary gland, the endocrine pancreas and the stomach. The molecular mechanism by which SST exerts its inhibitory effects on cell secretion, is still a matter of intense study and may vary between the different cell types. However, it is generally accepted that after binding its receptors, SST or SST analogs, active an intracellular trasductional message that downregulates the enzyme Adenilate Cyclase, which in turn inactives Protein Kinase A (PKA), leading to an intracellular decrease of both cAMP and Ca^{2+}. As reported by literature this signalling is mainly responsible for the secretion inhibition, with some effects due to the activation of phosphatases such as calcineurin (Bousquet, C. *et al*. 2001). Another intracellular pathway activated by this neuropeptide, in fact, shows the upregulation of some phosphatases belonging to different families such as serin-threonin kinases (PTPases, SHP-1 and SHP-2). SST and its analogs also exert antitumor activity through direct and indirect mechanisms, acting through SSTRs, which are found on tumor cells and cells in the tumor microenvironment (Fig.10).

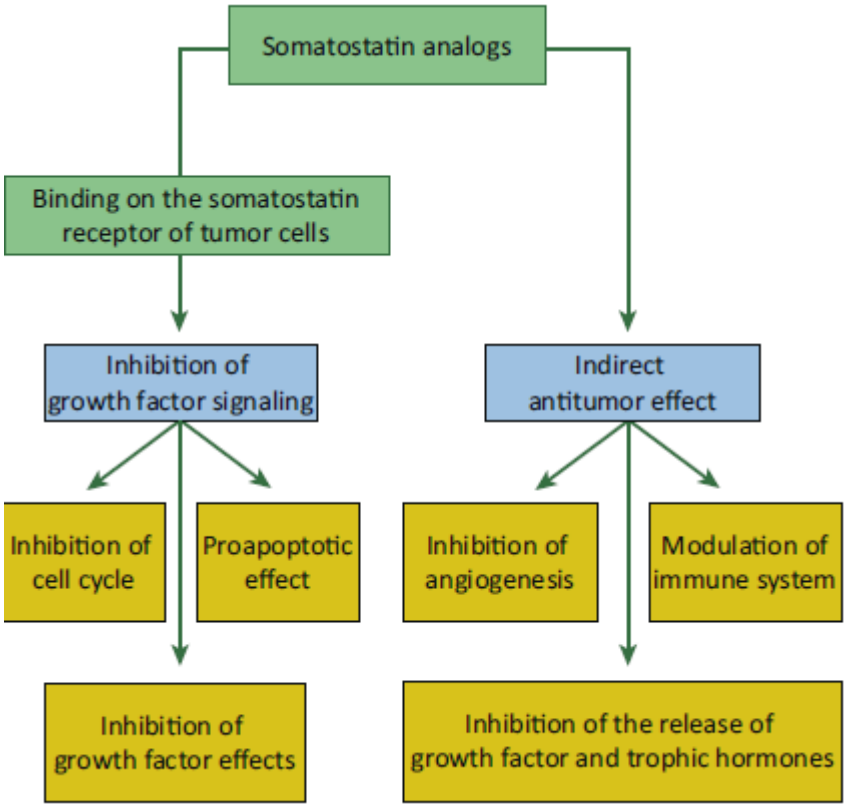


Figure 10. Antitumor effects of SST analogs. SST analogs exert antitumor activity through both direct and indirect mechanisms through SSTRs.

The direct anti-proliferative effects of SST and SST analogs are largely believed to be due to the activation of tyrosine phosphatases that dephosphorylate (and inhibit) growth factor receptors. In addition, the SST-mediated activation of phosphatases regulates more distal signalling pathways such as the MAPK pathway. Addition of SST (or synthetic analogs) to SSTRs expressing proliferating cells usually produces cell growth arrest at the G1 phase of cell cycle (Benali, N., *et al.* 2000). Interestingly, in some cells, activation of the SSTR2 and SSTR3 subtypes induced apoptosis and cell death rather than growth arrest through activation and upregulation of the tumor suppressor p53 and the pro-apoptotic protein Bax (Sharma, K. and C.B. Srikant. 1998). In fact, the expression of SSTRs in several human tumors was so pervasive that it helped to create an entire new field in oncology: peptide therapy. SST analogs have also been used in direct tumor reduction with 90Y radiolabeled analogs and in the symptomatic treatment of hormone secreting tumor (Reubi, J.C. 2003, Kaltsas, G.A. *et al.* 2005). Indirect antitumor effects of SSAs result from suppression of the secretion of growth or angiogenic factors. Angio-inhibitory action of SSTR2 in tumors (such as pancreatic cancer) involves the upregulation of the expression of thrombospondin-1 (TSP-1), a potent antiangiogenic factor. TSP-1 inactivates the angiogenic effects of VEGF and therefore plays a crucial role in SSTR2 tumor-suppressive activity on pancreatic tumor growth (Bevan, J.S. 2005) (Fig 11).

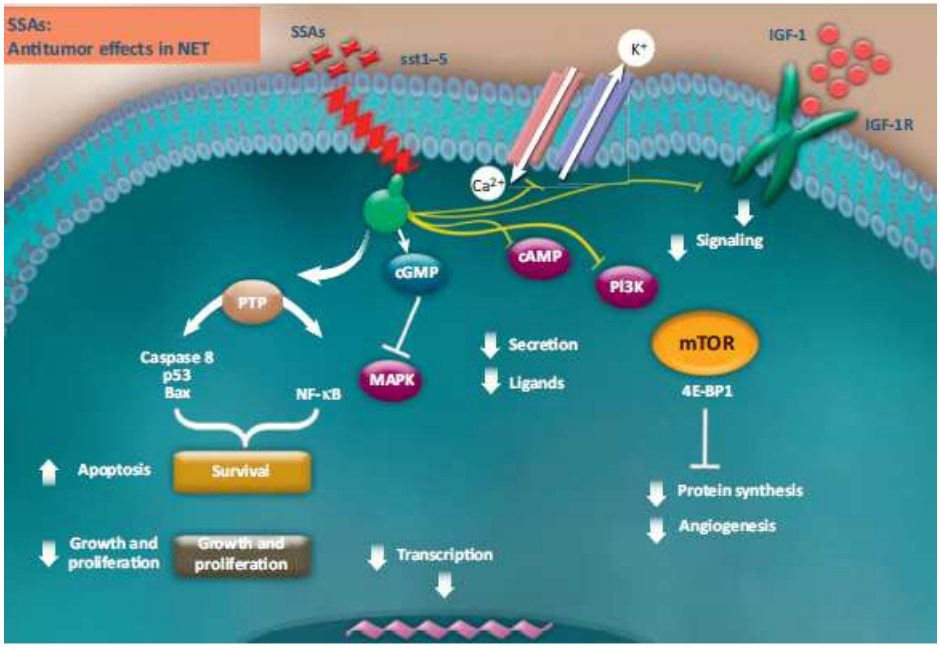


Figure 11. Mechanisms of antitumor activity of synthetic SST analogs in neuroendocrine tumors (NET).

After agonist activation, GPCRs are phosphorylated (involving protein kinase A, protein kinase C, and GPCR kinases) and internalized, probably via the formation of clathrin-coated pits (involving β-arrestins). The internalized receptors are then directed to endosomes in which they are dephosphorylated. Subsequently, the receptors are recycled back to the plasma membrane as functional (resensitized) receptors. GPCR downregulation results from lysosomal degradation of intracellular receptors, decreased mRNA 22 and receptor protein synthesis, as well as increased degradation via mobilization of membrane receptors directly to the lysosomal compartment (Jacobs S. and S. Schulz. 2008, Tulipano G. *et al*. 2004, Liu Q. *et al*. 2005). Actually, the presence and the role of SSTRs in CMM is not yet clarified because controversial results on SSTRs expression and the anti-proliferative effects of SST analogs in melanoma have been obtained.

Early in 1995, was hypothesized that malignant melanoma, being of neural crest origin, might contain SSTRs (Williams S. *et al*. 1997). The following effective finding that some melanoma contain SSTRs confirm that some subtypes of melanoma present neuroendocrine feature and implies some host control of melanoma growth. M Martinez-Alonzo *et al*. evaluated the SSTRs expression in 18 human skin melanoma cell lines. Their results, in agreement with previous studies, showing that SSTR2 is the most abundantly expressed SSTR in the majority of tumor types (Hofland Lj *et al*. 2001) and in cutaneous and uveal melanoma too (Navid Ardjomand *et al*. 2003, Lum SS. *et al*. 2001, Hofland Lj *et al*. 2001). They also test the effects of two SST analogs, octreotide and pasireotide (SOM230), on cell proliferation. The SAs investigated in this study, did not, however, significantly inhibit melanoma growth or induce cell death. Since the generally reported high expression of SSTRs in neuroendocrine tumors (NET), SAs have a pronounced role in the medical therapy for this class of tumors, especially pituitary adenomas and well-differentiated gastroentero pancreatic NET (GEP NET). The findings of

SSTRs in melanoma, lead to hypothesize the use of SAs could as a new possible promising therapeutic approach for this aggressive skin cancer. A deeper knowledge of the involvement of somatostatinergic system in melanoma may shed new light on the potential role of SSTRs as targets for adjuvant biotherapy in patients with advanced melanoma. On the basis of this evidence, the pourpose of the current study is the evaluation of SSTRs expression in cutaneous melanoma cell lines and investigating the role of SST analogs, particularly the pan-SSTRs agonist (pasireotide), as potential treatment in preclinical models of melanoma.

Aim of the Thesis

The incidence of CMM increased over time and current therapies produced disappointing results because of intrinsic tumor resistance and/or relapse due to a progressive escape. Therefore, oncogenesis of CMM need to be better addressed, in order to develop new treatment strategies.

Melanocytes originate from neural crest and can express SSTRs, suggesting a neuroendocrine features for some subgroups of CMM (Diakatou, E. et al. 2011, de Bruin C. et al. 2009), as most of neuroendocrine tumors. Given the neuroendocrine differentiation of some CMM, these tumors might be responsive to treatment with SST analogs. The rational to treat melanoma with SST analogs, alone or in combination with the *BRAF* inhibitor vemurafenib, is based on the well-known evidence that both drugs target the MAPK pathway, which has a critical role in melanoma development and progression, thus representing a primary therapeutic target. SST analogs, by binding SSTRs *via* interaction with Gi proteins, are able to inhibit adenyl cyclase (AC) activity and phosphatidylinositol metabolism, and to modulate MAPK pathway. This process results in anti-secretive effects in endocrine cells, as well as in increased apoptosis and inhibition of cell growth (Theodoropoulou M. et al. 2013.) On the other hand, vemurafenib is able to suppress the constitutive activation of MAPK pathway in melanoma cells harboring *BRAF* mutations. Therefore, the current study aimed at:

1. better understanding the role of SST analogs and SSTRs in *in vitro* models of CMM;
2. evaluating the expression of SSTRs in four CMM cell lines (A375, HMCB, M14 and COLO38);
3. investigating the *in vitro* effects of SST analogs pasireotide and octreotide, alone or in combination with vemurafenib, on cell viability, proliferation and cell cycle.

MATERIALS AND METHODS

3.1 Study methodology

In this study, we characterized the expression of SSTRs in four different human melanoma cell lines: A375, HMCB M14 and COLO38. The gene and protein expression and the intracellular localization of these receptors was explored. In all cell lines we tested the dose and time-dependent effects of the BRAF inhibitor (vemurafenib) and two SST analogs (pasireotide and octreotide) on cell viability. The effects of these drugs, alone or in combination on cell proliferation and cell cycle were explored.

3.2 Cell Lines and Culture Conditions

The human melanoma cell lines A375 and HMCB were purchased from American Type Culture Collection ATTC (Manassas, VA, USA) and COLO38 and M14 were kindly provided by Department of Oncological Immunology, Istituto Nazionale per lo Studio e la Cura dei Tumori "Fondazione Giovanni Pascale"-IRCCS-ITALIA, Naples, Italy. The HMCB cells were maintained in Minimum Essential medium and A375, COLO38 and M14 were cultured in Dulbecco's Modified Eagle Medium (MEM D-MEM GIBCO® Cell Culture Invitrogen.com) with non-essential amino acids, 10% Fetal bovine serum (FBS, Invitrogen Life Technologies, Carlsbad, CA, USA), 0,5 µg/ml amphotericin-B (Fungizone) (Invitrogen Life Technologies, Carlsbad, CA, USA) and 10 mM HEPES in a humidified incubator (37°C, 5% CO2). Cells were grown in adherent monolayer, were harvested with trypsin (0.05 %) and resuspended in culture medium. Cell viability always exceeded 95%. Media and supplements were obtained from Sigma Aldrich (Sigma-Aldrich Customer Support St. Louis, MO United States).

3.3 Drugs and reagents

SST analog pasireotide (SOM230) was obtained by Novartis Pharma AG (Novartis, Basel, Switzerland). Octreotide was purchased from Italfarmaco (ITALFARMACO, Milano, Italy). Vemurafenib (PLX4032, RG7204) was acquired by Selleckchem (Selleckchem.com Houston, USA). Pasireotide and vemurafenib were diluted in culture medium before use. Both drugs were used as a stock solution of 1nM (10^{-3}M) in sterile water, PBS and dimethyl sulfoxide (DMSO) 100% respectively. Anti-SSTR1 was kindly provided by Drs Herbert Schmid of Novartis AG (Basel, Switzerland), SSTR2-neutralizing antibody (UMB1) was purchased from Epitomics (abcam Inc. Cambridge, MA, USA) and SSTR5- neutralizing antibody (UMB4) was obtained by rabMAbs (abcam Inc. Cambridge, MA, USA).

3.4 RNA isolation and RT-qPCR

mRNA of melanoma cell lines, HMCB, A375, M14 and COLO38 was isolated by Dynabeads Oligo $(dt)_{25}$ kit (Dynal AS, Oslo, Norway). The poly (A^+) mRNA was eluted in H_2O (65°C) for 2 minutes twice. Twenty µl of cDNA were obtained in a Tris buffer (50mM, Tris-HCL ph 8.3) containing 100 mM DTT and 10 nM $MgCl_2$, 10 units RNase inhibitor, 2 units avian myelobastosis virus Super Reverse Transcriptase, oligo-dT (5 ng/ml) and 1 mM of each deoxinucleotide triphosphate in a final volume of 40 µl. After an incubation of 1 h at 42°C, the resulting cDNA was gently resuspended by pipetting and 160 µl of sterile H_2O were added. cDNA was used for quantification of mRNA levels of all investigated genes: B-actin, SSTR1, SSTR2, SSTR3, SSTR5. The primer sequences are shown in Table 1. All primers were purchase from Eurogentec (Eurogentec, Seraing, Liège, Belgium). RT-qPCR was performed by using Syber Green supermix (Maxima SYBR Green qPCR Master Mixes, Thermo Fisher Scientific Inc. Waltham MA), iCicling iQ5 (Bio-Rad Laboratories Headquarters 1000 Alfred Nobel Drive Hercules, CA) and iCycler iQ Optical System software 3.0 for real time amplification, according to the manufacturer's protocol. PCRs were carried out in a final volume of 12 µl, containing 1µM of primers, 1X Syber Green Mix and 5 µl of RT products. Newly synthesized cDNAs were screened for the expression of SSTR subtypes (SSTR1, SSTR2, SSTR3 and SSTR5). RT-qPCR cycling conditions: after an initial heating at 50°C and 94° C for 5 min, samples were subjected to 40 cycles of denaturation (94° C for 1 min), annealing (59° C for 2 min) and amplification (72° C for 1 min). Samples were normalized against the expression of the housekeeping gene β-actin. Reactions lacking reverse-transcriptase enzyme were used as control for genomic DNA contamination. Control reaction without cDNA was used to confirm lack of exogenous DNA contamination.

GENE	PRIMERS
SSTR1	Forward 5'-TGAGTCAGCTGTCGGTCATC-3' Reverse 5'-ACACTGTAGGCACGGCTCTT-3'
SSTR2	Forward 5'-TCGGCCAAGTGGAGGAGAC-3' Reverse 5'-AGAGACTCCCCACACAGCCA-3'
SSTR3	Forward 5'-CTGGGTAACTCGCTGGTCATCTA-3' Reverse 5'-AGCGCCAGGTTGAGGATGTA-3'
SSTR5	Forward 5'-CATCCTCTCCTACGCCAACAG-3' Reverse 5'-GGAAGCTCTGGCGGAAGTT-3'
β-actin	Forward 5'-AAACTGGAACGGTGAAGGTG-3' Reverse 5'TCAAGTTGGGGGACAAAAAG-3'

Tab 1. Primer sequences of SSTR1, SSTR2, SSTR3, SSTR5 and β-actin.

3.5 Immunocytochemistry (ICC)

The expression of SSTRs proteins in human melanoma cell lines was evaluated by ICC. Glass microslides were placed in Petri dishes (BD Falcon^{TM} Dish 100 x10 mm) and were coated for 30 minutes at 37°C with poly-lysine. A375, HMCB, M14 and COLO38 melanoma cells were plated on top of the prepared microslides at subconfluent concentration. After 24 hours, medium was removed and cells were fixed with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer, pH 6.9, for 40 minutes at room temperature (RT). After washing in PBS, cells were treated for 3 minutes with 50% methanol and for 3 minutes with 100% methanol. After another wash (1X TRIS /HCL/Tween 0,5%), the cells were treated with a 3% H_2O_2–PBS solution for 15 minutes at RT in the dark to quench endogenous peroxidase. After washing, cells were incubated at 4°C over night (ON) with SSTR1, SSTR2 and SSTR5 monoclonal antibodies (respective dilutions: SSTR1-SSTR2 1:1500, SSTR5 1:500 in antibody diluents (Biorbyt LLC, San Francisco, California, United States). The day after, the cells were incubated for 30 minutes at RT with HRP/anti-Rabbit/Mouse (Dako, Denmark). Bound antibodies were visualized by incubation with freshly prepared DAB (diaminobenzidine tetrahdrochloride) (Dako, Denmark). Slides were counterstained with hematoxylin and coverslipped. For negative controls, the primary antibody was omitted. The cells were observed under an inverted light field microscope (Leica DMIL) and the images were captured at 40x magnification with a Leica DFC 240 photo camera and LAS V 3.7 software.

3.6 MTT cell viability assay

In A375, HMCB, M14 and COLO38 melanoma cells, the effects of vemurafenib, octreotide and pasireotide on inhibition of cell viability was determined by MTT assay 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (Sigma-Aldrich Customer Support St. Louis, MO United States) according to the protocol provided by the manufacturer. Cells were plated in 100 µl medium supplemented with 10% FBS (complete growth medium) in 96-well plates (8000 cells/well). Cells were incubated at 37° C in a humidified 5% CO_2 atmosphere for 24 hours to allow full adhesion. After 24 hours, the medium was changed with serum-free medium for 12 hours, in order to synchronize melanoma cells to G1 phase of cell cycle. After 48 hours from seeding, cells were treated once with serial concentrations of vemurafenib (10^{-14}M to 10^{-6}M) and daily with serial concentrations of octreotide and pasireotide (10^{-13}M to 10^{-6}M) in complete medium. In brief, at the end of treatment (72 hours), 10 µl of MTT solution (MTT 5mg/ml in PBS) were added to each well and incubated at 37°C for 1 hour. MTT is a yellowish solution and it is converted to water insoluble MTT-formazan of dark blue color by mitochondrial dehydrogenases of viable cells. The blue formazan crystals in viable cells were solubilized in 100 µl/well of MTT solvent (10% HCl 1N in Isopropyl alcohol). The intensity of color in each well was quantified as optical density (OD) at 570 nm wavelength using Victor multi-plate reader (Victor 4X, Perkin Elmer, Massachusetts, USA). The percentage of cell viability inhibition in drug-treated cultured cells against the untreated control cells was calculated as follow:

*Inhibition (%): (drug-treated - untreated)/untreated*100*

The experiments were repeated three times, and each experiment was performed in quadruplicate.

3.7 Measurement of total DNA content: DNA assay

A375 and M14 melanoma cells were harvested with trypsin and 25000 cells/well were seeded in 1 ml of complete culture medium (10% FBS) in 24-well plates. The plates were then placed in incubator in 5% CO_2 at 37°C. After 24 hours, the complete medium was removed and the cells were starved with 1 ml of serum-free medium for 12 hours. After 48 hours from seeding cells were treated once with vemurafenib or daily with serial concentrations of octreotide and pasireotide (10^{-10}M to 10^{-6}M) in complete medium for 72 hours. After 72 hours of treatment, the medium was discarded and cells were harvested for DNA measurement. Measurement of total DNA content, representative of the number of cells, was performed using the bisbenzimide fluorescent dye (Hoechst 33258) (Boehring Diagnostics, La Jolla, CA), as previously described (De Martino MC *et al*. 2012). Data are expressed as percent of the control. The experiments were repeated three times and each experiment was performed in quadruplicate.

3.8 Analysis of cell cycle by flow cytometry: FACS

Evaluation of A375 and M14 melanoma cell cycle distribution was done using flow cytometry. Cells were seeded in 6-well plates at density of 5 x 10^4 and 15 x 10^4 cells respectively for A375 and M14 cell lines. After 24 hours, the complete medium was removed and the cells were starved with 1 ml of serum-free medium for 12 hours. Cells were then treated for 24 hours with vemurafenib, octreotide, pasireotide and a combination of vemurafenib and pasireotide. At the end of treatment cells were harvested and counted, then washed in PBS and stained with 50µg/ml propidium iodide for 60 min at RT. Possible double-stranded RNA was removed by incubation with 0.1 mg/ml RNAse (Invitrogen Life Technologies, Carlsbad, CA, USA). Untreated control cells were included. Flow cytometry analysis of the cell cycle was carried out immediately after staining. Cells were analysed by flow cytometry (FACScan, Becton Dickinson, San Josè, CA, USA) using the CyCLOPS Summit 3.1 (Cytomation, Fort Collins, CO, USA). Distribution of cells in the different cell cycle phases was evaluated by Mod Fit 2.0 (Verity Software HOUSe INC., Ranger, ME, USA). Each experiment was performed three times. Changes in cell cycle distribution after treatment with drugs are shown as mean ± SD of cells in the different cell cycle phases plotted.

Results

4.1 Expression of SSTRs in human cutaneous melanoma cell lines by RT-qPCR

Relative quantification for SSTRs mRNAs compared to the β-actin housekeeping gene expression, showed as mean± SD) was: SSTR1 32.5±1.1 and 33.7±2.7, SSTR2: 27±0.6 and 30.5±1.8, SSTR3: 31±2.9 and 34.4±1.1, SSTR5: 29±2 and 35.2±0.9, β-actin: 15±0.9 and 18.2±1.2 in A375 and HMCB cells respectively. In COLO38 and M14 cell lines, the SSTR1 expression was 32.1±2.9 and 30.5±2, SSTR2: 27.5±3.2 and 27.4±2.6, SSTR3: 29.3±4.4 and 29.1±2.9, SSTR5: 30.8±2.2 and 30±2.5, respectively (Fig.12). β-actin: 17.2±1.6 and 18.5±1.5 for M14 and COLO38 cell lines respectively.

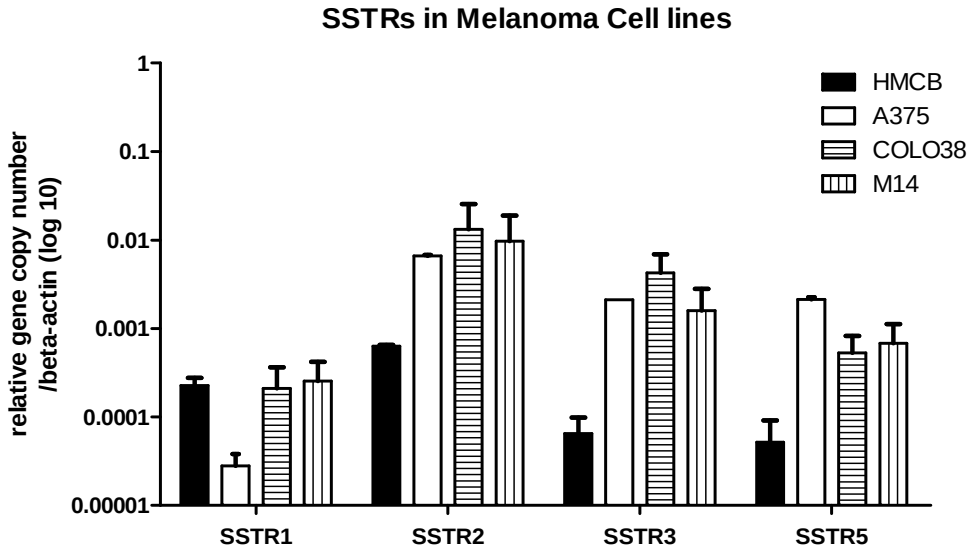


Figure 12. RT-qPCR expression of SSTRs in melanoma cell lines (HMCB, A375, COLO38 and M14).Values represent mean and SEM per gene assayed in duplicate in three independent experiments. Expression level are normalized against the housekeeping gene β-actin. All values are expressed as Log 10 scale.

4.2 Protein expression of SSTRs in melanoma cell lines by ICC

The protein expression and localization of SSTRs subtypes were evaluated by ICC. In particular, SSTR1, SSTR2 and SSTR5 expression was confirmed in all melanoma cell lines. A significant amount of staining was seen within the cytoplasm and in perinuclear region in all melanoma cell line tested (Fig.13). SSTRs are localized predominantly in cytoplasm at the condition tested. Particularly, a perinucear amount of staining for SSTR2 in COLO38 cells and for SSTR5 in HMCB and COLO38 cells was detected. SSTR1 seems to be present with a similar positivity in four melanoma cell lines, the staining for SSTR2 is stronger in HMCB and COLO38 melanoma cells than others two cell lines. SSTR5 is stronger in A375 and M14 melanoma cells than others two cell lines. The cells were scored based on the intensity of the staining. In particular, a score of +++, ++, + and 0 was assigned for strong, moderate, mild and no staining, respectively (Tab.2). (Gatto F. *et al*. 2013). As positive for STTR1, SSTR2 and SSTR5 control bronchial carcinoid H272 cells and hepatocellular carcinoma were used, respectively (Fig. 14). Negative control (Fig.15).

Cell line	SSTR1	SSTR2	SSTR5
A375	++	+	+++
HMCB	++	++	++
COLO38	+++	+	+
M14	+++	+++	+++

Table 2. ICC Score of melanoma cells based on the intensity of staining. SSTR1: A375 strong (+++), HMCB moderate (++), COLO38 strong (+++), M14 strong (+++); SSTR2: A375 mild (+), HMCB moderate (++), COLO38 mild (+), M14 strong (+++); SSTR5: A375 strong (+++), HMCB moderate (++), COLO38 mild (+), M14 strong (+++).

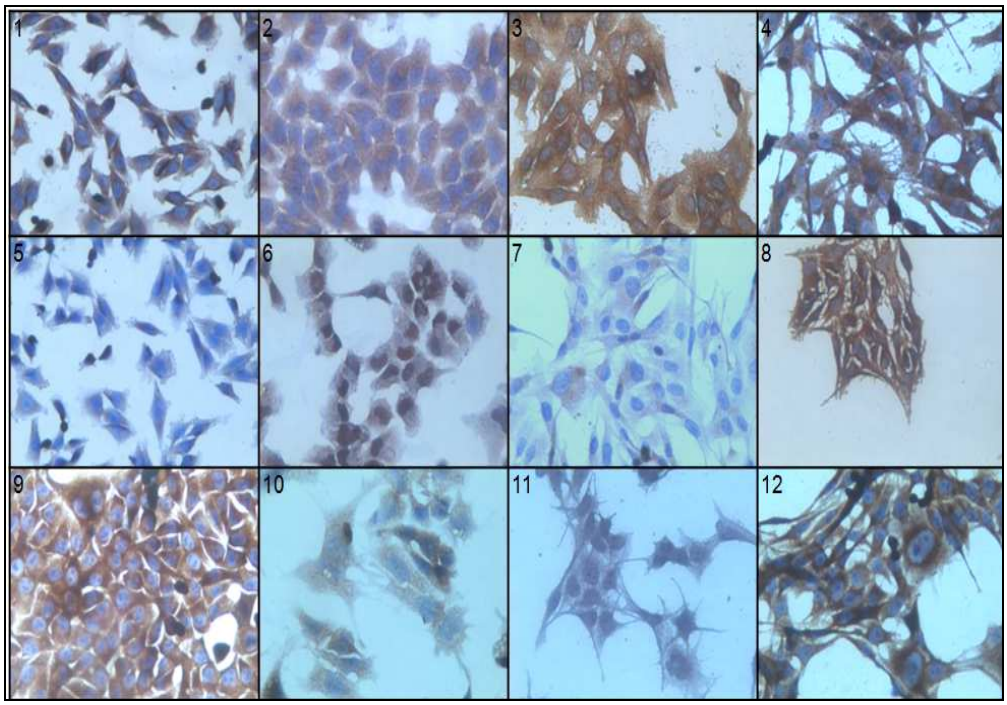


Figure 13. ICC for SSTR1, SSTR2 and SSTR5 in melanoma cell lines. 1-4 SSTR1 in A375, HMCB, COLO38 and M14 cell lines; 5-8 SSTR2 in A375, HMCB, COLO38 and M14 cell lines; 9-12 SSTR5 in A375, HMCB, COLO38 and M14 cell lines.

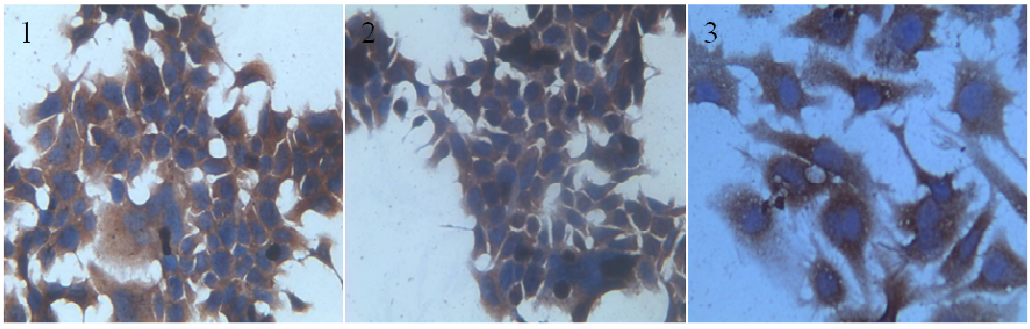


Figure 14. ICC for SSTR1, SSTR2 in bronchial carcinoid (H727) and SSTR5 in hepatocellular carcinoma (HepG2) cell lines. 1-2 SSTR1 and SSTR2 in H727 cell line. 3 SSTR5 in HepG2 cells. H727 and HepG2 cells were used as positive control for SSTR1, SSTR2 and SSTR5 expression.

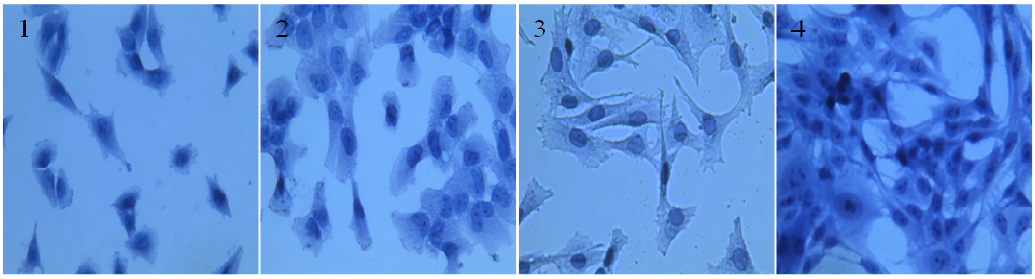


Figure 15. Negative control for SSTRs in A375, HMCB, COLO38 and M14 melanoma cell lines by ICC (1-2-3-4). Negative control refers to control in which the cells were incubated with antibody diluent, without the primary antibody included.

4.3 Effect of pasireotide on cell viability in melanoma cell lines

In melanoma cell lines, pasireotide showed different effects on cell viability. In particular, daily administration of pasireotide inhibited A375 and M14 viability at all tested concentrations: a significant maximal inhibition was achieved at 10^{-7}M (41% $p<0.001$ and 44% $p<0.001$ *vs* control for A375 and M14 cells, respectively) (Fig.16). Conversely, in COLO38 and HMCB cell lines pasireotide showed no effect on viability inhibition (Fig.17).

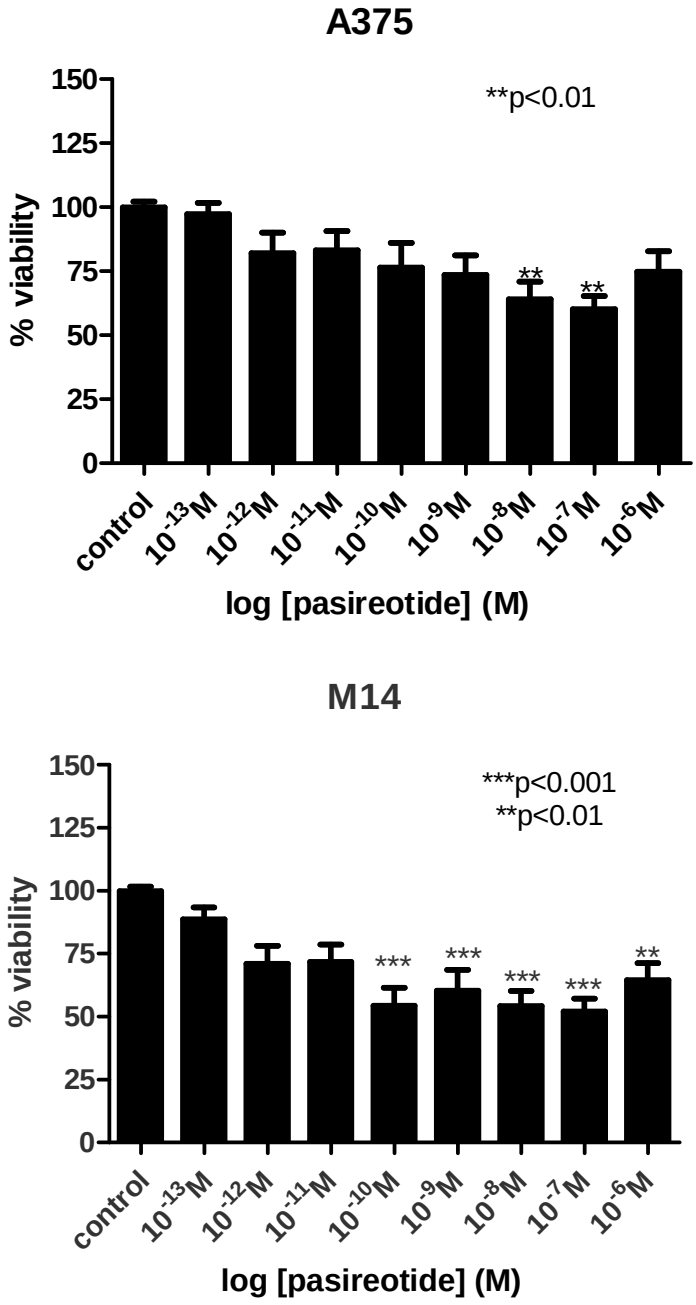


Figure 16. Inhibition of cell viability after 72 hours of daily incubation with serial concentrations of pasireotide in A375 and M14 melanoma cell lines. The histograms show the percentage of viability inhibition relative to control cell viability measured using MTT assay. The values represent the mean±SEM in quadruplicate of three different experiments. Control is set as 100%.

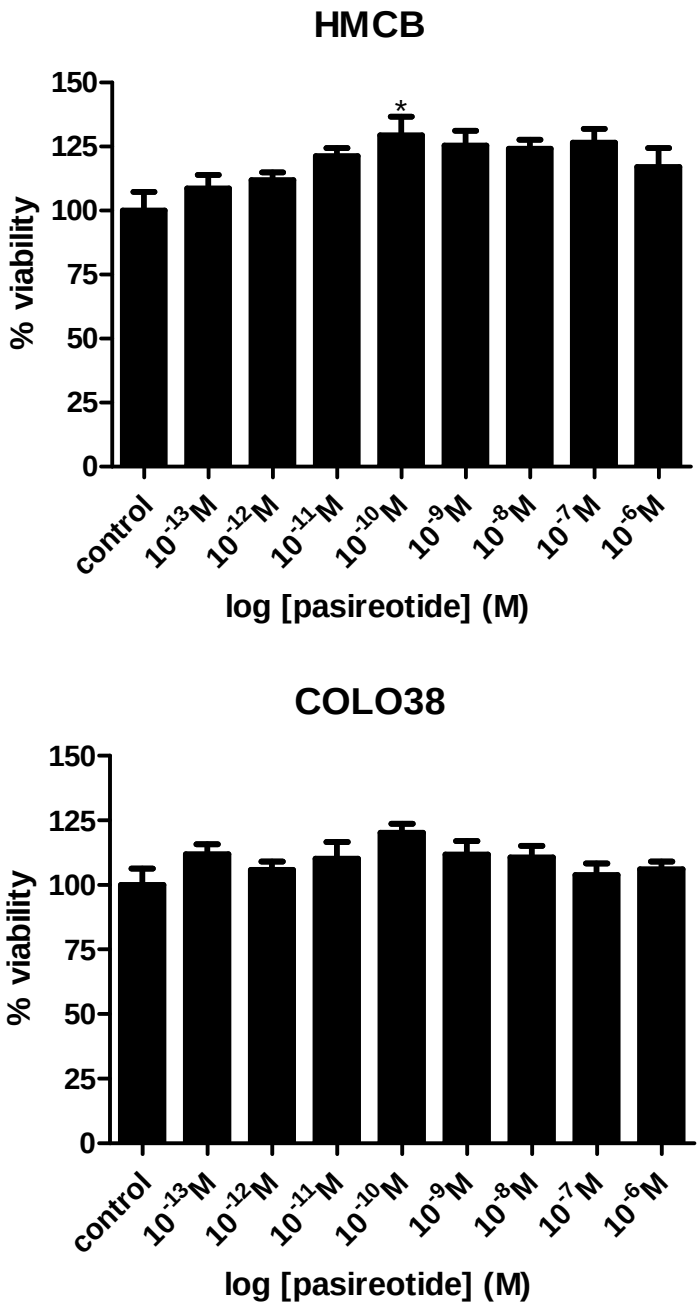


Figure 17. Inhibition of cell viability after 72 hours of daily incubation with serial concentrations of pasireotide in HMCB and COLO38 melanoma cell lines. The histograms show the percentage of viability inhibition relative to control cell viability measured using MTT assay. The values represent the mean±SEM in quadruplicate of three different experiments. Control is set as 100%.

4.4 Effect of octreotide on cell viability in melanoma cell lines

In melanoma cell lines, octreotide showed different effects on cell viability. In particular, daily administration of octreotide inhibited A375 viability: a significant maximal inhibition was achieved at 10^{-6}M (54.15% $p<0.001$ *vs* control) after 72 hours of treatment (IC_{50} of $2.91*10^{-11}$M) In M14 cell line the highest inhibitory response was observed after 72 hours of daily administration of octreotide 10^{-8}M and IC 50 of $7.768*10^{-11}$M (Fig.18). Conversely, in COLO38 and HMCB cell lines octreotide showed no effect on viability inhibition at any of the tested drug concentrations (Fig.19).

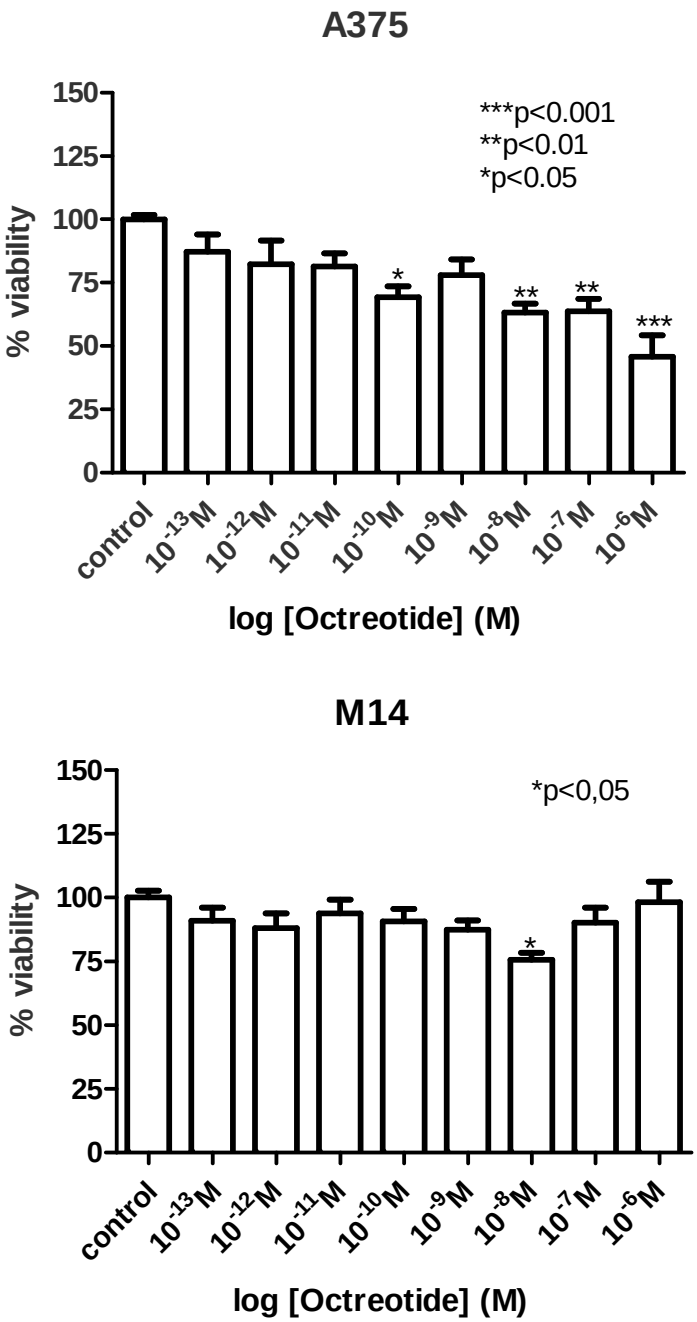
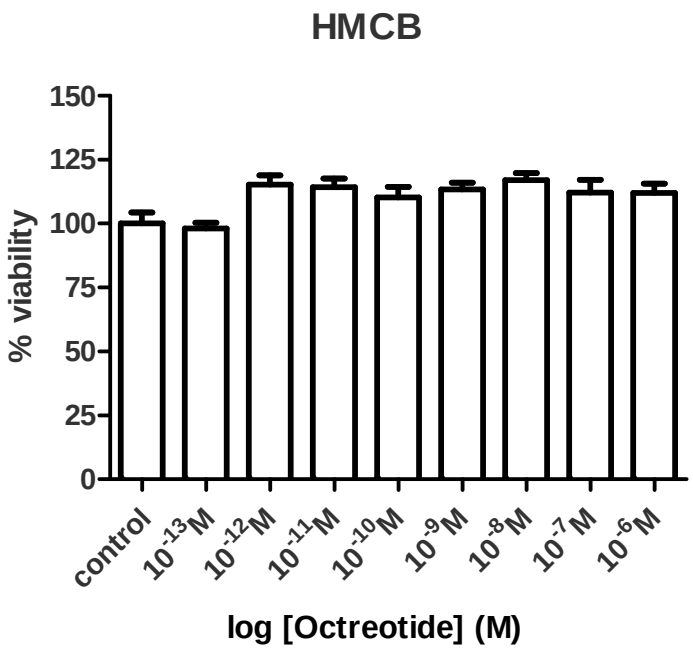


Fig 18. Inhibition of cell viability after 72 hours of daily incubation with serial concentrations of octreotide in cutaneous melanoma cell lines (A375 and M14). The histograms show the percentage of viability inhibition relative to control cell viability measured using MTT assay. The values represent the mean±SEM in quadruplicate of three different experiments. Control is set as 100%.



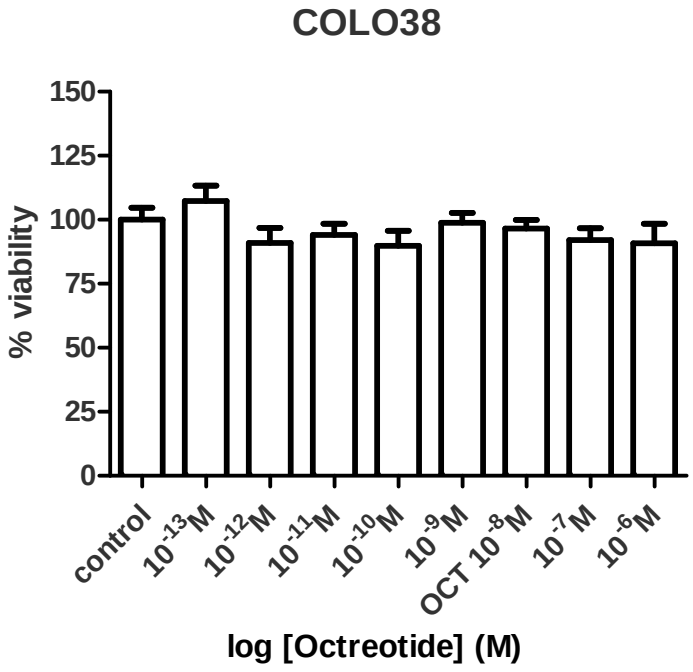


Figure 19. Inhibition of cell viability after 72 hours of daily incubation with serial concentrations of octreotide in malignant melanoma cell lines (HMCB and COLO38). The histograms show the percentage of viability inhibition relative to control cell viability measured using MTT assay. The values represent the mean±SEM in quadruplicate of three different experiments. Control is set as 100%.

4.5 Effect of vemurafenib on cell viability in melanoma cell lines

Vemurafenib showed different effects on cell viability in melanoma cell lines. (Fig.20 and Fig 21 .The graphs show the effects of vemurafenib in A375, M14, HMCB and COLO38 melanoma cell lines). In particular, in A375 cell line vemurafenib significantly suppressed the cell viability in a dose and time-dependent manner. The A375 cell viability is significantly inhibited at the maximal concentration tested (10^{-6}M) (55.52%, 65.52%, 82.89%, $p< 0.001$ *vs* control) after 24, 48 and 72 hours of treatment respectively, with IC_{50} of 10^{-8}M at all time of treatment assayed. In HMCB cell line, vemurafenib is able to significantly inhibit cell viability at the maximal concentration tested (10^{-6}M) (44.31%, $p<0.001$, 37.16%, $p<0.01$, 36.05%, $p<0.01$ *vs* control) after 24, 48 and 72 hours of treatment respectively, with IC50 of $9.2*10^{-12}$M, $5*10^{-13}$M and $3.2*10^{-12}$M after the different times of treatment. In M14 cell line the highest inhibitory response was observed after 72 hours of treatment and with the highest concentration of vemurafenib (10^{-6}M), with a mean inhibition of viability of 29.97% (IC50 of $4.33*10^{-8}$M). In COLO38 cell line, the maximal inhibition of cell viability was achieved with 10^{-6}M of vemurafenib (42.08% *vs* control) after 48 hours of treatment with IC50 of $1.33*10^{-7}$M. In M14 and COLO38 cell lines the inhibition of cell viability observed after treatment with vemurafenib was not statistically significant.

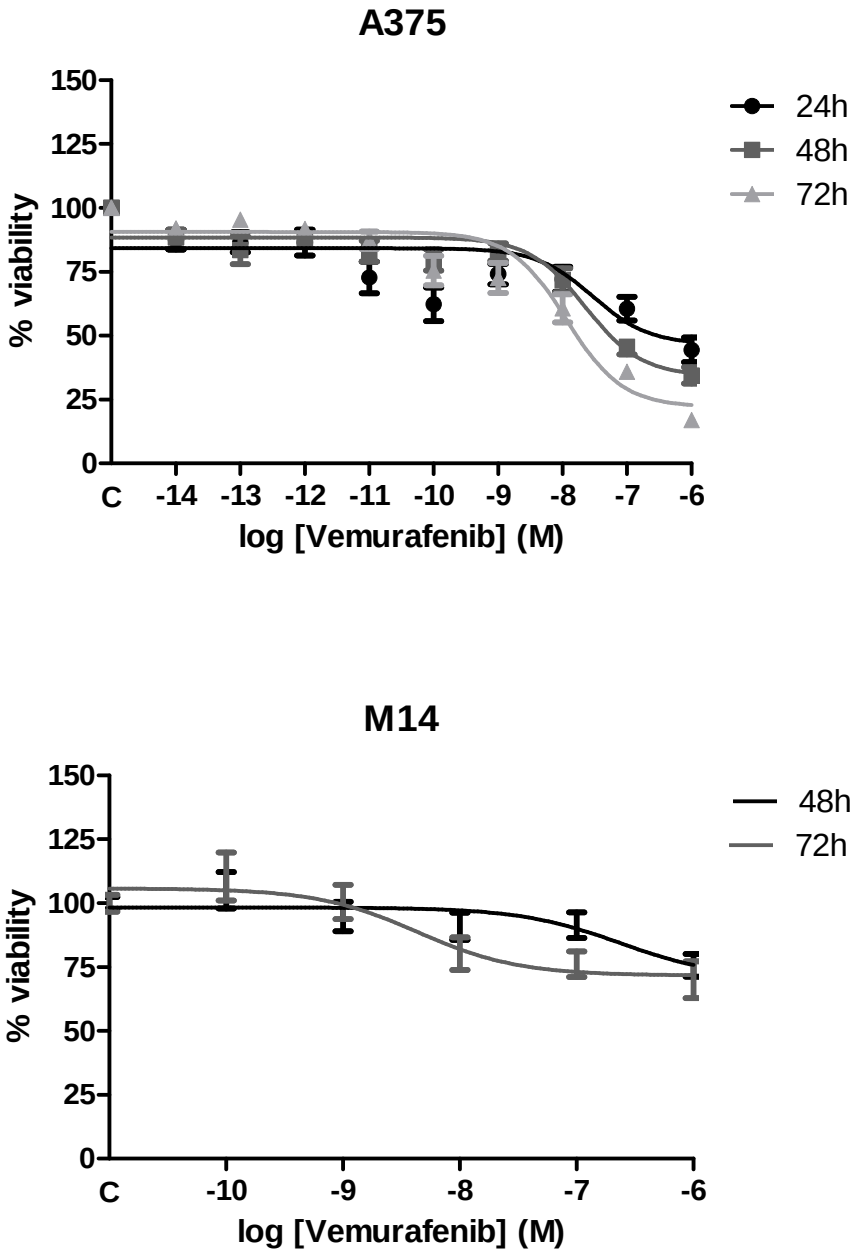


Figure 20. Effect of vemurafenib on melanoma cell lines viability (A375, M14) by MTT assay. Data are expressed as the percentage of control and represent the mean±SEM. The maximal significant inhibition was achieved with vemurafenib 10^{-6}M (55.52%, 65.52%, 82.89%, p< 0.001 *vs* control in A375 cells). In M14 cell line vemurafenib inhibited the cell viability only at 10^{-6}M (29.97%). Control is set as 100%. ***P<0.001, **P<0.01, *P<0.05.

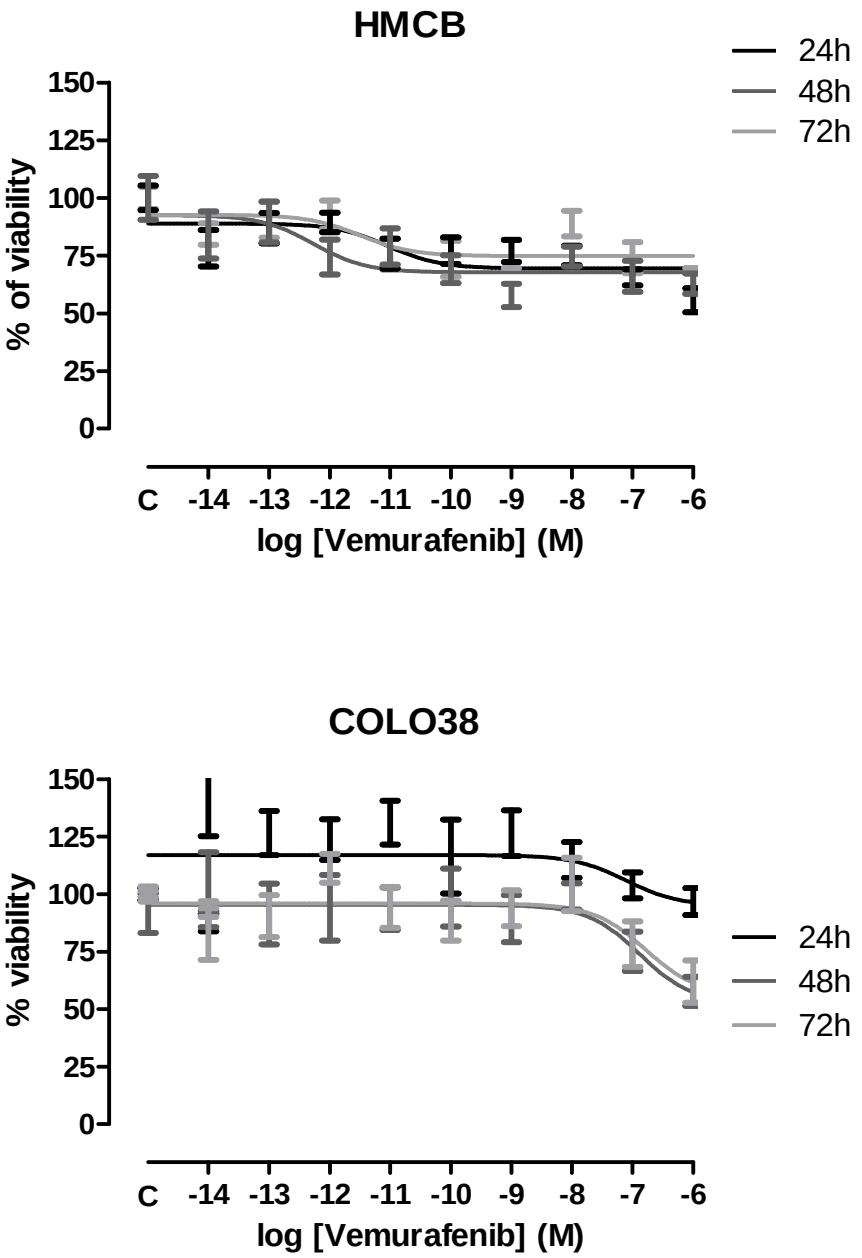
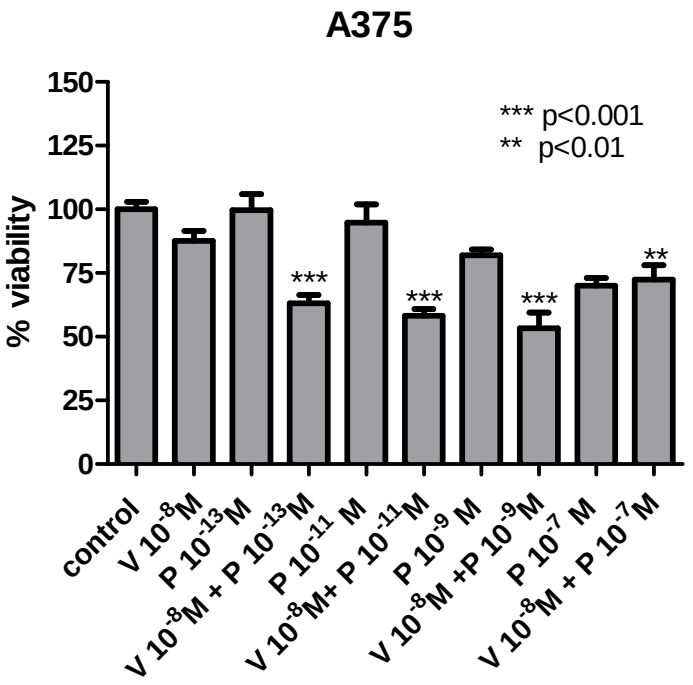
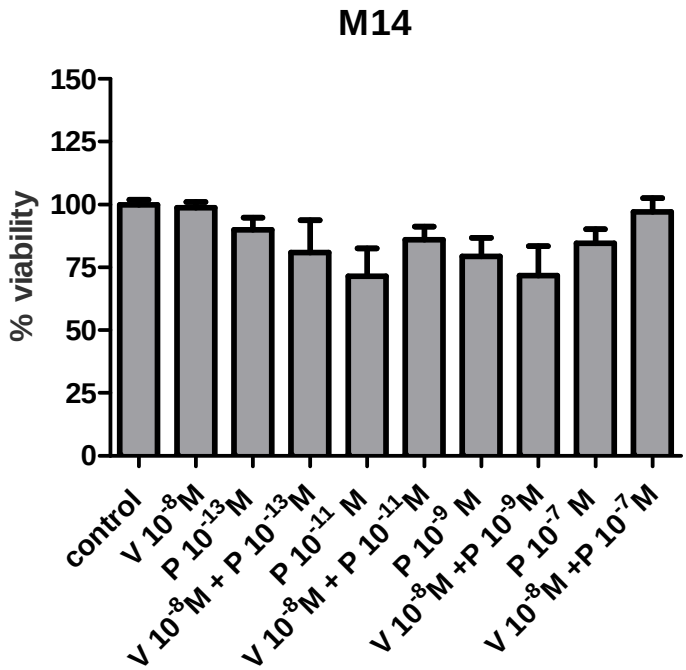


Figure 21. Effect of vemurafenib on melanoma cell lines viability (HMCB and COLO38) by MTT assay. Data are expressed as the percentage of control and represent the mean±SEM. In HMCB cell line, vemurafenib is able to significantly inhibit the cell viability with the maximal concentration tested (10^{-6} M) (44.31%, $p<0.001$, 37.16%, $p<0.01$, 36.05%, $p<0.01$ *vs* control) after 24, 48 and 72 hours of treatment respectively. In COLO38 cell line, the maximal inhibition of cell viability was achieved with 10^{-6} M of vemurafenib (42.08% *vs* control) after 48 hours of treatment. Control is set as 100%. ***P<0.001, **P<0.01, *P<0.05.

4.6 Effect of combined treatment with vemurafenib and pasireotide on cell viability in melanoma cell lines

Combined treatment with pasireotide and vemurafenib showed different effects on cell viability of four melanoma cell lines. In particular, in A375 cell line, the combined treatment with vemurafenib and daily administration of pasireotide for 72 hours of treatment significantly inhibited the cell viability *vs* control (vemurafenib 10^{-8}M + pasireotide 10^{-13}M 36.3% $p<0.001$, vemurafenib 10^{-8}M + pasireotide 10^{-11}M 41.77% $p<0.001$, vemurafenib 10^{-8}M + pasireotide 10^{-9}M 41.79% $p>0.001$, vemurafenib 10^{-8}M + pasireotide 10^{-7}M 27.62% $p<0.01$). Pasireotide 10^{-7}M was able to significantly inhibit the cell viability (29.97% $p<0.001$ *vs* control). The combined treatment with vemurafenib and pasireotide showed a significant suppression of cell viability at all tested concentrations except for pasireotide 10^{-7}M *vs* vemurafenib 10^{-8}M (vemurafenib 10^{-8} M + pasireotide 10^{-13} M 24.48% $p<0.01$, vemurafenib 10^{-8} M + pasireotide 10^{-11} M 29.33% $p<0.001$, vemurafenib 10^{-8} M + pasireotide 10^{-9} M 34.19% $p<0.001$) and *vs* pasireotide (pasireotide 10^{-13}M *vs* vemurafenib 10^{-8} M + pasireotide 10^{-13} M 36.64% $p<0.001$, pasireotide 10^{-11} M vs vemurafenib 10^{-8} M + pasireotide 10^{-11} M 36.58% $p<0.001$, pasireotide 10^{-9} M *vs* vemurafenib 10^{-8} M + pasireotide 10^{-9} M 28.56% $p<0.001$). In M14, HMCB and COLO38 melanoma cell lines, combined treatment with vemurafenib and pasireotide induced only a moderate inhibition of cell viability at all tested concentration. This inhibition did not, however, exceeded 50% of the control proliferation under any conditions (Fig.22).


A375
*** p<0.001
** p<0.01
% viability
150
125
100
75
50
25
0
***
***
***
**
control
V 10-8M
P 10-13M
V 10-8M + P 10-13M
P 10-11 M
V 10-8M+ P 10-11M
P 10-9 M
V 10-8M +P 10-9M
P 10-7 M
V 10-8M + P 10-7M


M14
% viability
150
125
100
75
50
25
0
control
V 10-8M
P 10-13M
V 10-8M + P 10-13M
P 10-11 M
V 10-8M + P 10-11M
P 10-9 M
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P 10-7 M
V 10-8M +P 10-7M

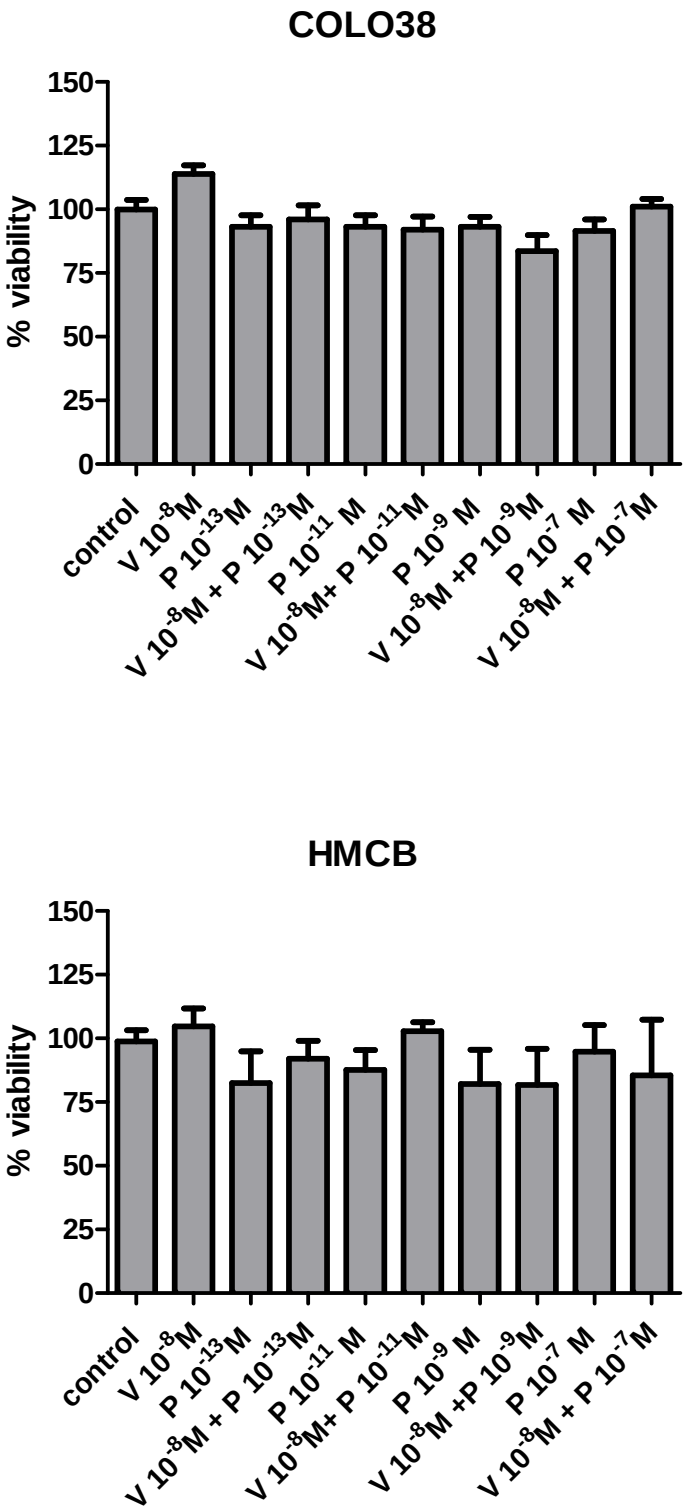
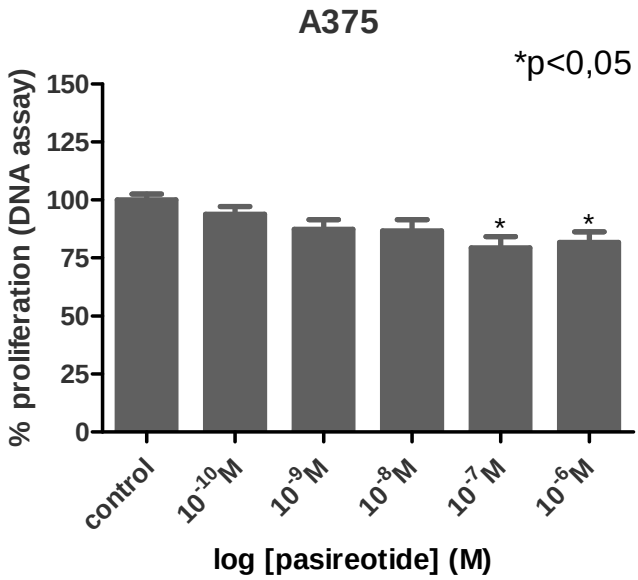


Figure 22. Effect on melanoma cell lines viability (A375, M14, COLO38 and HMCB) after 72 hours of treatment with vemurafenib and pasireotide in combination (MTT Assay). Pasireotide was administrated every day. Data are expressed as the percentage of control and represent the mean±SEM of three different experiments in quadruplicate. Control is set as 100%.

4.7 Effect of pasireotide on melanoma cell lines proliferation

The daily administration of pasireotide for 72 hours in melanoma cell lines A375 and M14 was able to inhibit cell proliferation in a dose-dependent manner. In A375 cells the maximal significant inhibition of cell proliferation, was achieved with pasireotide 10^{-7}M and 10^{-6}M (18.32% and 20.57% $p<0.05$ *vs* control, respectively), as measured by DNA assay(Fig.23). Also in M14 cells pasireotide significantly suppressed cell proliferation at the concentration of 10^{-7}M and 10^{-6}M (23.51% and 21.02% $p<0.05$ *vs* control, respectively) (Fig.24).



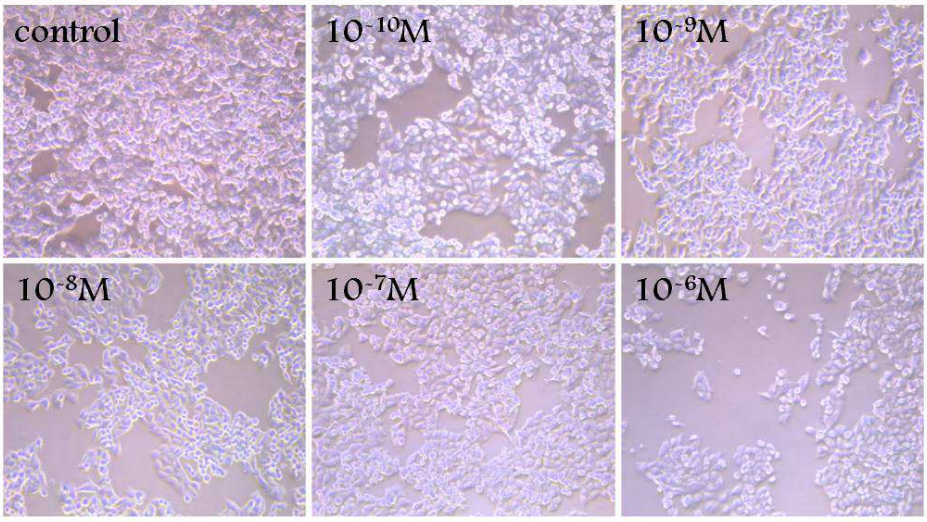


Figure 23. Effect of daily administration of pasireotide for 72 hours on melanoma cell lines proliferation in A375 melanoma cells. Data are expressed as the percentage of control and represent the mean±SEM. Control is set as 100%. Images under the graph represent A375 cells treated for 72 hours with serial concentrations of pasireotide (10^{-10}M -10^{-6}M). The pictures are acquired at 10X magnification.

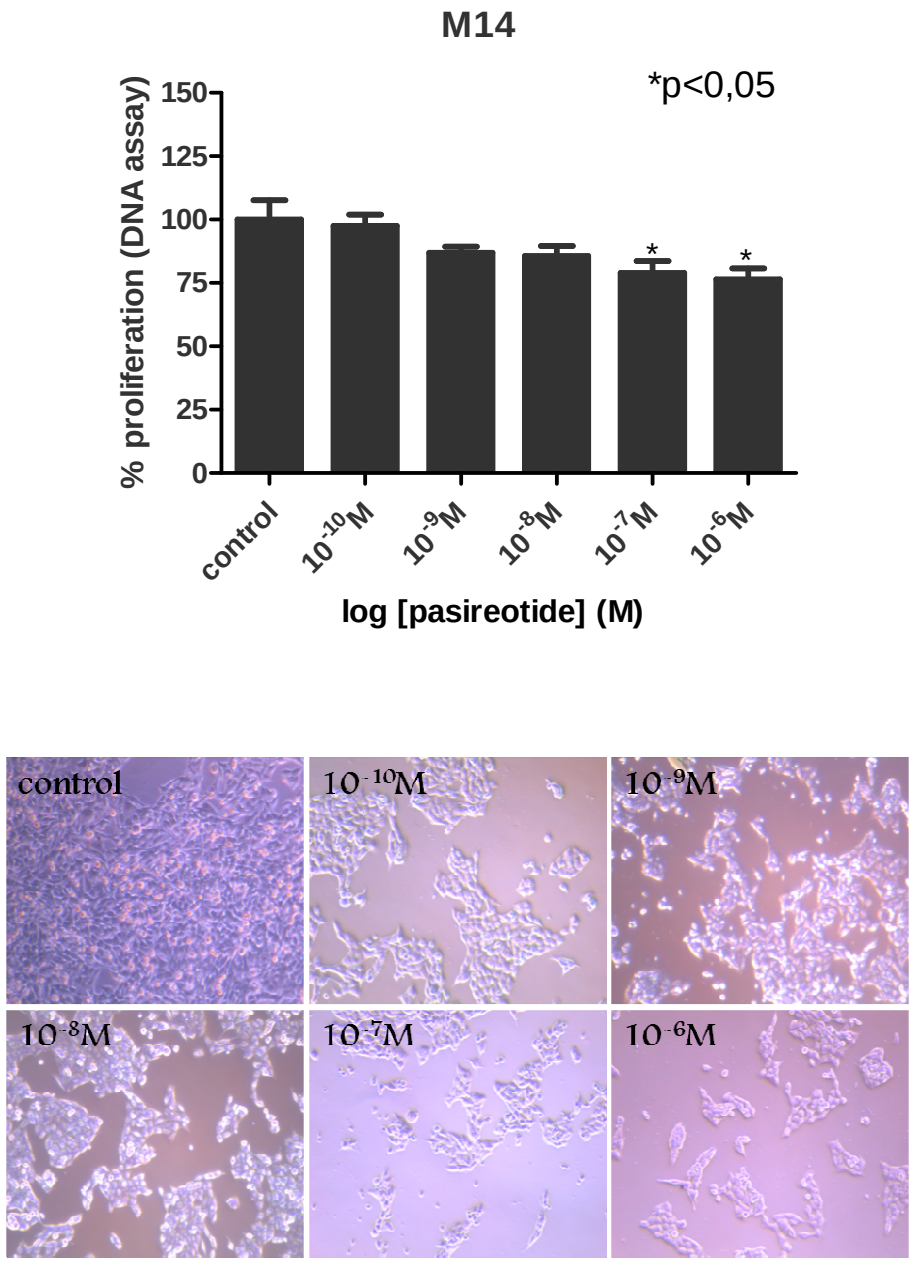


Figure 24. Effect of daily administration of pasireotide for 72 hours on melanoma cell lines proliferation in M14 melanoma cells. Data are expressed as the percentage of control and represent the mean±SEM. Control is set as 100%. Images under the graph represent M14 cells treated for 72 hours with serial concentrations of pasireotide (10^{-10}M -10^{-6}M). The pictures are acquired at 10X magnification.

4.8 Effect of octreotide on melanoma cell line proliferation

In melanoma cell lines the SST analog octreotide did not show any effect on cell proliferation. The cell proliferation of A375 and M14 melanoma cells line was not inhibited by daily treatment with serial concentration of octreotide (10^{-10}M-10^{-6}M) for 72 hours (Fig.25 and Fig.26)

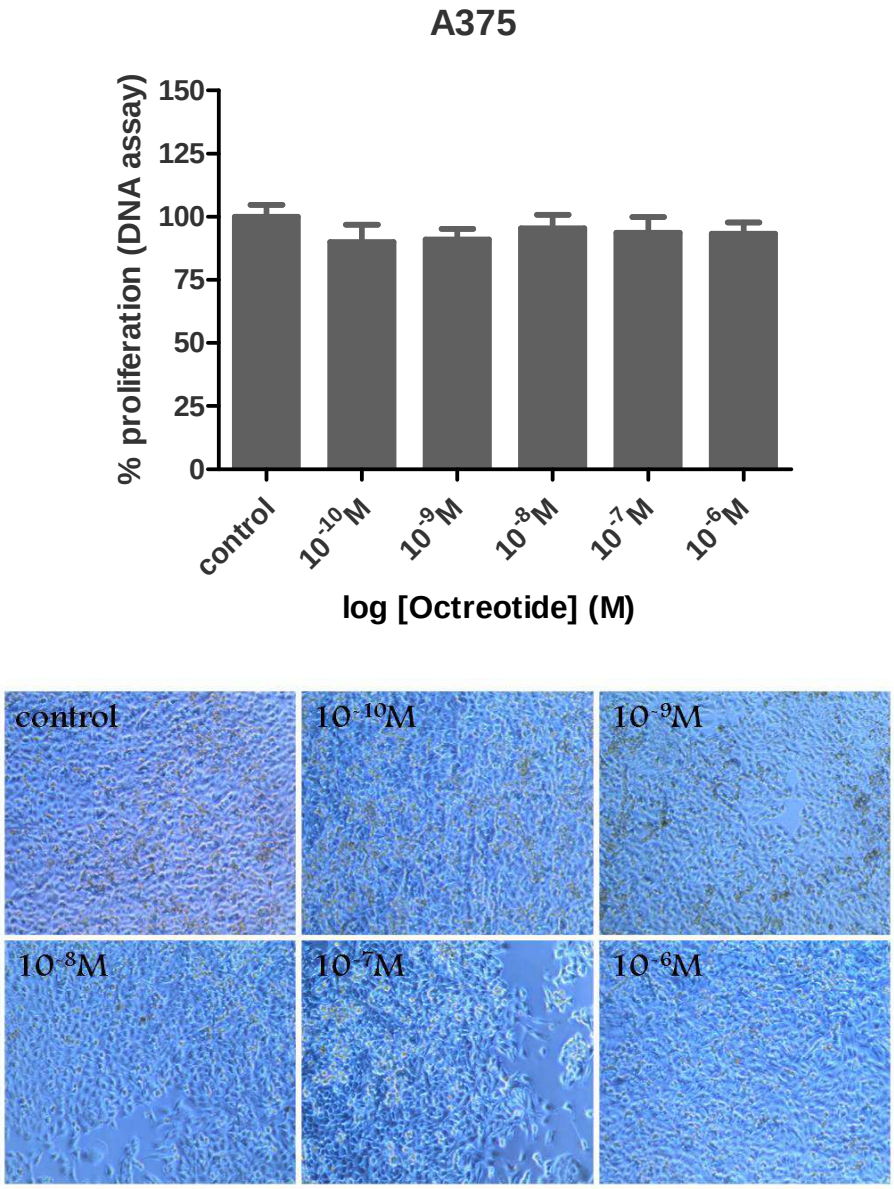


Figure 25. Effect of octreotide on A375 cell line proliferation by DNA assay. Data are expressed as the percentage of control and represent the mean±SEM of three different experiments in quadruplicate. Control was set as 100%. Images under the graph represent A375 cells treated for 72 hours with serial concentrations of octreotide (10-^{10}M -10^{-6}M). The pictures are acquired at 10X magnification.

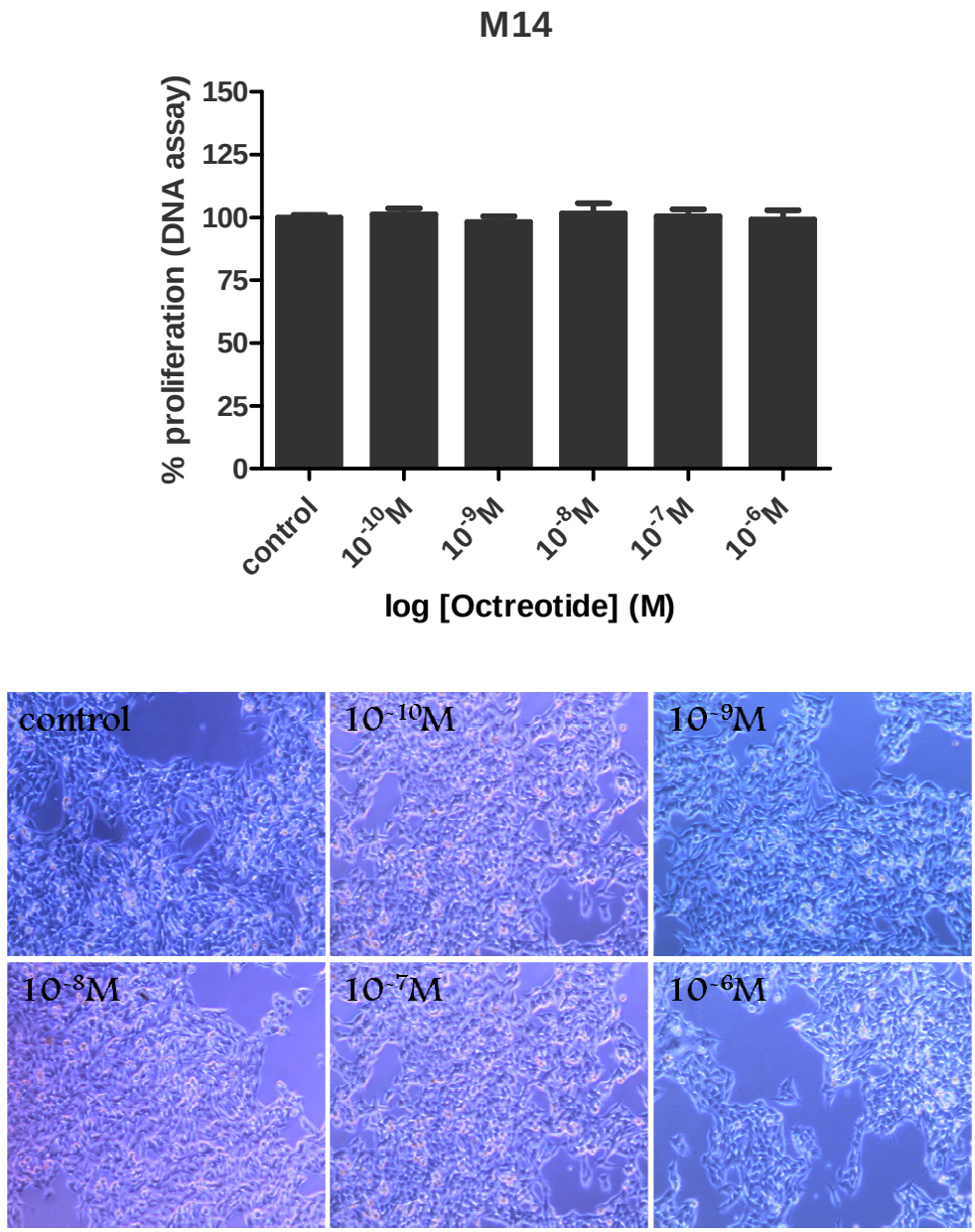


Figure 26. Effect of octreotide on M14 cell line proliferation by DNA assay. Data are expressed as the percentage of control and represent the mean±SEM of three different experiments in quadruplicate. Control was set as 100%. Images under the graph represent A375 cells treated for 72 hours with serial concentrations of octreotide (10^{-10}M -10^{-6}M). The pictures are acquired at 10X magnification.

4.9 Effect of vemurafenib on melanoma cell lines proliferation

In melanoma cell lines, vemurafenib showed different effects on cell proliferation. Treatment of A375 with vemurafenib for 72 hours achieved a significant inhibition of cell proliferation with vemurafenib at 10^{-8}M, 10^{-7}M and 10^{-6}M (18.97%, 30.81% and 33.06% *vs* control $p<0.001$) as measured by DNA assay (Fig.27). In M14 cell line vemurafenib induced a inhibition of cell proliferation only at 10^{-6}M concentration (15.26% *vs* control $p<0.01$) (Fig.28).

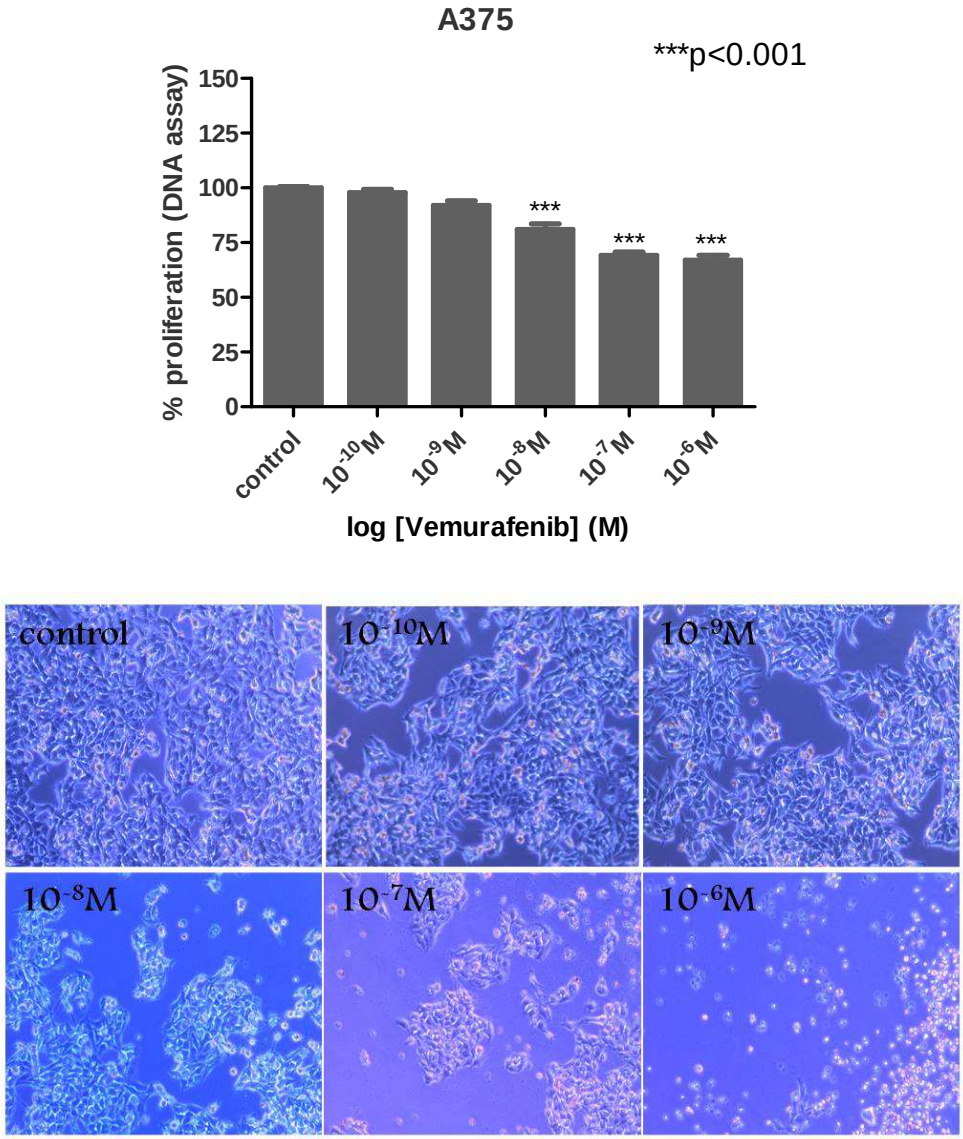


Figure 27. Effect of vemurafenib on A375 cell line proliferation by DNA assay. Data are expressed as the percentage of control and represent the mean±SEM of three different experiments in quadruplicate. Control was set as 100%. Images under the graph represent A375 cells treated for 72 hours with serial concentrations of Vemurafenib (10-^{10}M -10^{-6}M). The pictures are acquired at 10X magnification.

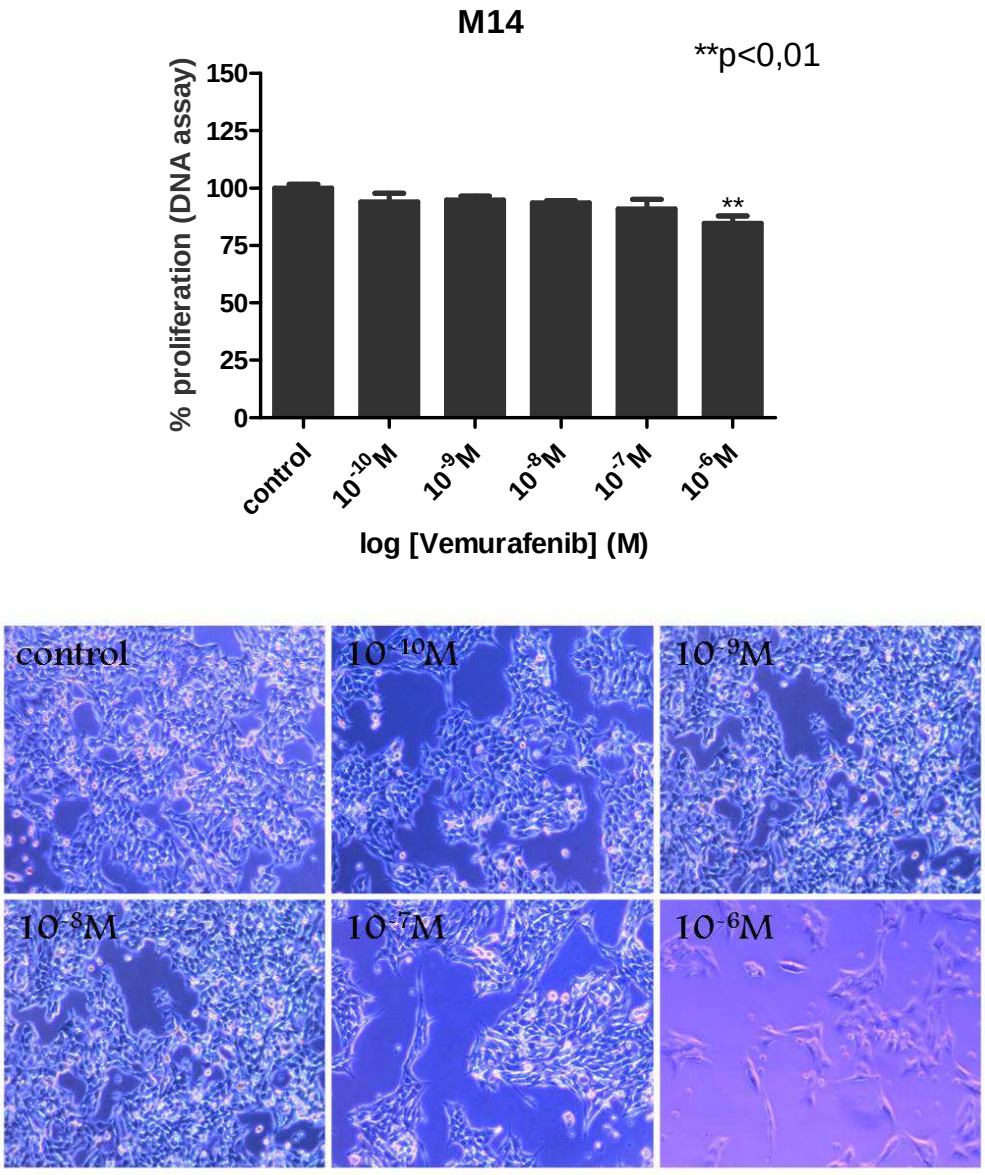
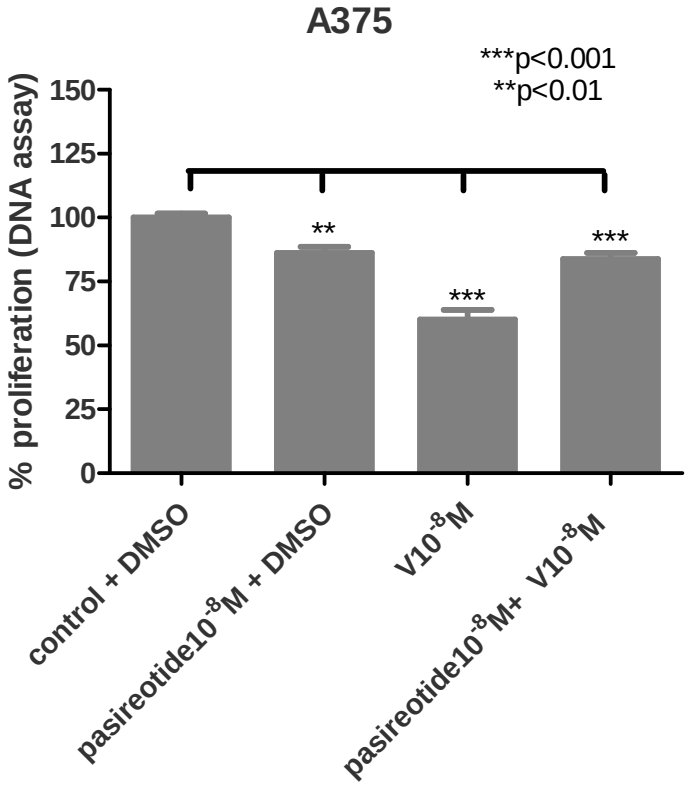
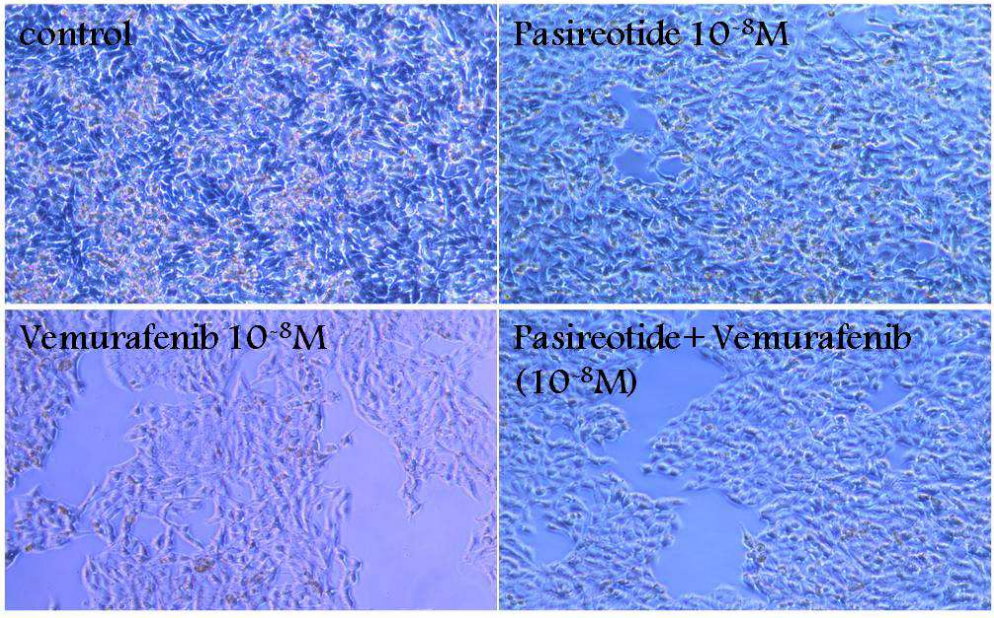


Figure 28. Effect of vemurafenib on M14 cell line proliferation by DNA assay. Data are expressed as the percentage of control and represent the mean±SEM of three different experiments in quadruplicate. Control was set as 100%. Images under the graph represent M14 cells treated for 72 hours with serial concentrations of Vemurafenib (10-^{10}M -10^{-6}M). The pictures are acquired at 10X magnification.

4.9 Effect of combined treatment with vemurafenib and pasireotide on melanoma cell line proliferation

Combined treatment with pasireotide + vemurafenib did not show additive effects on cell proliferation, as measured by DNA assay, compared to the single treatment with each drug in A375 and M14 melanoma cell lines (Fig. 29) In particular, the combined treatment with vemurafenib and pasireotide administered daily, for 72 hours, was not able to increase the inhibitory effect on melanoma cell proliferation in A375 and M14 cells compared to vemurafenib and pasireotide. Both single treatment and combination of vemurafenib and pasireotide (10^{-8}M) were only able to inhibit significantly cell proliferation compared to control (13.16% $p< 0.01$, 39.76% $p<0.01$ and 16.22% $p<0.01$ *vs* control for pasireotide, vemurafenib and vemurafenib + pasireotide, respectively in A375 cells; 14.88% $p<0.001$, 19.84% $p<0.001$ and 15.64% $p<0.001$ *vs* control for vemurafenib, pasireotide and vemurafenib + pasireotide, respectively in M14 cells).


A375
***p<0.001
**p<0.01
% proliferation (DNA assay)
150
125
100
75
50
25
0
**
***
***
control + DMSO
pasireotide10-8M + DMSO
V10-8M
pasireotide10-8M+ V10-8M


control
Pasireotide 10-8M
Vemurafenib 10-8M
Pasireotide+Vemurafenib
(10-8M)

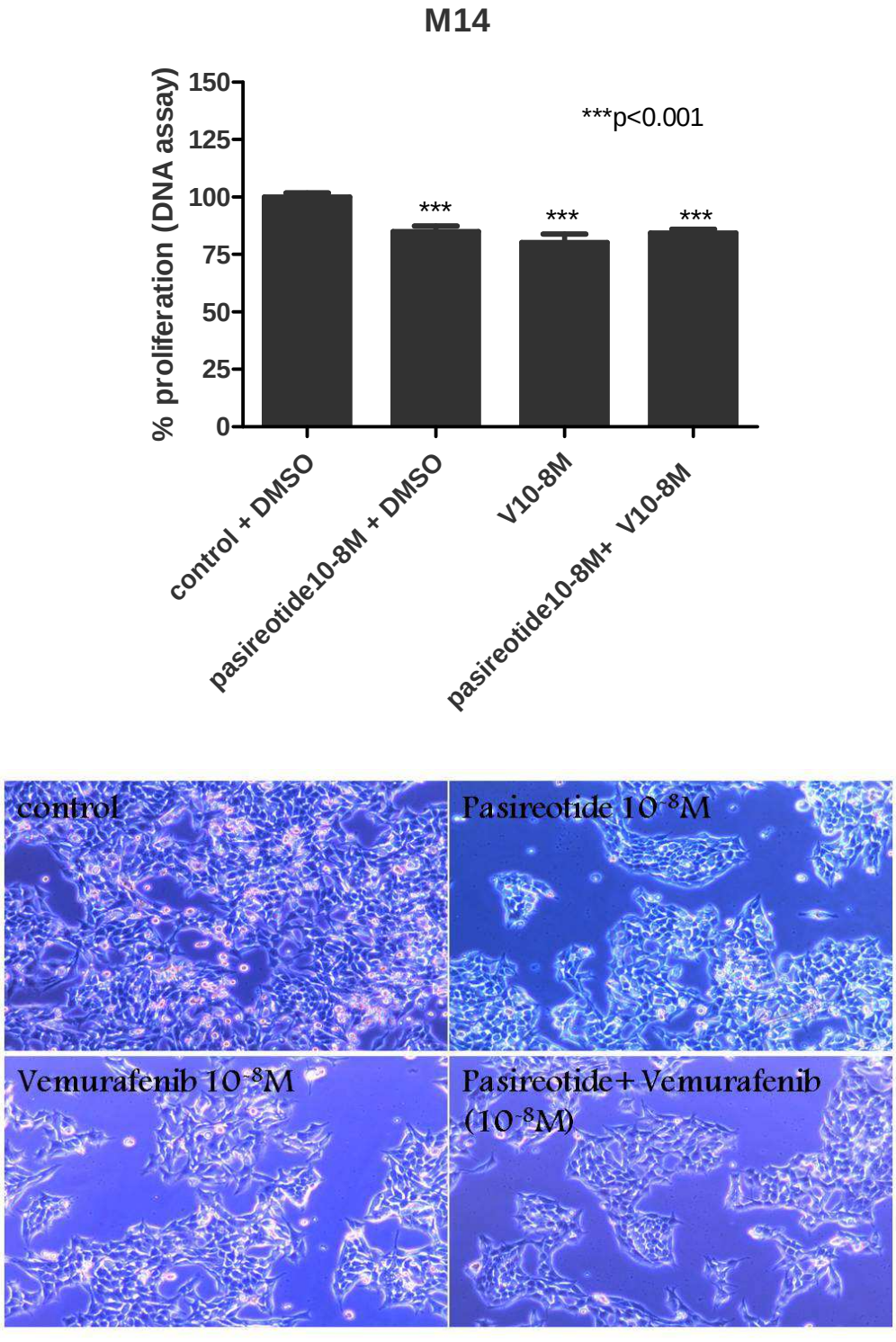


Figure 29. Effect on melanoma cell lines proliferation (A375 and M14) of 72 hours of combined treatment with vemurafenib and pasireotide at a concentration of 10^{-8}M (DNA Assay). Pasireotide was administrated every day. Data are expressed as the percentage of control and represent the mean±SEM. Control is set as 100%. Images under the graphs represent A375 and M14 cells respectively, treated for 72 hours with serial concentration of combined treatment with vemurafenib and pasireotide (in control and pasireotide 10^{-8}M, DMSO was added as vehicle). The pictures are acquired at 10X magnification.

4.10 Analysis of cell cycle by FACS

To determine whether the observed effects on cell viability and proliferation were elicited by gross changes in cell cycle progression such as cell cycle phase blocks, treated and untreated cells (A375 and M14) were stained with propidium iodide (PI) following a 24 hours incubation with pasireotide, vemurafenib and the combination of them. All drugs are used at the concentration of 10^{-8}M. The staining with PI offers an instantaneous depiction of cell cycle. Flow cytometry analysis revealed that: in A375 cells, treatment with pasireotide induced a moderate reduction of cells % in G2/M phase compared to untreated cells after 24 hour of treatment, vemurafenib induced an increase of cells % in G0-G1 phase and the combined with psireotide and vemurafenib treatment determinate a reduction of cells % in phase S (Fig.30). In M14 cells, treatment with pasireotide induced a moderate reduction of cells % in G2/M phase, vemurafenib strongly increase the cells % in G0-G1, moderate reduced the cells % in G2/M and strongly reduced the cells in % S phase. The combined treatment with pasireotide and vemurafenib induced a reduction of cells % in G2/M and S pahases (Fig.31). The following tables summarize the cells % distribution of cells in cell cycle phases after 24 hour of treatment with each compounds. (Tab.3 and Tab.4 for A375 and M14 cells, respectively). No big alterations of the cells cycle was detected after treatment, but a strong decrease of the number of treated cells suggest a anti-proliferative effects of tested compounds.

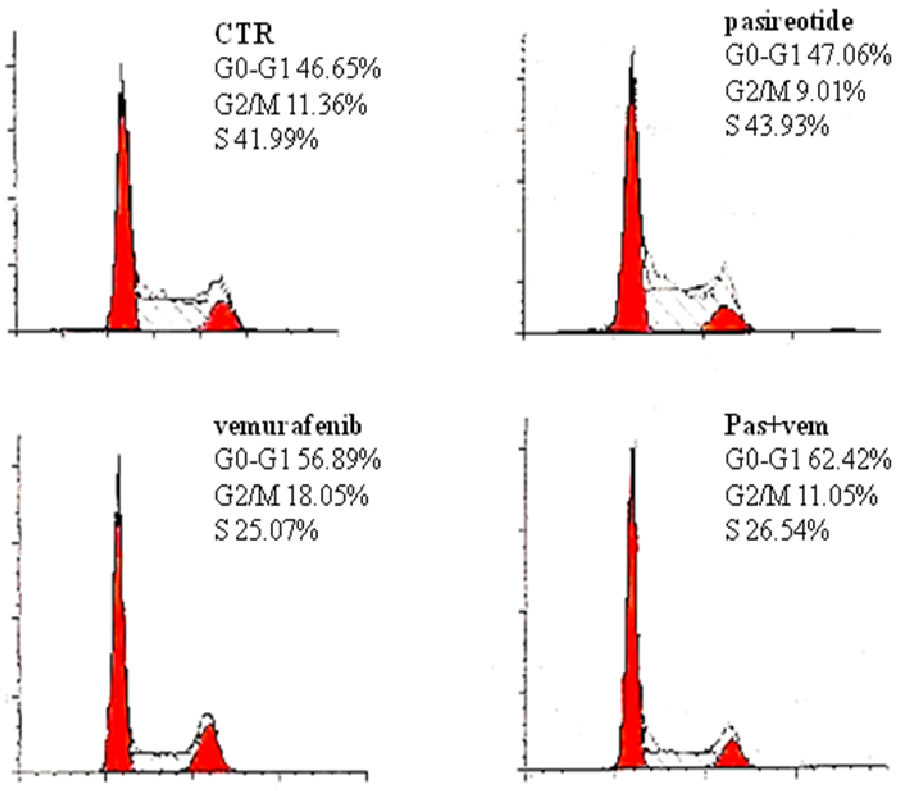


Figure 30. Analysis of A375 cell cycle by FACS. A375 cells are treated with pasireotide, vemurafenib and pasireotide+vemurafenib at dose of 10^{-8}M for 24 hour. M14 ells were stained with Propidium iodide (PI) (50µg/µl).

A375	G0/G1	G2/M	S
CTR	46.65	11.36	41.99
Pasireotide	47.06	9.01	43.93
Vemurafenib	56.89	18.05	25.07
Pas+Vem	62.42	11.05	26.54

Table 3. Table summarizes the relative distribution of A375 cells (%) in the different phases of the cell cycle after 24 hours of treatment with pasireotide, vemurafenib and pasireotide + vemurafenib. All tested drugs are at the concentration of 10^{-8}M.

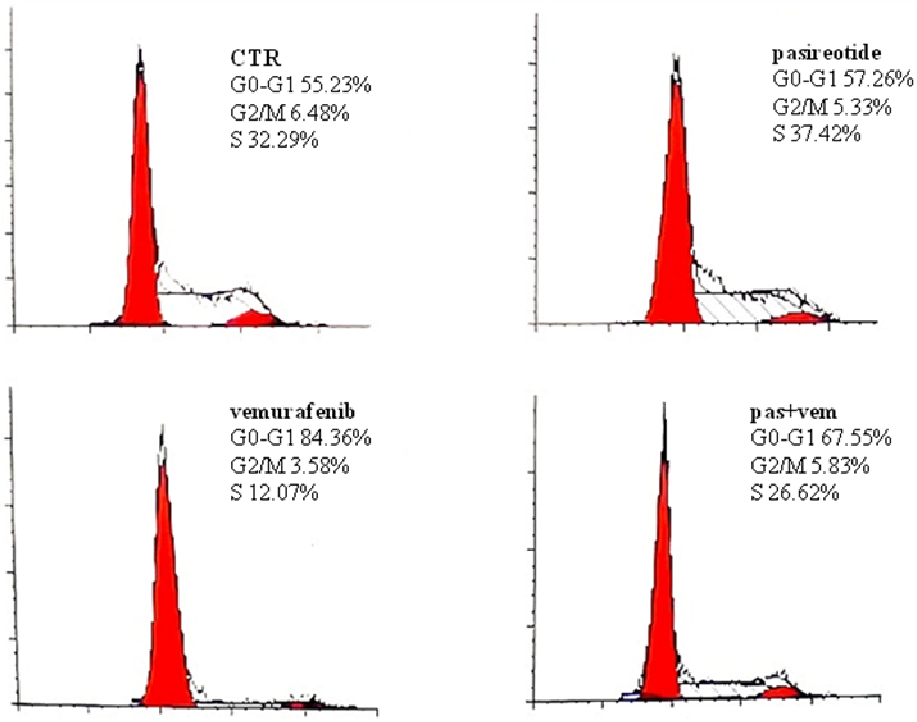


Figure 31. Analysis of M14 cell cycle by FACS. M14 cells were treated with pasireotide, vemurafenib and pasireotide+vemurafenib at dose of 10^{-8}M for 24 hour. M14 ells were stained with Propidium iodide (PI) (50µg/µl).

M14	G0/G1	G2/M	S
CTR	55.23	6.48	32.29
Pasireotide	57.26	5.33	37.42
Vemurafenib	84.36	3.58	12.07
Pas+Vem	67.55	5.83	26.62

Table 4. Table summarizes the relative distribution of M14 cells (%) in the different phases of the cell cycle after 24 hours of treatment with pasireotide, vemurafenib and pasireotide + vemurafenib. All tested drugs are at the concentration of 10^{-8}M.

Discussion

This study shows that four different types of human cutaneous melanoma cell lines have different mRNA and protein expression pattern of SSTRs. In two of these cell lines, treatment with SST analogs, particularly pasireotide a multi-ligand receptors, inhibits cell viability and proliferation in a concentration-dependent manner.

CMM remains a devastating disease with poor prognosis, despite the great efforts to develop new treatments and to understand the molecular mechanisms underlying its oncogenesis and progression. The median survival time for patients with metastatic melanoma is 8-9 months, and the 3-year-survival rate is less than 15 percent (Balch CM. *et al*. 2009). The incidence and mortality of CMM, in contrast to many other tumor types, continues to increase worldwide. Surgical resection remains the mainstay of therapy for localized disease, whereas new treatment options are required for CMM. Molecular targeted therapies have been promisingly developed during the last years to improve the response rate, to reduce the adverse effects and toxicity and to overcome the onset of resistance to the current antineoplastic therapies. The understanding of the driven genetic aberrations in melanoma has allowed the development of new treatment approach for patients with this malignancy. Nevertheless these molecular targeted therapies are predicted to be effective only in a subset of patients, that are generally those presenting the appropriate alteration in the molecular targets and not presenting any activated mechanism of resistance (Arnedos M. *et al*. 2014). Additionally after a certain time, also responsive patients can develop resistance, rising the requirement of new alternative therapeutic strategies. In the field of new therapeutic approaches, the introduction of SST analogs represents an attractive perspective because of SST ability to control hormonal secretion and inhibit progression in neuroendocrine tumors (Ruscica M. *et al*. 2013). The expression of membrane receptors in several tumors represent an interesting option for target therapy also because these receptors after ligand binding can be internalized thus they can carry into tumor cells radionuclide-conjugated or chemotherapeutic-conjugated ligands. The use of radionuclides-conjugated SST analogs in patients with neuroendocrine tumors testify the role of SSTRs in diagnosis and treatment of human neoplasia. The role of SSTRs in cutaneous melanoma has been scantily described. Indeed only few studies evaluated SSTRs expression in human samples, the role of radionuclides-conjugated SST scintigraphy and SST analog treatment in melanoma. Octreotide most clinically developed SST analog, has an higher affinity for SSTR2. SSTR2 is abundantly expressed in cutaneous melanoma as well as in other tumor types including neuroendocrine tumors (Hofland L. and S.W. Lamberts. 2001, Lum SS *et al*. 2001). Lum SS. *et al*. showed by RT-PCR and Southern blotting that SSTR1-2-3-4-5 are expressed in 96%, 83%, 61%, 57% and 9%, respectively, in malignant melanoma biopsies from 17 patients. Moreover, they demonstrated that ^{111}In-Pentetreotide scintigraphy (OctreoScan) was able to successfully image 63% of melanomas in a subgroup of evaluated patients. According to Martinez Alonso *et al*., SSTR2 mRNA is the predominant SSTR mRNA expressed in 18 skin melanoma cell lines including 4 primary tumor-derived and 14 metastatic tumors-derived (12 cutaneous and subcutaneous and 2 visceral). They observed

that all melanoma cell lines express the mRNA of at least one of the four SSTRs. The majority of cell lines express at the highest levels SSTR2, followed by SSTR1, SSTR3 and SSTR5. The current study confirms by relative mRNA quantification of SSTRs, that SSTR2 is the highest expressed SSTR subtype in four melanoma cell lines (A375, HMCB, M14 and COLO38). However, the relative expression of the other SSTR investigated was different compared with previous reports being mRNA levels of SSTR5 and SSTR3 higher than SSTR1 in three of the four evaluated cell lines. Taking in account that the melanoma cell lines tested in our study are different from those used in the above motioned studies, these results support the existence of an heterogeneous expression of SSTRs in CMM. In all cell lines the protein expression of SSTR1, SSTR2 and SSTR5 was confirmed by ICC. Few data are available about SSTRs protein expression in uveal melanoma. Valsecchi ME. *et al.* demonstrated that 46% of patients with uveal melanoma (14 of 30 patients) were positive by Octreoscan. IHC was performed on 10 Octreoscan-positive patients. All of them expressed SSTR2. In another series of uveal melanoma the expression of SSTRs observed by IHC, was 60%, 92%, 28% and 52% for SSTR2A, SSTR2B, SSTR3 and SSTR5, respectively. Additionally patients presenting an high SSTR2 protein expression levels had a better survival compared to those with low levels, suggesting that SSTR2 protein expression could be useful as marker of prognosis (Ardjomand N. *et al*). To our knowledge the current study is the first report on the SSTR1, 2 and 5 protein *expression* in preclinical model CMM. The evaluation of protein expression by ICC is interesting because it also suggests the protein subcellular localization. The present study suggests that at the condition tested, SSTRs are predominantly expressed in cytoplasm. SSTRs are membrane-associated receptors but SSTR-ligand complexes undergo cellular internalization with progressive translocation from the cytoplasm to the nucleus (C.A. Hornick *et al*. 2000, D.D. Klisovic *et al*. 2001, J.C. Reubi *et al*. 2001). Renzo Cescato *et al*. evaluated by IHC the SSTR2, SSTR3 and SSTR5 receptor internalization after agonist or antagonist treatment (R. Cescato *et al*. 2006). They observed that SSTR2 and SSTR3, in untreated cells (HEK 293, human embryonic kidney) are localized exclusively to the cell surface. Conversely, even in untreated cells SSTR5 is not only localized to cell-surface but also in intracellular perinuclear and cytoplasmatic regions. Furthermore, their study showed that SSTR2 and SSTR3 agonists, but not SSTR2 and SSTR3 antagonists, can trigger receptor internalization. The authors suggested that SSTR5 intracellular distribution might depend by its particular trafficking. Indeed SSTR5 might either not be internalized upon ligand binding, either it might undergo to a rapid recycling after internalization as result of a massive recruitment to the cell surface from an intracellular SSTR5 reserve pool (Stroh T. *et al*. 2000). Therefore, it is possible to speculate that in untreated melanoma cell lines SSTRs are localized predominantly in cytoplasmatic region, but that at least partially after SST analogs stimulation, they might be recruited to the plasma membrane. To confirm this hypothesis western blotting experiments are ongoing (data not shown). Preliminary results shows that SSTR5 might be recruited to plasma membrane upon pasireotide binding suggesting that the different trafficking of SSTR2 and 5 might explain the stronger antiproliferative effects observed with pasireotide compared to octreotide in the two responsive melanoma cell lines. These results will clarify the different sub-cellular distribution of SSTRs before and after SST analogs stimulation.

Additionally, to detect SSTR subtypes position in the different cellular compartments in melanoma cells, confocal and electron microscopy has also been planned. Tejeda M. *et al*. demonstrated that TT-232, a SST analog with high affinity for SSTR1 and SSTR4, had an anti-proliferative effects (tumor growth inhibition and survival time prolongation) in B-16 rodent melanoma and in HT-18 human lymphoid melanoma xenograft models. Schwab RE at al. also tested the anti-proliferative efficacy of TT-232 in human melanoma cell lines and tumors. They investigated the effect of TT-232 in seven melanoma cell lines and two xenograft CB17-SCID mice. T-232 strongly inhibited proliferation of all cell lines *in vitro* and tumor viability *in vivo*. Martinez-Alonso *et al*. investigated the effect of SST analogs, octreotide and pasireotide, in 18 primary and metastatic human cutaneous melanoma cell lines. Both of the SST analogs inhibited melanoma cell proliferation in a concentration-dependent manner. Both SST analogs induced only a moderate inhibition of melanoma cell viability (maximal inhibition observed with 10^{-6}M after 3 days of treatment: 19.85% and 18.55% *vs* control, respectively). In the present study, the four melanoma cell lines used had a different sensitivity to the two SST analogs tested. Octreotide is a synthetic SST analog with high binding affinity for SSTR2. Although SSTR2 was the most expressed SSTR subtype at mRNA level and it was clearly expressed also at protein level in all cell lines tested, octreotide inhibited cell viability only in A375 and M14 cells. The maximal significant inhibition was achieved with 10^{-6}M (54.15% $p<0.001$ and 24.32% $p<0.01$ *vs* control; $IC_{50}2.9*10^{-11}$M and $7.7\ 10^{-11}$M, respectively). Octreotide did not inhibited cell proliferation in the four melanoma cell lines used. Pasireotide is a novel multireceptor SST analog, which binds with high affinity to all SSTRs except to SSTR4 (Weckbecker G. *et al*. 2002). In contrast to octreotide, pasireotide exhibits particularly high subnanomolar affinity to SSTR5 (Ma P. *et al*. 2005). Pasireotide significantly inhibited, in a dose dependent-manner, viability and proliferation in two melanoma cell lines (A375 and M14). Both these cell lines presented a strong protein expression of SSTR5. Conversely, pasireotide did not have any effects in two melanoma cell lines (HMCB and COLO38) that had weak SSTR5 protein expression. These results suggest that pasiretotide might at least in part exerts is action through SSTR5 binding and that strong protein expression of SSTR5 could be predictive of response to this drug in human cutaneous melanoma cells. These results are in agreement with previous reports that suggest that pasireotide has stronger antisecretive and antiproliferative effects than octreotide in cells and tissues that express SSTR5 other than the SSTR2 and 1 receptor subtype (Schmid HA. 2007). This is also in line with the different affinity profile of pasireotide and octreotide for the various SSTRs (Tab.5).

Binding affinities of somatostatin, octreotide and pasireotide for the five sst receptor subtypes (sst_{1-5}) (Bruns et al., 2002; Schmid and Schoeffter, 2004)

Compound	sst_1	sst_2	sst_3	sst_4	sst_5
Somatostatin (SRIF-14)	0.93	0.15	0.56	1.50	0.29
Octreotide	280.0	0.38	7.10	>1000	6.30
Pasireotide	9.3	1.0	1.5	>1000	0.16
Octreotide/pasireotide	30	0.4	5	-	40

Results are IC_{50} values (nmol/L).

Table 5. Relative binding affinity of SST analogs for the five SSTRs.

Additionally pasireotide might be stronger than octreotide because trafficking of SSTRs upon octreotide and pasireotide stimulation strongly differ (Cescato R. 2006). Some studies have suggested that Octreotide induces rapid internalization of SSTR2 and subsequent ß-arrestin binding and receptor degradation into endocytic vesicles potentially determining receptor desensitization (Pöll F. *et al*. 2010, Hipkin RW. *et al*. 1997). Pasireotide induces the recruitment of a functionally distinct pools of ß-arrestin that bind to SSTR2 in unstable ß-arrestin-SSTR2 complexes. Consequently, SSTR2 recycles rapidly to the plasma membrane after endocytosis in pasireotide-treated cells, but not in octreotide-treated cells. Compounds with high affinity to both SSTR2 and SSTR5, show distinct internalization properties at these 2 receptors: a strong SSTR2 internalization and rapid recycling but no SSTR5 internalization (Cescato R. 2006).

In both responsive melanoma cell lines the maximal significant inhibition, with daily administration of pasireotide was achieved with 10^{-7}M (about 40% for viability and 20% for proliferation). The IC_{50} of pasireotide was $8.3*10^{-12}$M and $5.16 *10^{-13}$M in A375 and M14 cells respectively that are within the range of tolerability, as determined in clinical trials (Cmax about 10^{-7}M) (Beglinger C. *et al*. 2012). The effects of pasireotide on cell viability observed at the tested condition were stronger than the effects on cell proliferation. In melanoma cell lines the different effects of octreotide and pasireotide on cell viability (measuring metabolic activity of cells) and proliferation (measuring the effective number of cells) could depend by the different techniques used and by the fact that the inhibition of metabolic activity is a precocious event that could precede the inhibition of cell proliferation.

Preliminary results of the FACS analysis suggest that the antiproliferative effects of pasireotide in A375 and M14 could at least in part depend by an inhibition of cell cycle.

Vemurafenib, a selective *BRAF* V600 mutant kinase inhibitor, was approved by FDA in August 2011 based on the BRIM3 Phase III study showing improved clinical outcomes compared to dacarbazine (Chapman PB. *et al*. 2011. 2012). The objective response rate for vemurafenib was 48% after five months and an overall survival of 84% after six months. It represents a major breakthrough in targeted therapeutics of advanced melanoma. Unfortunately, the benefit of this agent is limited by the frequent and rapid onset of resistance. Multiple mechanisms of resistance have been described, including elevated expression of the kinase CRAF (Montagut C. *et al*. 2008), activating mutations in N-RAS, MEK1, or AKT1 (Nazarian R. *et al*. 2010, Wagle N. *et al*. 2011, Lo

RS. 2012) aberrant splicing of *BRAF* (Poulikakos PI. *et al*. 2011), activation of phosphatidylinositol-3-OH kinase (PI3K) via the loss of *PTEN* (Paraiso KH. *et al*. 2011), and persistent activation of receptor tyrosine kinases, including platelet-derived growth factor receptor b (PDGFRb), insulin-like growth factor IR (IGF-IR), and EGF receptor (EGFR) (Villanueva J. *et al*. 2010, Girotti MR. *et al*. 2013). Since these mechanisms of resistance to monotherapy have been reported, recent therapeutics efforts have focused on increasing MAPK inhibition trough combined therapies. The RAS/RAF/MEK/ERK signaling pathway is central to the pathogenesis of cutaneous melanoma (McCubrey. *et al*. 2007) and for this reason it represents a primary therapeutic target. SST analogs also could exert their anti-proliferative effects by modulating MAPK signaling (Theodoropoulou M. *et al*. 2013). Investigating the function of each SSTR in several tumor types has provided a wealth of information about the common but also distinct signaling cascades that suppress tumor cell proliferation, survival and angiogenesis, this provide the rationale for developing multi-receptor-targeted SST and combination with signaling targeted agents such as *BRAF* inhibitors. Vemurafenib significantly inhibited A375 cell viability and proliferation in a time and dose dependent manner. These results are consistent with the well documented BRAF V600E mutation in A375 melanoma cell line. In HMCB twenty-four hours treatment with vemurafenib significantly affected cell viability at maximal concentration, but this effect was lost with time. In agreement with previous reports melanoma cells with wild-type *BRAF* are not responsive to vemurafenib (Poulikakos PI. *et al* 2010, Hatzivassiliou G. *et al*. 2010). At condition tested Vemurafenib did not significantly inhibit cell viability and cell proliferation in M14 and COLO38. The genetic background and potential mutations other than BRAF may affect the response to these drugs.

In A375 the combined treatment with vemurafenib and pasireotide had significant additive effects on cell viability but not on cell proliferation. In M14 combined treatment with vemurafenib and pasireotide had no significant additive effects on cell viability and cell proliferation. In HMCB and COLO38 combined treatment with vemurafenib and pasireotide had no significant additive effects on cell viability while cell proliferation was not investigated. In pasireotide sensitive cells (A375 and M14), the loss of additivity may be due to the activation of escape pathways and/or to the capability of vemurafenib to affect the SSTRs trafficking.

Taking together, the results of the current study have a potential translational value since the expression of SSTRs might indicate the potential use of SST analogs, radio-labeled SST analogs, SST analogs conjugate with chemotherapic agents and SSTR scintigraphy in the management of a subset of patients with CMM. This study encourages further studies to better define the role of SST pathway in diagnosis, prognosis and as potential target for treatment in human CMM.

Conclusion

Our data provide evidence for SSTRs expression in CMM supporting the hypothesis that this tumor might have some neuroendocrine features. The pan-SSTRs agonist pasireotide significantly inhibits cell viability and proliferation in two human CMM cell lines (A375 and M14), with greater effects as compared to octreotide. These data suggest that SST analogs, particularly pasireotide, may have a role in the treatment of patients with CMM. The cell lines responding to pasireotide present a higher protein expression of SSTR5, suggesting that strong SSTR5 protein expression could be predictive of responsiveness to this drug in CMM. Three day-treatment with vemurafenib significantly inhibits cell viability and cell proliferation only in one CMM cell line (A375) with a well characterized *BRAF* mutation. Combined treatment with vemurafenib and pasireotide do not have additive inhibitory effects in most of cases, suggesting that the combination of these treatments does not give major advantages.

This study has a potential translational value since the expression of SSTRs might indicate the potential use of SST analogs, radio-labeled SST analogs, SST analogs conjugate with chemotherapic agents and SSTR scintigraphy in the management of a subset of patients with CMM. These results encourage further studies to better define the role of SST pathway in diagnosis and prognosis, and as potential target for treatment in human CMM.

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