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REZISTENCE MELANOMŮ K LÉČBĚ VINCA ALKALOIDY

Diplomová práce

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DIFFERENTIAL RESISTANCE OF MELANOMA TO VINCA–ALKALOIDS

Diploma thesis

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Lucie Rozkydalová

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Abstrakt

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Název práce: Rezistence melanomů k léčbě vinca-alkaloidy

Maligní melanom (MM) představuje nejnebezpečnější a velmi agresivní kožní nádor s rychlým rozvojem lékové resistence, která je hlavní překážkou úspěšné léčby MM. Na základě předchozích studií (microarray datová analýza), byl jako jedna z potencionálních příčin selhání léčby vinca alkaloidy (VAs) vybrán gen KIT, který hraje důležitou roli v patofyziologii melanomu. KIT byl zvolen kvůli úplnému potlačení jeho exprese v rezistentních buněčných liniích (CAL1R–VAs) v porovnání s parentálními buňkami (CAL1–wt). Kromě toho KIT také interaguje s proteiny NF–κB a cyklinem D1–2, které jsou zahrnuty v rezistenci melanomu uvnitř molekulární sítě vybudované softwarem IPA. Ačkoliv bylo potvrzeno potlačení exprese genu KIT v rezistentních CAL1R–VAs buněčných liniích (qRT-PCR), represe KIT prostřednictvím siRNA transfekce neukázala žádný efekt na *in vitro* senzibilitu CAL1-wt buněk k VAs. To značí, že KIT není přímo zahrnut v rezistenci melanomu, ale mohl by být biomarkerem rezistence k VAs. Toto zjištění je potřeba potvrdit skrze biopsii a mohlo by být velmi nápomocné k optimalizaci a individualizaci léčby.

Abstract

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Title of diploma thesis: Differential resistance of melanoma to vinca–alkaloids

Malignant melanoma (MM) represents the most dangerous and very aggressive skin tumor with fast development of drug resistance which is the main obstacle in successful treatment of MM. According to previous studies (microarray data analysis), KIT gene, which plays key role in melanoma pathophysiology, was chosen as one of the potential causes of failure of treatment by vinca alkaloids (VAs) because of its complete underexpression in melanoma CAL1 resistant cells (CAL1R–VAs) in comparison with parental cells (CAL1–wt). Moreover, KIT also interacted with NF–κB and cyclin D1–2 proteins involved in chemoresistance of melanoma – inside molecular network built using IPA software. Although KIT underexpression in resistant CAL1 R–VAs cell lines were confirmed (qRTPCR), KIT repression using specific siRNA transfection did not show any effect on *in vitro* sensibility of CAL1–wt cells to VAs. It signifies that KIT is not directly involved in melanoma resistance but could be a biomarker of resistance to VAs. This last point remains to be studied trough biopsies analysis and could be helpful to optimize and individualize treatment.

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1. List of Abbreviation

AKT	v-akt murine thymona viral oncogene homolog
ARF	alternate open reading frame
BCG	bacillus Calmette-Guerin
Bcl-2	B-cell CLL/lymphoma 2
BRAF	v-raf murine sarcoma viral oncogene homolog 1
CAL1–wt	parental CAL1 cell line
CAL1R–VAs	CAL1 cell lines resistant to vinca alkaloids
CAL1R–VCR	CAL1cell line resistant to vincristine
CAL1R–VDS	CAL1 cell line resistant to vindesine
CAL1R–VRB	CAL1 cell line resistant to vinorelbine
CCND1	cyclin D1
CDK4	cycline-dependent kinase 4
CPDs	cyclobutanepyrimidine dimers
CTLA–4	cytotoxic T–lymphocyte antigen 4
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate–buffered saline
DSBs	double-strand breaks
FBS	fetal bovine serum
GNAQ	guanine nucleotide binding protein q polypeptide
GNA11	guanine nucleotide binding protein alpha 11
GSH	glutathione
GSTs	glutathione S-transferases

GSTM1	glutathione S–transferase 1
HR	homologous recombination
IC_{50}	the half maximal inhibitory concentration
IFN-α	interferon-α
IL-2	interleukin 2
INK4A	cyclin-dependent kinase inhibitor p16
IPA	Ingenuity pathway analysis software
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
MAPK	RAS mitogen–activated protein kinase pathway
MDR	multidrug resistance
MITF	microphtalmia-associated transcription factor
MM	malignant melanoma
MRP1	multidrug resistance protein 1
NHEJ	non-homologous end-joining factor
NRAS	neuroblastoma RAS viral oncogene homolog
NTsiRNA	non–targeting small interfering RNA
PD–1	programmed cell death 1 protein
PI3K	phosphoinositide 3 kinase inhibitor
PTEN	phosphate and tensin homolog
p53	protein 53
qRT–PCR reaction	quantitative real–time reverse transcriptase polymerase chain
RAS	retrovirus associated sequence oncogene

RT	reverse transcription
RTKs	receptor tyrosine kinases
siRNA	small interfering RNA
TP53	tumor protein p53
UV	ultraviolet radiation
VAs	vinca – alkaloids
VCR	vincristine
VDS	vindesine
VRB	vinorelbine
6-4 PPs	6-4 photoproducts

2. Introduction

Malignant melanoma (MM) is generally one of the most dangerous and aggressive tumors with alarming increase of incidence worldwide. The huge danger of MM is not based only on increasing incidence but also on its tendency to make metastasis to almost every organ (1) and on fast development of drug resistance which present the biggest reason of failure of treatment (2).

In cases when the systematic chemotherapy is needed, the number one in treatment of MM is currently targeted therapy but either this new personalized treatment approach is not saved from developing of drug resistance (3). And just in this moment, there is need of classical therapy where vinca alkaloids (VAs) indisputably belong to. And that's why it is still actual and important to dedicate the research to vinca alkaloids and to try to find the mechanism/s of chemoresistance to VAs or genetic and molecular pathways which are involved in.

For realization of our aim we decided to use transcriptomic analysis which is giving us an overview about entire cells and differentially expressed genes via analysis of RNA.

3. Theoretical part

3.1. Malignant melanoma

Malignant melanoma is the most dangerous and aggressive form of skin tumour. In spite of cutaneous melanoma accounts for only 4% of all skin cancers it causes 90% of skin cancer mortality (4). Its incidence in most developed countries has risen faster than any other cancer type and it is one of the most common causes of cancer and cancer death between the ages of 20 – 35 (1). The increasing incidence of malignant melanoma (MM) is not the only one problem of this kind of skin cancer, another one is chemoresistance to anticancer drugs and treatment in general and strong propensity for dangerous metastasizing to almost any organ (1,2). But melanoma prognosis would not be so dismal with early detection before malignant melanocytes become invasive.

3.1.1. Definition

Melanoma is malignant tumour that arises from neoplastic proliferation of melanocytes – specialized pigment cells which are located on the basement membrane of epithelial surfaces and produce melanin, the pigment responsible for skin and hair color. Melanoma primarily involves the skin, but it can also affect eyes and rarely meninges, gastrointestinal tract and mucous membrane of mouth and genital (1,4,5). In comparison with other epithelial skin tumours for melanoma is not typical aggressive local destruction of skin but the high risk of fast hematogenous or lymphogenous metastasizing (4).

3.1.2. Epidemiology

In general, melanoma together with nonmelanoma skin cancer belong to the most common types of cancer in white population (6) and during last four decades cutaneous melanoma is the most rapidly increasing cancer worldwide (7). Incidence among dark-skinned ethnic groups is 1 per 100,000 per year or less but it is up to 50 per 100,000 per year among fair-skinned population (6).

The annual increase of melanoma has been estimated between 3% and 7%, depending on various populations. It can be expected that despite the increasing incidence rate, a further decrease in melanoma mortality may develop as a result of more screening activities, improvement of early diagnosis and a lot of campaigns with primary prevention (7).

Besides skin color, one of the most important factors for increasing incidence is geographic zone. The highest incidence rates are in Australia, New Zealand and the southern states of United States of America. Incidence rates in European countries are still lower but during last decades it increases too (7).

People with lighter pigmentation, an inability to tan, blond or red hair, or blue eyes, and many pigmented lesions (including freckles, common and clinically atypical moles) have a higher risk of melanoma than the general population (Figure 1). Another risk factor is a history of familial melanoma; according some studies melanoma arises in these persons 10 times often than among the general population (8).

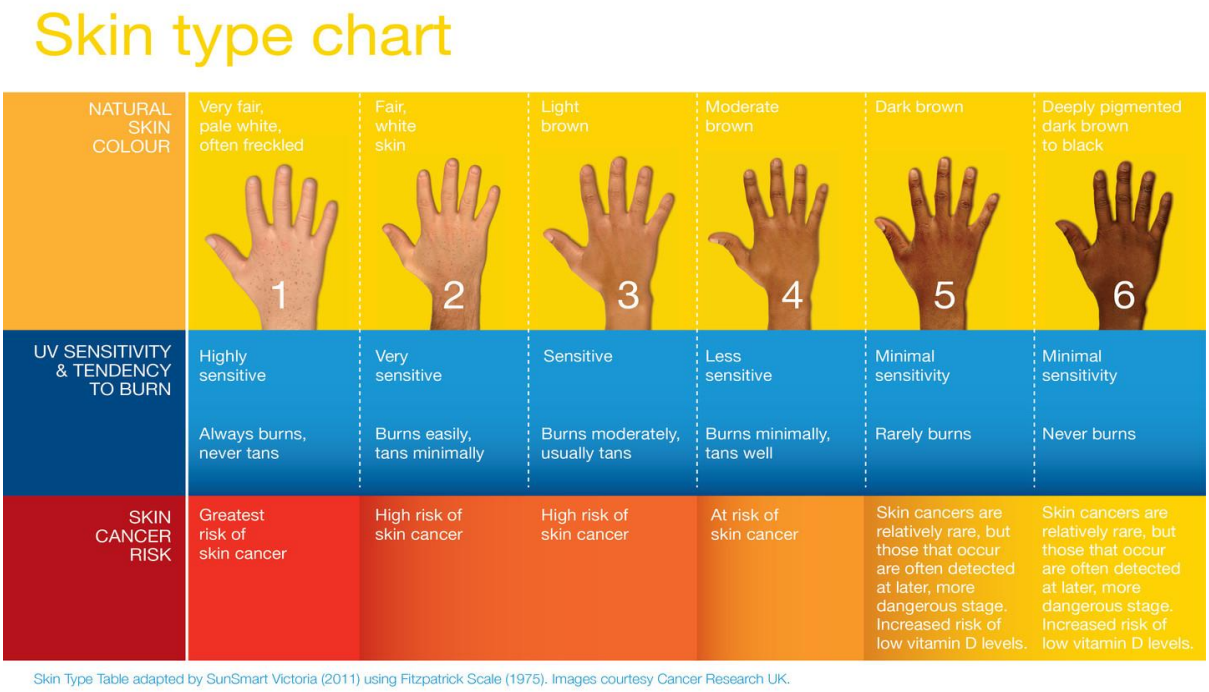


Figure 1 Skin types and their skin cancer risk.

Copy from: http://www.sunsmart.com.au/skin_type%20/%202685 (27/3/2013)

The sun exposure and severe sunburns, especially during childhood belong to most important and strong exogenous factors which increase a risk of cutaneous melanoma (8).

Sex is not very significant general risk factor because the male/female ratio varies in melanoma databases in different countries – in countries with a high cutaneous melanoma incidence preponderance of men is observed and the other way around in countries with a lower incidence, a higher ratio of women patients with melanoma can be found (7). But the anatomic site of MM varies according to sex. (7).

3.1.3. Different subtypes of melanoma

The cutaneous melanoma is divided to main four classical subtypes. These subtypes are distinguished by clinical and histopathological features according to epidermiological parameters and particular patterns of mutation (4) (Figure 2).

Superficial spreading melanoma (SSM) represents approximately 70% of skin melanomas and it is the most often subtype in patients between the ages of 30 – 50 (9). In most cases it is flat or mildly raised, bigger than 6 mm and often with multiple colors and pale areas of regression (4).

Nodular melanoma is a primarily nodular, exophytic and brow–black with often inclination to ulceration and bleeding. It is characterized by an aggressive vertical phase, with a short or absent horizontal growth phase. Thus, an early identification in an intraepidermal stage is almost impossible (4). The lesion can be amelanocytic, which makes identification more difficult too. The incidence of this subtype is 15 – 30% of skin melanomas (9).

Lentigo maligna melanoma arises often after many years (at least 10 – 15) without metastasizing from lentigo maligna. The lesion of lentigo maligna is usually dark brown or black color with size 1 – 3 cm and it is located on the sun–damaged faces of elderly individuals in the age of 65 and more. After developing of lentigo maligna melanoma the color of lesion changes to blue–black (9).

Acral lentiginous melanoma is the least often subtype in white populations, but the most common subtype in populations with darker skin color (e.g., Africans, Asians and Hispanics). This subtype of melanoma is not associated with sun exposure (4,9).

Figure 2 Different subtypes of melanoma. 1 – Superficial spreading melanoma, 2 – Nodular melanoma, 3 – Lentigo maligna melanoma, 4 – Acral lentiginous melanoma. Modify: http://courses.washington.edu/hubio567/melanoma/types.htm (2/12/2012)

In addition to these main types, there are several rarer variants of melanoma which constitute less than 5% of cases, e. g. desmoplastic or amelanotic melanomas (4).

3.1.4. Pathophysiology

The skin is the largest organ of the body with surface area of 1,6 – 2 m^2 in adults (10). It has several functions, the most important being to form a physical barrier to the environment, allowing and limiting the inward and outward passage of water (skin contains about 72% of water (10)), electrolytes and various substance while providing protection against micro–organisms, ultraviolet radiation, toxic agents and mechanical insults (11). Understanding how skin can function in these many ways starts with understanding the structure of the 4 layers of skin – the epidermis, dermis and subcutaneous tissue (12) (Figure 3).

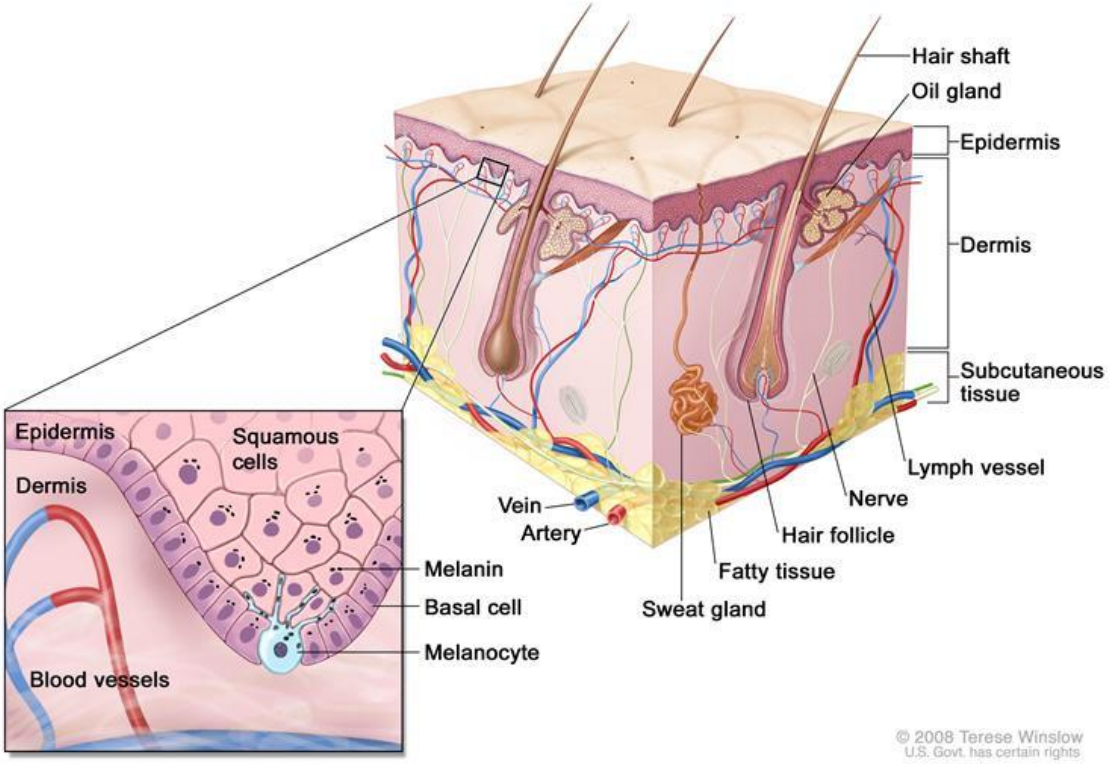


Figure 3 Anatomy of the skin, showing the epidermis, dermis and subcutaneous tissue. Melanocytes are in the layer of basal cells at the deepest part of the epidermis. Copy from: http://www.cancer.gov/cancertopics/pdq/treatment/melanoma/Patient/page1 (27/3/2013

From melanoma point of view the most important skin layer is outer layer – epidermis. The epidermis varies in thickness from 0,05 mm on eyelids to 0,8 – 1,5 mm on the soles of the feet and palms of the hand (11). It is made up of cells called keratinocytes which are stacked on top of each other forming different sub-layers (13) – stratum basale, stratum spinosum, stratum granulosum, stratum licidum and stratum corneum. Keratinocytes move from the lower sub–layer upwards to the surface and so these sub–layers are form by the different stage of keratin maturation (11). There are three types of specialized cells in epidermis. One of them are the melanocytes produced in stratum basale (12).

The normal major function of melanocytes is the synthesis, storage and transfer of melanin pigments to surrounding epithelial cells as a response to sunlight. The regulation of growing up of melanocytes is regulated by epidermal keratinocytes which maintain homeostasis. In response to ultraviolet (UV) radiation, keratinocytes secrete

factors that regulate melanocyte survival, differentiation, proliferation and motility, stimulating melanocytes to produce melanin and resulting in the tanning response (14). Thereby, melanocytes have a key role in protecting our skin from the damaging effects of UV radiation and in preventing skin cancer (5). When this regulation by keratinocytes does not work, a dysplastic nevus arises.

It means that sun's UV radiation plays really important role in development of skin cancer. Sunlight consists of two types of ultraviolet radiation – UVA and UVB. Both UVA and UVB radiation contribute to freckling, skin wrinkling and development of skin cancer. In spite of the fact that UVA radiation penetrates deeper into the skin (Figure 4), UVB radiation is more powerful and dangerous because of its damaging effect to the DNA of skin cells (15).

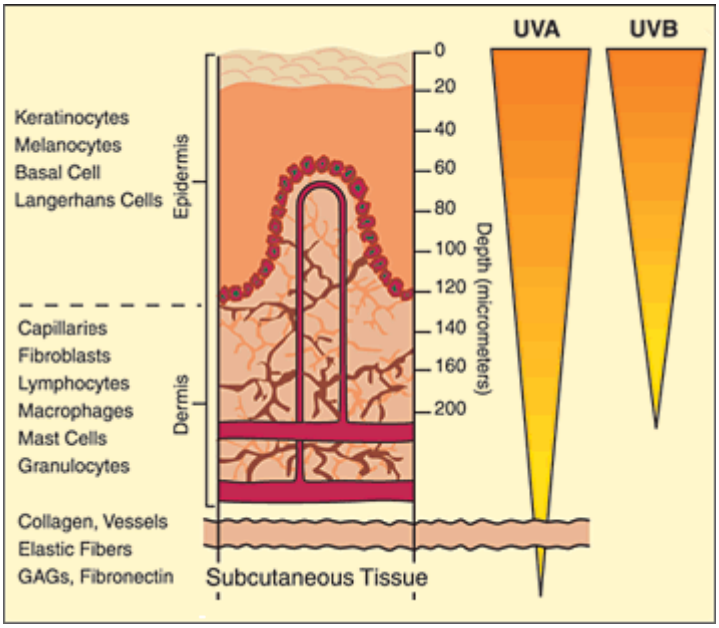


Figure 4 Penetration of UV radiation into the skin. Copy from: http://www.dermatology.ucsf.edu/skincancer/General/prevention/UV_Radiation.aspx# (27/3/2013)

Radiation damage to DNA is potentially dangerous to cells, since a single photon hit may have a carcinogenic or lethal effect. Several different types of DNA damage by UV have been identified. However the two major classes of mutagenic DNA lesions induced by UV radiation are cyclobutanepyrimidine dimers (CPDs) and 6–4 photoproducts (6–4 PPs), and their Dewar valence isomers (16). In normal conditions these damages are repaired by DNA repair mechanisms as e.g.: the simplest and oldest

one – photoreactivation; base or nucleotide excision repair; mutagenic repair or lesion bypass; recombinational repair and there are some alternative repair pathways too. In case that these mechanisms do not work and DNA damages (especially base dimers (Figure 5)) stay not repaired, it stops replication of DNA and it can have fatal effects (16,17).

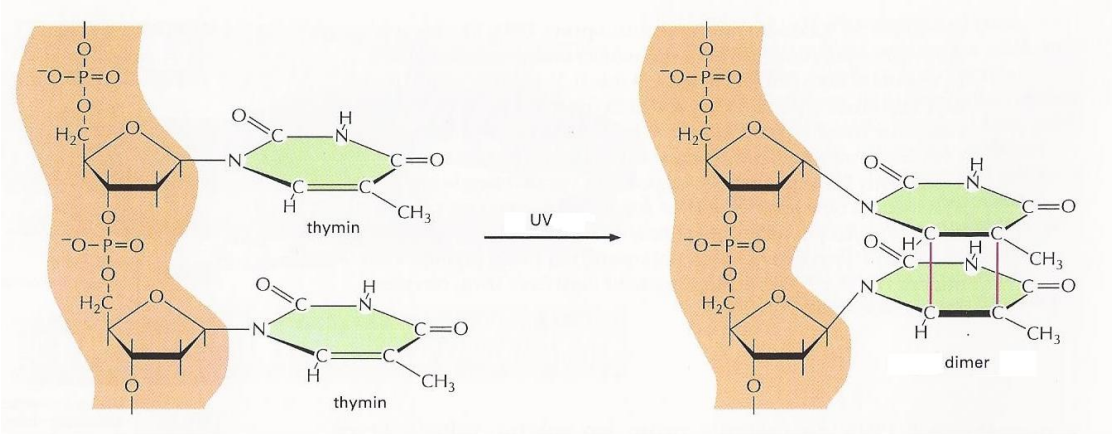


Figure 5 Formation of thymine dimer as a result of DNA dammage by UV radiation. Modify: Základy buněčné biologie, Alberts et al. (1998)

But UV is not only one exogenous cause of MM. The process of development of melanoma is multifactorial and the sequence of events in which normal melanocytes transform to melanoma cells is only poorly understood (18). It can arise either from already preexisting pigment lesion such as a congenital, acquired or atypical nevus or *de novo* – determination of origin is difficult because the most of patients cannot remember if they had some pigment lesion before or not.

Anyway melanocytic moles, benign clusters of melanocytes, have drawn special attention as potential precursor lesions (1). The most often warning sign of MM is newly–emerged changing pigment lesion. The rule ABCDE for clinical evaluation was established:

- A = asymmetry (the pigment lesion is not symmetrical)
- B = border irregularity
- C = color change or variegation

- D = diameter (diameter larger than 6 mm or growing lesion is characteristic of melanoma but even the pigment lesion smaller than this diameter can be malignant)
- E = evolution

There are five stages of tumor progression which have been suggested (19):

- benign melanocytic nevi
- melanocytic nevi with architectural and cytologic atypia (dysplastic nevi)
- primary malignant melanoma–radial growth phase
- primary malignant melanoma–vertical growth phase
- metastatic malignant melanoma

3.1.5. Staging: from anatomical to molecular staging

Cutaneous melanoma is divided into five (0 – IV) groups according to histopathological stage. Every group has its several own subgroups – stage 0 is *in situ* tumour (not grown deeper than the epidermis); stages I and II are without any regional or distant metastases and these two stages are divided into subgroups according to Breslow's tumour thickness (≥ 4 mm) and ulceration (present or absent); stage III is associated with high risk of regional lymph node metastases (the micrometastases, the macrometastases and satellite or in-transit metastases) and stage IV with distant metastases.

Next to this histopathological staging there are other two scales describing development of melanoma tumors – Breslow's depth and less reliable Clark's level (Figure 6). Breslow's thickness is defined as the total vertical height of the melanoma, from the granular layer to the area of deepest penetration into the skin. Level of invasion so called Clark's level refers how deep the tumor has penetrated into the layers of the skin and is only of independent significance for thin tumors (≤ 1 mm thickness) (4,20). Together with presence of histologically recognized ulceration and mitotic rate, Breslow's depth and Clark's level belong to the most important histological prognosis factor for primary melanoma without metastases (4).

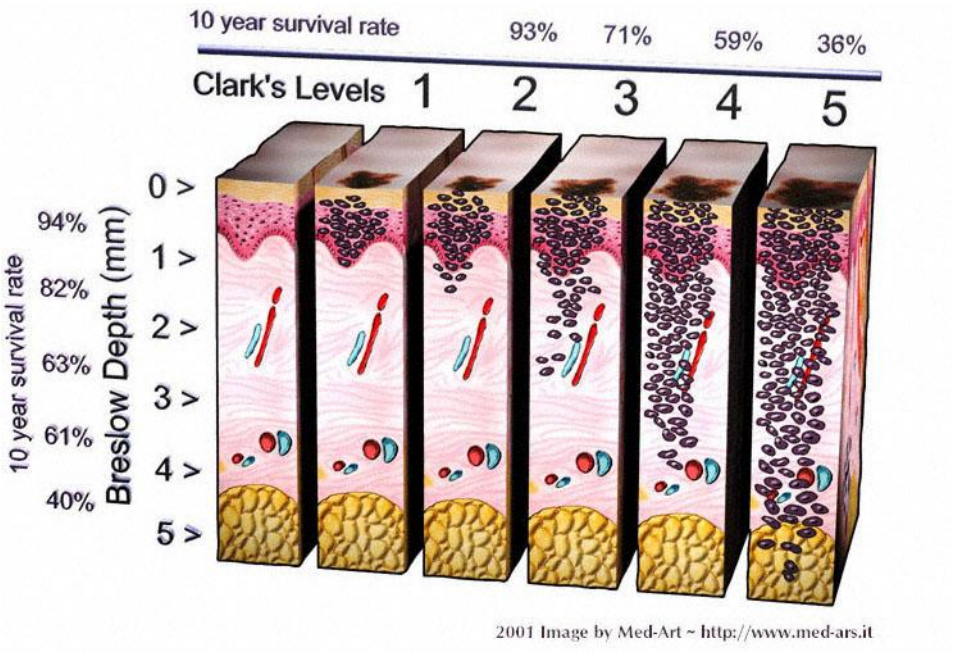


Figure 6 Stage of melanoma dividing according to Breslow's depth and Clark's level with prognosis in particular stage. Copy from: http://www.ilmelanoma.com/en/melanoma_epidemiology/staging-of-

Melanoma provides one of the best examples how genetics and environment interact in the pathogenesis of cancer (1) . The main role in risk of melanoma is played by genetic factors. Cellular pathways with high frequency of mutations include the p53 and retinoblastoma pathway and the RAS mitogen–activated protein kinase (MAPK) pathway which activate with BRAF and NRAS oncogenes. Mutation of these two genes is the most common mutation in superficial spreading and nodular melanomas. Besides BRAF and NRAS, mutations of TP53 and KIT take part in development of mucosal and acral lentiginous melanoma. Mutation in these genes may be inherited or causes by UV energy (14). A melanoma molecular disease model is based on the most of these genes and dividing melanoma into subtypes according to mutations in them (Table 1).

Table 1 Melanoma molecular subtypes:

Detailed subtypes	Pathway(s)	Key gene/ biomarker(s)
1.1	MAPK	BRAF
1.2		BRAF/PTEN

1.3		BRAF/AKT
1.4		BRAF/CDK4
2.1	c–KIT	c–KIT
3.1	CNAQ GNA 11	GNAQ
3.2		GNA 11
4.1	NRAS	NRAS
5.1	MITF	MITF
6.1	AKT/PI3K	PTEN
6.2		AKT
6.3		PI3K
7.1	CDK	ARF/INK4A
7.2		CDK4
7.3		CCND1/Cyclin D1
8.1	P53/BCL	Bcl-2
8.2		P53
Modify: Vidwans SJ, Flaherty KT, Fisher DE, Tenenbaum JM, Travers MD, et al. (2011) A Melnoma Molecular Disease Model. PLoS ONE. 2011 Mar 30;6(3):e18257		

3.2. Treatment of malignant melanoma

There are several different types of treatment, which can be used alone or in combination, either simultaneously or sequentially: surgery, radiotherapy and drugs (2). The approaches to treatment are rapidly changing according to new pieces of knowledge in molecular profile of melanoma. The larger understanding of molecular and genetic levels is not important only for treatment (21) but also for the early detection which has significant influence for overall survival of patients (4).

The type of treatment depends on the stage and location of the melanoma lesion and on patient's overall health. While early stages of melanoma can be successfully treated by surgical excision, advanced stages are uniquely refractory to current therapies (21).

3.2.1. Surgical therapy

The standard treatment in all case of primary melanoma is surgical excision of tumour lesion. Tumor lesion has to be removed with sufficient excision of margins of

normal surrounding skin which is depending on tumour thickness (Table 2) (4). But no more than 2 cm of normal skin needs to be removed from all sides of melanoma in stage I (histopathological staging). Wider margins make healing more difficult and without effect on prognosis (22).

Table 2 Recommended minimal excision margins for melanoma:

Tumor thickness (Breslow's depth)	Excision margin (cm)
in situ	0,5
≤ 2.0 mm	1
≥ 2.0 mm	2
Copy from: Garbe C. et al. Diagnosis and treatment of melanoma. European consensus – based interdisciplinary guideline – Update 2012, Eur J Cancer (2012)	

Melanomas in unusual sites (e.g., in the nail bed or nail matrix, on the fingers, and on the soles of the feet) are uncommon and require special surgical attention (8).

In case of distant metastases, complete operative removal of metastases is therapy of choice together with radiation and systemic therapy.

3.2.2. Radiation therapy

Radiation therapy of the primary tumour is very rarely indicated, performed exclusively in patients in whom surgery is impossible or not reasonable. This therapeutic approach is used in case of extensive skin metastases in combination with surgical therapy, painful bone metastases with loss of structural stability and compression of the spinal canal and brain metastasis which are usually deadly during 3 – 5 months(4).

3.2.3. Adjuvant therapy

Adjuvant therapy is offered for patients with primary melanoma (tumours thicker than 2 mm and stage II and III) and regional lymph node metastases, who are without distant metastases, but at the high risk of recurrence and further tumour spreading (4).

Over the past 25 years, adjuvant therapy for immediate–risk and high–risk patients have shifted from regional therapy, systemic imunostimulants such as Bacillus Calmette–Guerin (BCG) and Corynebacterium parvum, or pharmacologic

immunomodulators such as levamisole, to recombinant DNA–produced biologic agents such as IFN–α, granulocyte–macrophage colony–stimulating factor, and antibodies that have immunoregulatory function such as those that block cytotoxic T–lymphocyte–associated–antigen 4 (CTLA–4) (23).

Using various nonspecific immunostimulatory agents as BCG, cytokines as interleukin–2 (IL 2) or melanoma specific vaccines failed to show any therapeutic efficacy. Currently approach to adjuvant therapy is based on using immunotherapy with interferon α–the first substance with significant effect. The dose and duration of administering depend on histopathological stage (e.g.: low dose administered during 18 months in stage II–III) (4).

3.2.4. Systematic therapy of metastatic disease

Systematic therapy of metastatic disease is indicated to patients with inoperable regional metastases and distant metastases (histopathological stage IV). Despite the fact that about 90% of melanomas are diagnosed as primary tumors without any evidence of metastasis is really important to develop systematic therapy because of strong propensity of metastasizing, especially either lymphatic or the hematogenous route (4).

We can divide systematic therapy approach into three groups–classical chemotherapy using cytostatic drugs, biological targeted therapy and immunotherapy. The role of these therapeutical groups is to comply with both main goals of systematic therapy – prolongation of survival and reduction of tumour size or load with a resultant increase in symptom–free course or a decrease in symptoms. Disadvantage of chemotherapy is no influence on prolongation of survival (4).

The conventional classification system is based on histopathological patterns but this way of dividing of melanoma is not too appropriate for choice of effective treatment. Now, in genomic era, when it is clear that melanoma is composed of several biologically distinct subtypes, each with unique genetic and clinical features, and each likely to respond differently to any therapeutic strategy (24), personalized therapy becomes number one in treatment of MM. Because of big genomic heterogeneity of melanoma, it was really important to understand melanoma on its molecular and genetic level for successful personalized targeted therapies. One of the recent approaches to the treatment of melanoma is based on big variability of MM in molecular level and dividing melanoma into 8 groups according to different melanoma molecular subtypes

(e.g., mutation in BRAF gene or in KIT pathway, with RAS gene abnormalities or with abnormalities in the AKT signaling pathway). This approach is called Melanoma molecular disease model (21) and it enables to choose the most appropriate therapy targeted exactly to each subtype.

Numerous targeted inhibitors have already been developed and are under clinical investigation. But among approved drugs (both in USA and European Union) is vemurafenib which is inhibitor of BRAF. Vemurafenib blocks the mutated BRAF protein, turning off the rapid cells growth and causing cell death in tumors with the BRAF mutation (25). It achieves a high rapid tumor response rate roughly 50% (in patients with BRAF mutation, more exactly V600 mutation) and a significant prolongation of survival in comparison to dacarbazine. It has minor systemic (e.g.: fatigue) but major cutaneous side–effects, including photosensitivity, development of epithelial tumors and seldomly melanomas. Problem of resistance appears also in case of vemurafenib and it is a frequent event (4).

Currently dabrafenib (inhibitor of BRAF) and trametinib (inhibitor of MEK) are in clinical trials as well as combination BRAF and MEK inhibition. Another drug in clinical trials is imatinib – cKIT inhibitor which is testing for using in melanomas arising in sun–protected sites.

Another way how to treat melanoma is trough immune system. Monoclonal antibody ipilimumab was the first approved (both in USA and in EU) immunotherapy. It is the first and only approved therapy for metastatic or inoperable melanoma and it has a benefit for overall survival of patients. Ipilimumab blocks CTLA–4 which plays a role in either slowing down or turning off the body's immune system which in turn reduces its ability to fight and destroy cancer cells (26).

Other immune–active compounds, PD–1 antibodies, are currently in clinical trials.

Although classical anticancer drugs are not the first–choice drugs and it could seem that the approach of classical chemotherapy is obsolete in our genetic and molecular age but it is not truth. The drugs which are developing now, e.g., targeted compounds, they are defeated by chemoresistance as well as any other drugs and after the only way for continuing with treatment is conventional chemotherapy.

Chemotherapy can lead to regression of tumors and a reduction in tumour–related symptoms. In monotherapy dacarbazine is longest–established number one with the one of the biggest response rate (depending on the dose even 23%) following temozolomide (dacarbazine derivative), fotemustine and vindesine. Because of comparatively easy development of chemoresistance, polychemotherapy or chemoimunotherapy have been established. The most common combination is dacarbazine, vindesine and cisplatin (depending on the dose, response rate even 45%), another drugs used in polychemotherapy are carmustine, carboplatin or paclitaxel. The combination of multiple chemotherapeutic agents achieves higher remission rates than monotherapy. The combination with cytokines (interferon–α and IL–2) did not bring any positives, even the tolerability of monotherapy is worsened (4).

3.3. Vinca alkaloids

The vinca alkaloids are subset of natural product anticancer drugs originally derived from Periwinkle plant (lat.*Vinca rosea*) – vincristine (VCR; Figure 7) and vinblastine and their semisynthetic analogues vindesine (VDS; Figure 8A) and vinorelbine (VRB; Figure 8B). They are widely used in treatment of various tumours because of their antiproliferative activity which is a result of depolymerization of mitotic spindle microtubules.

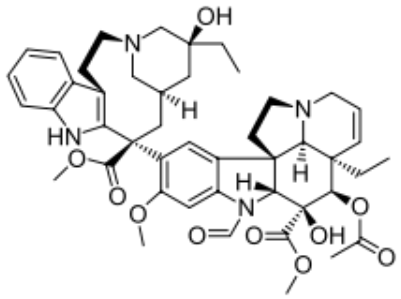


Figure 7 Formula of vincristine.
Copy from: http://en.wikipedia.org/wiki/Vincristine (24/1/2013)

The interaction of the Vinca alkaloids with tubulin is based on binding to tubulin and at high concentration they can inhibit polymeration of tubulin into microtubules, at the lowest effective they kinetically stabilize microtubules by reducing the rate of tubulin addition and loss at microtubule ends (27). The normal function of microtubules is providing both structure and flexibility the cells need to divide and replicate–cells cannot divide without microtubules (28).

Antiproliferative activity of vinca alkaloids is due predominantly or entirely to inhibition of mitotic spindle function. In spite of expectation, inhibition of proliferation by vinblastine and vincristine is due to spindle microtubule perturbation, not due to depolymeration of the microtubules (27). Different vinca alkaloids have their own

unique functions, e.g., vinblastine and vinorelbine inhibit angiogenesis which is one of the essentials steps in developing of cancer (28).

In spite of expectable reduction in using of vinca alkaloid in treatment because of new molecular approaches they are in the second most–used class of cancer drugs and they will remain among the fundamental cancer therapies (28).

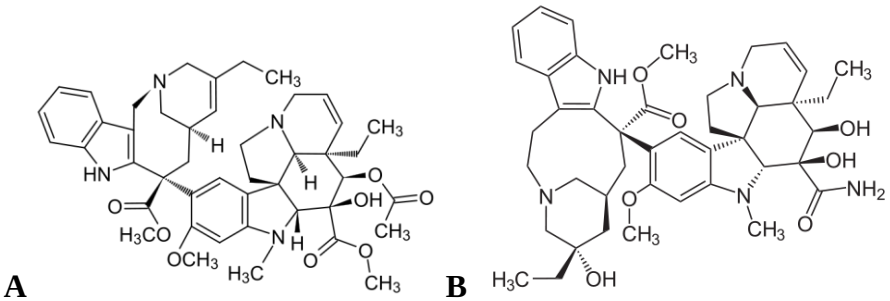


Figure 8 Formula of vinorelbine (A) and vindesine (B). Copy from: http://en.wikipedia.org/wiki/Vinorelbine, http://en.wikipedia.org/wiki/Vindesine (24/1/2013)

3.4. Mechanisms of cancer drug resistance

The drug chemoresistance is over–time serious clinical problem in the treatment of disease in general, not only in cancer, especially in case so aggressive and easily metastasizing tumors like melanomas doubtless are and it presents big limitation for successful treatment (2).

There are the two main types of drug resistance – primary and acquired resistance. Both primary (caused by some inherent characteristic which prevents the drug from working) and acquired drug resistance are the major factors in failure of many forms of chemotherapy. The acquired drug resistance appears very fast and has become more common with more effective therapy and it is caused by previous chemotherapy and based on a lot of different cellular and molecular mechanisms (29).

By exposing cells to one drug, resistance to this single drug or class of drugs can occur, but cells can also simultaneously become cross–resistant to number of anticancer drugs which are not related by chemical structure or mechanism of action, this type of resistance is called multidrug resistance (MDR).

Chemotherapy (both dacarbazine monotherapy and polychemotherapy) is an extremely ineffective and unsatisfactory means of treating malignant melanoma due to the drug resistance characteristic of this disease, which either is intrinsic at onset or develops during applications of cytostatic drugs (30). The intrinsic resistance is responsible for low response rate to cytostatics from the beginning of treatment, e.g.: only 23% in case of dacarbazine (4).

Various hypotheses have been proposed to account for the phenomenon of drug resistance. Altered transport of the drug across the plasma membrane (efflux pump and reduced uptake of drugs), enhanced DNA repair mechanism, alteration of enzyme of DNA topoisomerase and apoptosis play important role in cancer drug resistance and can be considered to potential mechanisms of resistance (2).

Ironically, some of the mechanisms that could be utilized by cancer cells to resist cytotoxic drugs are probably evolved in normal cells as a defense mechanism against environmental carcinogens (2). For example, previous studies have shown participation of Glutathione S–transferases M1(GSTM1) and Multidrug resistance protein 1 (MRP1) in chemoresistance of malignant melanoma to treatment by VAs, especially in case of VCR (31).

3. 4. 1. Altered transport across the plasma membrane

One of the major mechanism of MDR is the expression of an energy–dependent drug efflux pump, known alternatively as P–glycoprotein (also known as multidrug resistance protein), transmembrane protein–member of ATP binding cassette family, which normally protect tumor cells from cytotoxicity of anticancer drug (generally all xenobiotics), especially natural product anticancer drug such as anthracyclines or vinca alkaloids.

Reduced uptake of drugs is caused by mutation that eliminate of modify cell surface molecules which are normally use for pinocytosis or endocytosis of nutrients and other essential low molecular weight molecules. This mechanism of resistance is specific for nutrient analogs and structurally related compounds (29).

3.4.2. Enhanced DNA repair mechanism

DNA is under constant attack from endogenous (e.g., free radicals generated by cellular metabolism) and exogenous toxins. Thus it is not surprising that cells have

developed multiple mechanisms to ensure DNA integrity. The results of attack by anticancer drugs are DNA double–strand breaks (DSBs) which can be repair by either homologous recombination (HR) which uses undamaged sister chromatid as a template or non–homologous repair pathway (NHEJ) which repairs DSBs during V(D)J immunoglobulin recombination and T–cell receptor rearrangement (32).

3.4.3. Alteration of enzyme of DNA topoisomerase

Another type of non–P–glycoprotein–mediated MDR, also called atypical MDR, is due to alteration of the function of enzyme of DNA topoisomerase II. The reduced activity of the enzyme is responsible for decreased chemosentivity to topoisomerase II inhibitors, mostly natural product anticancer drug with the exception the vinca alkaloid (33).

3.4.4. Apoptosis

Inactivation of apoptosis, also called programmed cell death, is a "hallmark of cancer", an obligate ritual in the malignant transformation of normal cells–trough inactivation of apoptosis cells enhance their chances of survival and increase their resistance to chemotherapeutic agents. However, inactivation of cell death is not unique to melanoma, it is general mechanism in all cancer type, but melanoma is extremely recalcitrant (34).

Apoptosis is under control two really important genes – p53 (a tumor suppressor) and bcl–2 (a key inhibitor of cell apoptosis). According to Melanoma molecular disease model–subtype 8, aberrations and mutations in these two genes play key role in melanoma pathogenesis (21).

It is known that apoptosis plays very important role in melanoma resistance and maybe this involvement could be key mechanism.

3.4.5. Melanoma chemoresistance to VAs

Major mechanism of multidrug resistance in cultured cancer cells was the expression of an energy–dependent drug efflux pump, known alternatively as P–glycoprotein or the multidrug transporter (29). P–glycoprotein catalyzes the energy–dependent export of number of structurally different substances, such as VAs (30).

Expression of P–glycoprotein has been studied in primary and metastatic lesions of melanoma as well as in melanoma cell lines, but no significant melanoma–specific P–glycoprotein up–regulation could be detected (30).

Tumor cells may become refractory to treatment with VAs by a variety of mechanism. These include alterations in the structure of tubulin proteins or increased expression of the multidrug resistance protein MDR1 (35). Among the systems involved in cancer resistance, xenobiotic detoxification by phase II glutathione conjugation reaction plays a crucial role. The conjugation of electrophilic molecules, including anticancer drug, with glutathione (GSH) is catalyzed by a multigene family of enzymes–the glutathione S–transferases (GSTs) (31).

Previous works of the laboratory have shown that GST M1 and MRP1 are differentially involved in melanoma resistance to VAs. GSTs and MRP1 can act, sometimes in synergy (e.g.: in case of vincristine), to protect tumor cells from cytotoxicity of anticancer drugs (31). Indeed coordinated expression/activity of GST M1 and MRP1 are required for resistance to vincristine (VCR), vindesine (VDS) whereas only expression of GST M1 is necessary for resistance to vinorelbine (VRB).

4. Aims of the diploma thesis

Resistance to chemotherapy is the major issue in MM treatment. This chemoresistance can be due to several different types of biochemical changes associated with gene alterations (33). That's why we decided to identify new molecular determinants and pathway which can be involved in chemoresistance of malignant melanoma (MM) to VAs. For the achievement of our aims we decided to perform a global transcriptomic analysis on resistant MM cell lines (CAL1R–VAs) produced by long – term exposure of CAL1–wt to VAs.

On the basis of previous results of DNA microarray we decided to investigate genes which are significantly over- or under–expressed in resistant cell lines in comparison with the parental cell lines. In my diploma thesis I focus at under–expressed KIT gene. Besides its potential role in drug resistance and generally in melanoma, this gene is also interesting as one of the molecular target in biological therapy.

5. Experimental part

5.1. Material

5.1.1. Drugs and chemicals

Vincristine (100μM in H_2O) and Vinorelbine (100μM in H_2O) were from Sigma–Aldricht (St. Quentin–Fallavier, France). Vindesine (100μM in H_2O) was from laboratory Eurogenerics (Boulogne – Billancourt, France).

5.1.2. Cell lines

Cells are cultivated at 37°C in a fully humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS), 1% of L–glutamine (2mM) and 1% of antibiotics (mix of penicillin 100U/ml and streptomycin 0,1g/L).

Parental human malignant melanoma cell line (CAL1–wt) was from metastases of human malignant melanoma. Resistant CAL1 cells (CAL1R-VAs) were produced from CAL1–wt by continuous exposure (6 – 12 month) to VAs (IC_{50}; 4nM). CAL1 cell lines resistant to VCR, VDS and VRB are called CAL1R–VCR, CAL1R–VDS and CAL1–VRB, respectively.

5.2. Methods

5.2.1. Quantitative real–time reverse transcriptase PCR

In order to confirme expression level and to ensure efficiency of inhibition of expression by siRNA, quantitative real–time reverse transcriptase polymerase chain reaction (qRT–PCR) was used.

Extraction of total cellular was carried out using TRIzol® reagent according to the manufacturer's procedure (Invitrogen ™, Villebon sur Yvette, France). The quality of every sample was verified by agarose gel electrophoresis and the quantity was measured by spectrophotometer BioPhotometer (Eppendorf, Hamburg, Germany). Reverse transcription (RT) of mRNA was done using standard procedure.

KIT expression was analyzed by qRT–PCR using standard procedures and specific primers (Figure 5) on a LightCycler instrument (Roche Diagnostics, Meylan, France) using the kit QuantifastTM SYBER® Green PCR (Qiagen–Coourtabouf,

France). The amplification was performed in 45 cycles according to following protocol: 1) initial predenaturation 95°C/ 10 min; 2) every cycle is divided into 3 phases – denaturation 95°C/ 10s, annealing 60°C/ 6s and extension 72°C/10s.

The specificity of amplification was verified by post–PCR melting curves analysis and the crossing threshold (C_T) was determined for each amplification curves. As a calibrator CAL1–wt cell line was used and all results were normalized with β–actin gene (positive control) and expressed as a ratio which was evaluated using quantification based on C_T: $normalized\ ratio = 2^{\Delta C_T\ KIT} / 2^{\Delta C_T\ \beta-actine}$

Table 3 Sequences of primers using in qRT – PCR

Gene	Direction	Sequences of primers (5'→3')	Size of amplicon
β-actin	sense:	AGAAGGATTCCTATGTGGGCG	101
	anti – sense:	CATGTCGTCCCAGTTGGTGAC	
KIT	sense:	ACCTGCTGAAATGTATGAC	141
	anti – sense:	CAGTTTGCTAAGTTGGAGTA	

5.2.2. Small–interfering RNA and transfection reagents

In order to analyze involvement of KIT in MM acquired resistance to VAs we inhibited expression of this gene using siRNA transfection. Both non–targeting siRNA (negative control) and specific siRNA were from Dharmacon (Dharmacon; Epsom, United Kingdom). Transfection was realized with Lipofectamine 2000 according to the manufacture's protocol (Invitrogen™, Villebon sur Yvette, France).

5.2.3. Western blot analysis

To evaluate efficiency of KIT knock–down on proteins was used for Western blot analysis.

Cells were washed with Dulbecco's phosphate–buffered saline (DPBS) and incubated in a RIPA lysis buffer (Sigma–Aldrich; St. Quentin–Fallavier, France) for 5 min at 4°C. Afterwards the lysates were centrifuged at 8 000g for 10 min at 4°C and the supernatants were frozen at – 80°C.

Protein levels of the supernatants were determined according to Bradford with Coomassie Plus Protein Assay kit (Thermo Fisher Scientific; Brebiéres, France). Protein

extracts (20 μg) were separated by sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE) electrophoresis in gel containing 10% of acrylamide and transferred onto nitrocellulose membranes. The membranes were saturated overnight in solution of TBS–Tween 20 (0,1%)–BSA (5%) at 4°C. After that the membranes were washed with solution of TBS-Tween 20 (0,1%) three times, followed incubation with rabbit polyclonal primary antibodies, anti–KIT (dilution 1/ 500), and as a control anti–β–actin (dilution 1/ 1 000) (Abcam^{TM}, Paris, France) for 1 hour at room temperature. Then, the membranes were washed again with solution of TBS–Tween 20 (0,1%) and incubated with anti–rabbit polyclonal secondary antibodies IgG–HRP (dilution 1/ 20 000) (Abcam^{TM}, Paris, France). Finally, the membranes were washed with solution of TBS–Tween 20 (0,1%) and the antibodies were revealed by autoradiography (kit of detection of chemoluminescence ECL; Amersham, Velizy–Villacoublay, France).

5.2.4. Cytotoxicity assay

To test implication of KIT in acquired resistance to VAs was assessed viability assay based on the neutral red.

48 hours after transfection of siRNA cells were seeded into 96–well microtiter plates with density 7 500 cells/ well in 100 μl/ well of DMEM supplemented with 10% of FBS and 1% of L–glutamine. After 24 hours of incubation, thus 72 hours after transfection, cells were exposed VAs (50μl/ well of scale from 0 to 2000mM) during 4 hours in DMEM supplemented with 1% of FBS and 1% of L–glutamine, after that the medium with VAs was changed in 150μl of fresh DMEM supplemented with 10% FBS and 1% of L–glutamine. After 72 hours, cells were incubating in present of neutral red (0,33g/L, Sigma–Aldrich) during 3 hours at 37°C. Afterwards, cells were washed with DPBS and destained with solution of glacial acetic acid (1%) and ethanol (50% v/v).

Absorbance was measured at 540 nm using a microtiter plate reader (Labsystems Multiskan MS^{TM}; PAA, Farnborough, United Kingdom). The effect of drugs on cell survival was expressed as a percentage of viability of treated–cells in comparison with control cells. IC_{50} was defined as a reduction of optical intensity over 50% and relative absorbance was counting according to following formula:

$$(average\ of\ absorbace\ in\ 6\ wells / average\ of\ absorbance\ in\ control\ 6\ wells\ ) \times 100$$

5. 2. 5. Statistical analysis

qRT–PCR of KIT mRNA and confirmation of siRNA efficiency were repeated three times. Viability assay was repeated three times for all conditions, six wells for every concentration. Data are presented as mean ± SD. Student's t test used to access statistical significance. Differences of $p < 0.05$ were considered statistically significant.

6. Results

6.1. Data analysis

To understand the results in a good way it is necessary to present method where the results for confirmation came from–DNA Microarray data analysis (it is not explained in the chapter Materials and methods because it was not real part of my work). In general, DNA Microarray is modern method based on analysis of gene expression. It enables overview on entire genome because of thousands of probes which represent individual sequences of transcripts and enables hybridization after connection with mRNA samples (labeled with fluorescent dye). After hybridization, laser light is used to excite fluorescent dye and fluorescent emission is measured. For our analysis DNA, Microarray using Affymetrix HG–U133 Plus 2.00 GeneChip with 54 120 probes, corresponding to over 47 000 transcripts, was chosen for its comprehensive coverage of the human genome. In order to understand mechanism that leads to melanoma resistance against VAs, this global transcriptomic analysis was performed on melanoma CAL1–wt cell line becoming resistant after long exposure to VAs. After RNA hybridization, results was analyzed via software Expression Console 1.1 and Microarray Affymetrix Software 5.0 (MAS5) (36).

A comparison of expression profiles among different cell lines was first performed, using Cluster and TreeView software. These softwares generated gene clusters and arborescent hierarchical dendrogram. According to this dendrogram, expression profiles of parental cell line and resistant CAL1R–VRB and expression profiles of resistant cell lines CAL1R–VCR and CAL1R–VDS were similar (Figure 9). Interestingly, this result confirms the precedent laboratory works about the differential resistance of melanoma to vinca alkaloids (31).

In order to choose genes potentially involved in melanoma chemoresistance, microarrays results were analyzed using a without *a priori* method. Ratio of emission intensity between CAL1–wt and CAL1R–VAs was first calculated and genes were divided to two groups–over (ratio $>$ 2) and under–expressed (ratio $<$ 0.5) in the 3 resistant cell lines in comparison with parental cell line. Because of big number of genes in this extent, the cut of ratio was changed–ratio $>$ 10 for over–expressed genes and ratio $<$ 0.1 for under–expressed genes. Finally, 18 genes remained – 3 over–expressed

and 15 underexpressed. Differential resistance between CAL1R cell lines was also study (data not show).

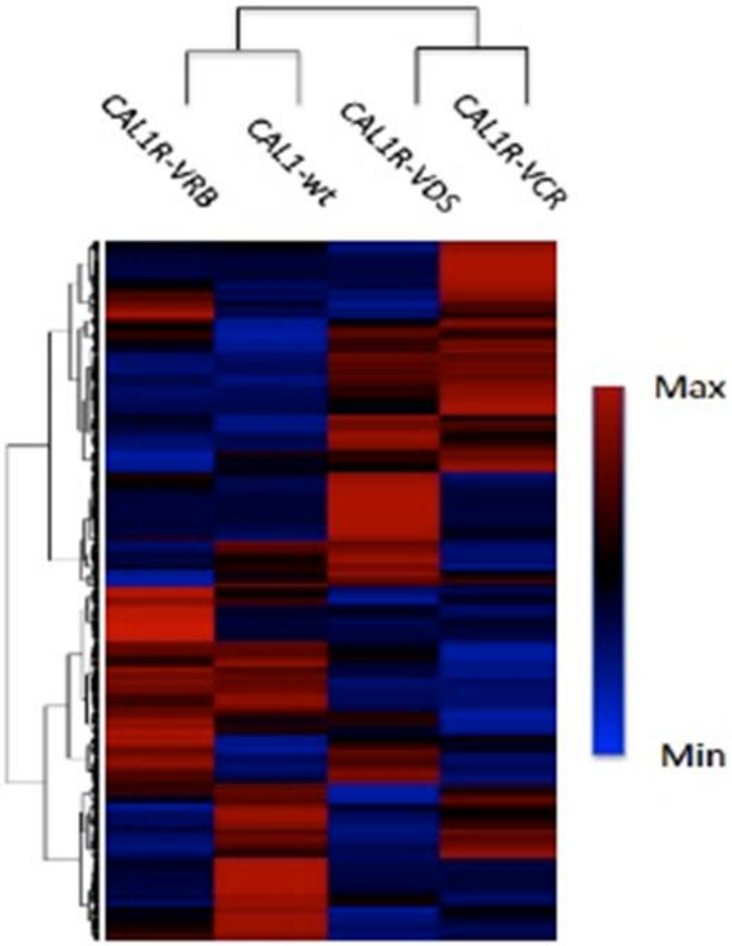


Figure 9 Dendogram of similarity of CAL1 cells expression profiles. The expression levels of transccripts are visualized by a linear gradient from red (expression level maximum) to blue (expression level minimum)

Then, Ingenuity Pathway Analysis (IPA) software version 9.0 was used to produce graphical representation of the molecular relationships between genes (maximum 35/ network) based on bibliographic databases (Integuity® Knowledge Base). A P–score value which gives probability of random generating [$P - score = 2\log_{10}(P - value)$] is associated with each network. Analysis of selected genes generated 1 network with a P–score of 27 (Figure 10). It showed that 12 from 18 selected genes mutually interacted and that they were associated with factor of transcription NF–κB and cyclin D1–two proteins involved in chemoresistance of malignant melanoma. IPA analysis also showed association with cellular function of

cytoskeleton–cell movement, development of hematopoietic system and migration of immune cells.

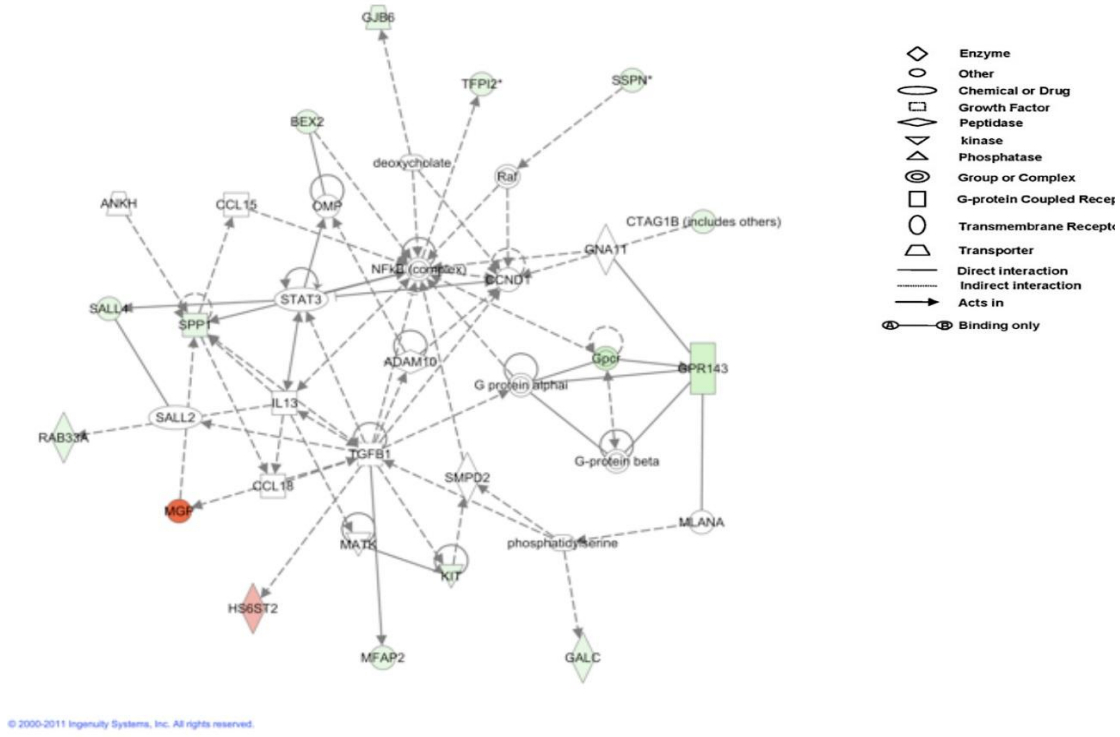


Figure 10 Network of intermolecular interaction of 18 selected genes generated by IPA. In red: overexpressed genes, in green: underexpressed genes.

After analysis of results the attention was first aimed at genes with the most significant modification of expressions, 3 over (MGP, HS6ST2 and SLITRK6) and 3 under (GPR143, KIT and SLC45A2) expressed genes in the 3 resistant cell lines. These genes were subsequently tested for their involvement in melanoma drug resistance to treatment by VAs. KIT gene, which play significant role in targeted therapy besides other functions, was chosen as a gene of interest and confirmation of these results was chosen such as aim of my thesis.

6.2. Functional analysis

To verify microarray data, qRT–PCR was performed. The level of KIT expression from DNA Microarray data analysis was compared with results from qRT–PCR. Both of these methods have shown that KIT was underexpressed in resistant CAL1R–VAs cell lines in comparison with CAL1–wt cell line (Figure 11).

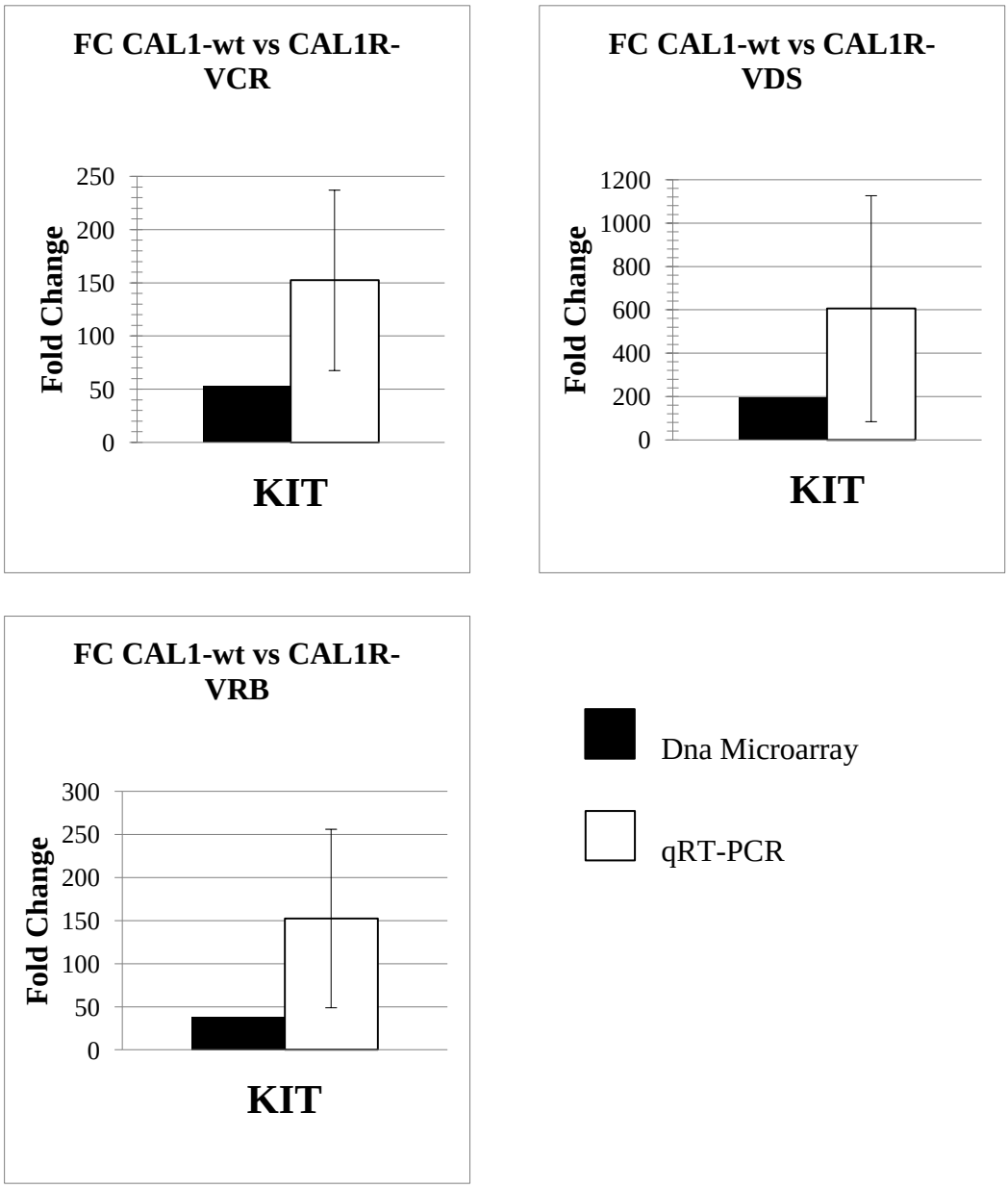


Figure 11 Levels of expression of KIT gene in resistant CAL1R–Vas cell lines. Comparison results from DNA Microarray and qRT–PCR. Levels of expression were expressed such a ratio of level of expression of KIT in parental CAL1–wt cell line and level of expression of KIT in resistant CAL1R–VAs cell lines.

To determine the role of KIT in drug resistance we decided to suppress expression of this gene by transfection of specific KITsiRNA in CAL1–wt. Initially, we had to find optimal conditions out for working of siRNA – which day and which concentration is the best for inhibition of gene expression. First, the determination of the kinetic of expression inhibition was realized using siRNA transfection in concentration

of 50 mM. Total RNA from transfected CAL1–wt cells was extracted during 3 days (24, 48 and 72h) after transfection and the level of KIT expression was determined by qRT–PCR. The extent of KIT expression inhibition level was determined using qRT–PCR and expressed in percentage (Figure 12). The best level of expression inhibition (65 %) was found on day 2. The determination of optimal concentration was performed at day 2, using similar protocol with different concentration (10mM, 20mM and 50mM). The concentration of 50mM was provided such as optimal concentration which induced sufficient inhibition of expression – 88.43 % (Figure 13).

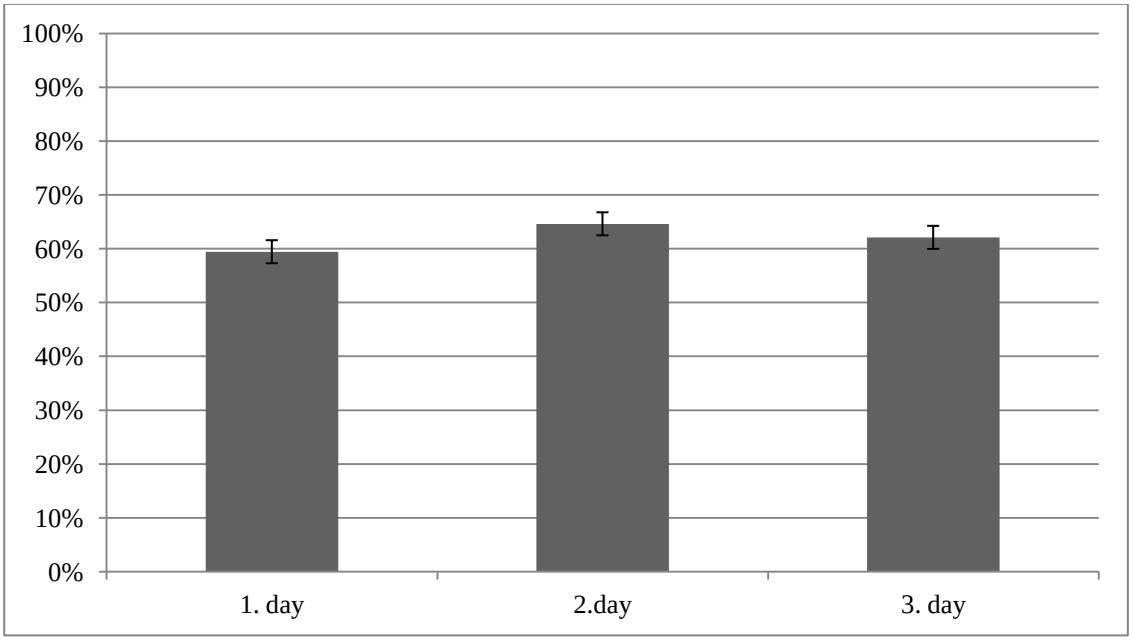


Figure 12 Effect of transfection KITsiRNA (50 nM) on expression of gene KIT in parental CAL1–wt cell line determined by qRT–PCR in day 1, 2 and 3 after transfection. The percentage of inhibition of expression was expressed according to formula: $100 - \left[100/\left(2^{\Delta C_T\,KIT}/2^{\Delta C_T\,\beta-actine}\right)\right]$ %.

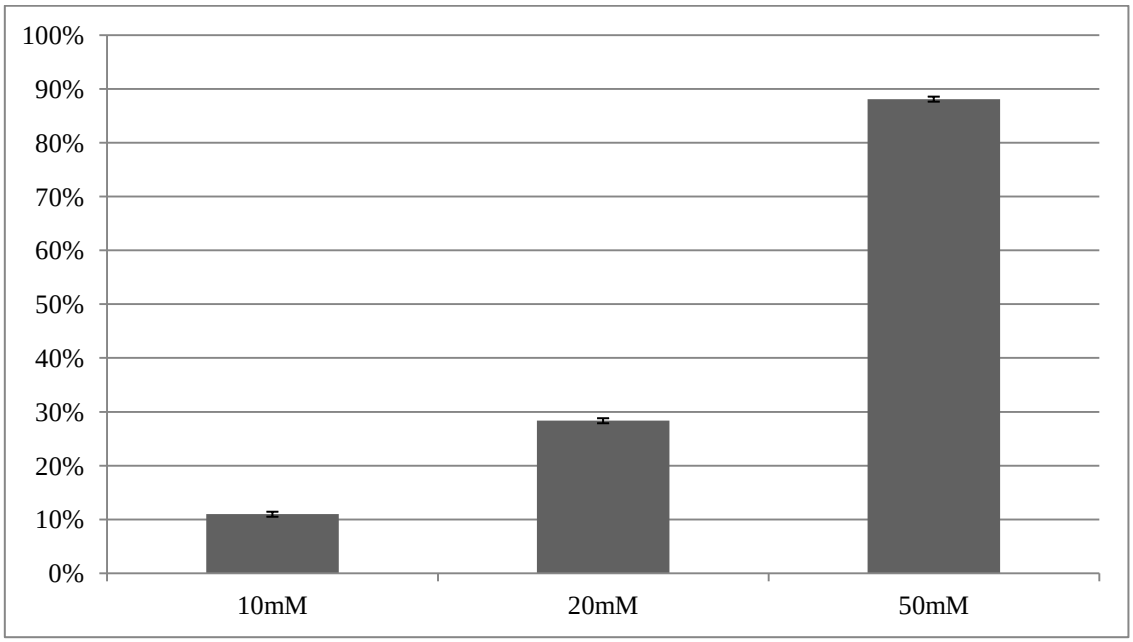


Figure 13 Effect of different concentrations (10mM, 20mM and 50 mM) on expression of gene KIT in parental CAL1–wt cell line determined by qRT–PCR day 2 after transfection of KITsiRNA. The percentage of inhibition of expression was expressed according to formula: $100 - \left[100/\left(2^{\Delta C_{T\,KIT}}/2^{\Delta C_{T\,\beta-actine}}\right)\right]$ %.

The effect of inhibition of KIT expression on protein level in parental CAL1–wt cell line was finally determined using Western blot analysis. Western blot was performed 72h after transfection of KITsiRNA (so 24h after day with maximal inhibition of RNA). A specific expression inhibition of KIT protein was observed (Figure 14). β–actin was used for standardization of results such as positive control, and NTsiRNA as negative control.

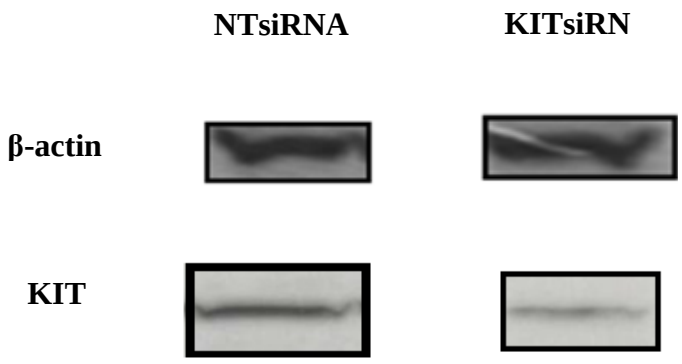
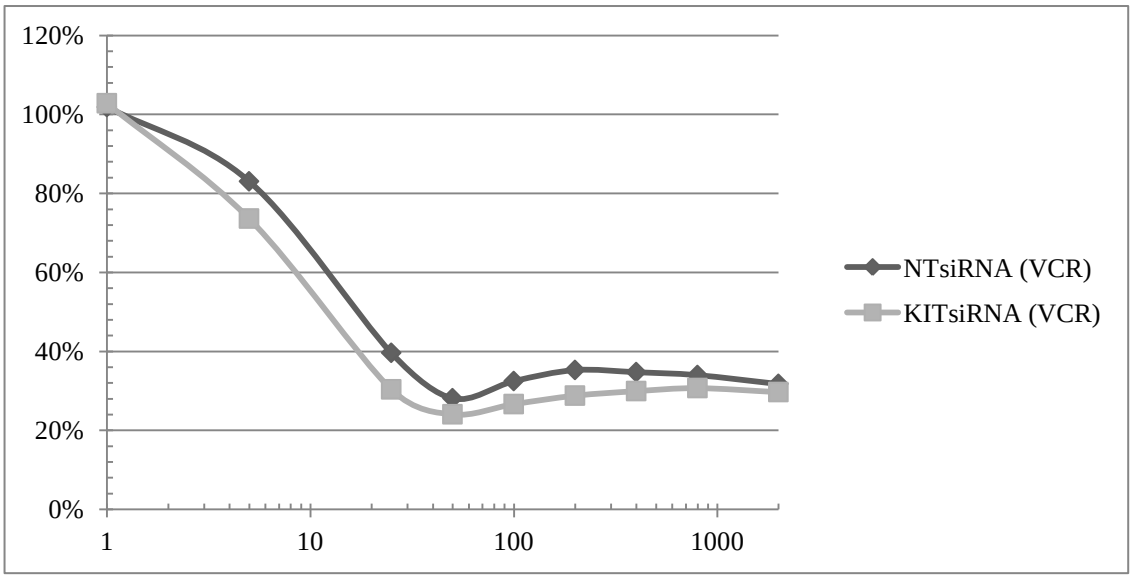


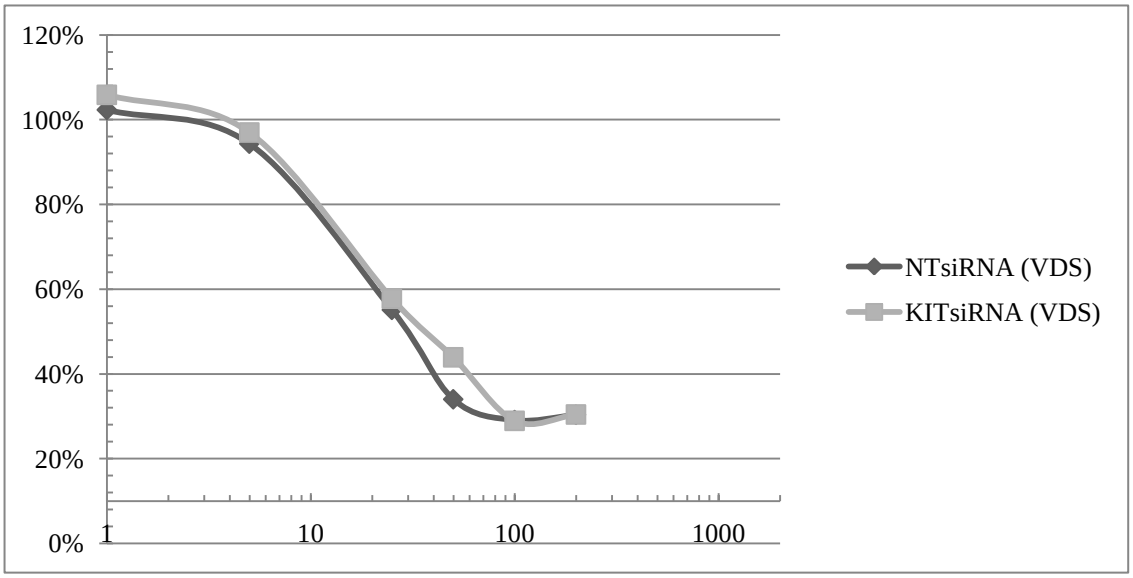
Figure 14 Effect of transfection of KITsiRNA and NTsiRNA (such a negative control) on expression of proteins coded by KIT in parental CAL1–wt cell line.

After setting optimal conditions for siRNA transfection, the effect of inhibition of KIT expression on sensibility of CAL1–wt VAs was measured using neutral red uptake cytotoxicity assay. The inhibition of KIT expression by siRNA did not have any significant effect on cell viability after treatment by VCR (Figure 14A), VDS (Figure 14B) and VRB (Figure 14C). In comparison with the control (NTsiRNA), the effect of inhibition of KIT expression on sensibility of parental cell lines CAL1-wt was expressed as IC_{50} ratio (Table 4).

A



B



C

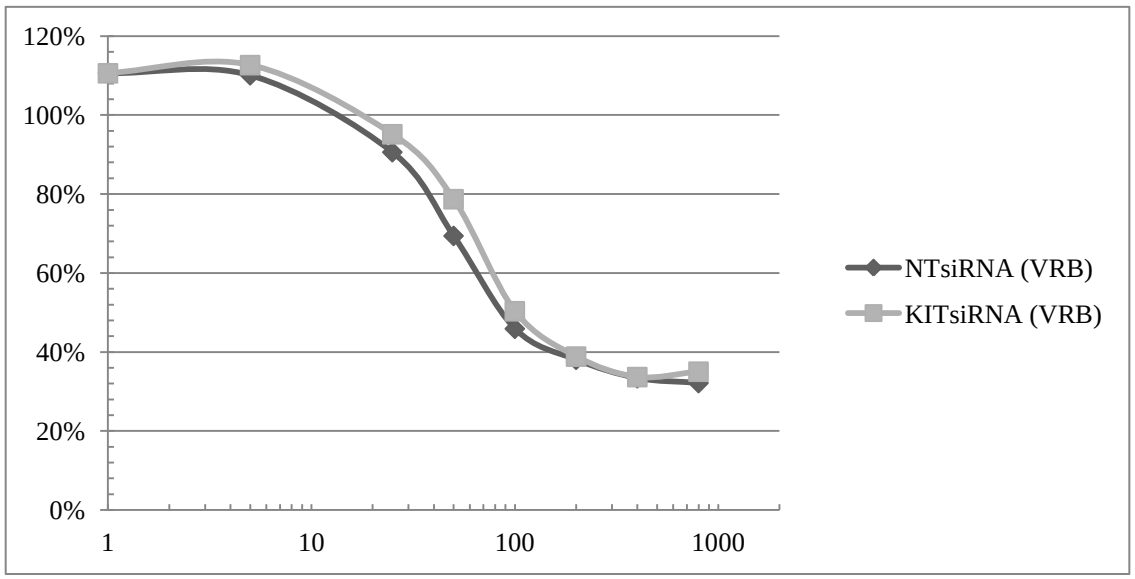


Figure 14 Effect of inhibition of KIT expression on viability of parental CAL1–wt cells treated by VCR (A), VDS (B) and VRB (C) after transfection of KITsiRNA and NTsiRNA (negative control).

Table 4 Effect of inhibition of KIT expression on CAL1–wt cell line sensitive to VAs expressed such as IC_{50}. Factor of resistance was expressed according to formula:

$$IC_{50}\ after\ KITsi\ RNA\ transfection\ /IC_{50}\ after\ KITsiRNA\ transfection$$

	IC_{50}		
	VCR	VDS	VRB
NTsiRNA	12	25	95
KITsiRNA	19	29	115
resistance factor	1.58	1.16	1.21

7. Discussion

Malignant melanoma, as the most dangerous skin cancer, still remains the aim of many research groups and treatment of MM represents a big therapeutic challenge. In addition to the synthesis of new chemical compounds, which could be effective in treatment, and looking for new therapeutic approaches, finding reasons of failure of classical approaches (where treatment by VAs undoubtedly belongs to) is really important for understanding mechanism of resistance development of this aggressive disease. In general, discovery of mechanism of resistance, both primary and acquired, could bring hope and get better prognosis for thousands of patients with melanoma and to stop constantly increasing incidence worldwide.

In order to understand mechanism that leads to melanoma resistance against VAs, a global transcriptomic analysis of melanoma CAL1–wt cell line becoming resistant to VAs after long exposure to VAs was proposed. Dual approach analysis was performed on the transriptom: firstly using Affymetrix genecheaps, secondly using IPA. Six genes were indentified: 3 completely unlighted on resistant cell lines (GRP143, KIT and SLC45A2) and 3 highly overexpressed (MGP, HS6ST2 and SLITRK6), interacted inside the same intermolecular network built by IPA. We selected one underexpressed gene in resistant cell lines in comparison with parental cell line–gene KIT and confirmation of results of DNA Microarray exactly for this gene was picked out as the aim of my thesis.

KIT (official full name v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogenes homolog), also known by other name, e.g., CD 117, SCFR, PBT or tyrosine–protein kinase Kit, belongs to a family of proteins called receptor tyrosine kinases (RTKs). The signaling pathways stimulated by the KIT protein control many important cellular processes such as cell growth and division (proliferation), survival and movement (migration). KIT protein signaling is important for development of certain cell types, including pigment cells (melanocytes), reproductive cells (germ cells), early blood cells (hematopoietic stem cells), immune cells called mast cells, and cells in the gastrointestinal tract called interstitial cells of Cajal (37).

KIT is protooncogen and its mutation or overexpression can lead to the cancer. According to melanoma molecular disease model, one of the subtypes is characterized

by alteration in the KIT pathway, specifically by genetic aberrations including mutations and/or increase in copy number of KIT receptor (21). Although KIT mutations seem to be rarer than BRAF and NRAS mutations, which are the most common in melanoma without chronic sun–damage, they may reflect the important role of KIT tyrosine kinase in melanocytes development.

Interestingly, microarray data showed a paradoxical drastic underexpression of KIT in resistant cell lines in comparison with the parental CAL1–wt cell line. These unexpected results, because of comparison with any types of cancer where KIT is overexpressed (e.g. in breast cancer, in meningioma, small cell lung cancer and gastrointestinal stromal tumor (38–41), were confirmed using qRT–PCR.

Intermolecular interaction analysis using IPA software has shown possibility of KIT importance in melanoma drug resistance to VAs because of KIT connection with NF–κB and cyclin D1. NF–κB plays important role in control of large number of normal cellular and organismal processes, including cellular growth and apoptosis (42). Cyclin D1 belongs to highly conserved cyclin family whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle (43). Vinca alkaloids depolymerize mitotic spindle microtubules, which is important for cell cycle, so that's why KIT connection with these proteins could be significant in melanoma drug resistance to VAs. Moreover, the ability to escape apoptosis appears to be the major mechanism of chemoresistance of malignant melanoma (34,44). Finally, numerous immunohistochemical studies have linked progressive loss of KIT expression with the transition from benign to primary and metastatic melanomas (45).

So functional analysis was performed in order to verify KIT involvement in melanoma resistance to VAs. In this context, KIT expression was inhibited by transfection of specific KIT siRNA in CAL1–wt. Then, the effect of inhibition of KIT expression on sensibility of CAL1–wt to VAs was measured using neutral red uptake cytotoxicity assay. Unfortunately, this test failed to show any significant effect on CAL1 resistance to VAs *in vitro*.

This type of results signifies that KIT is probably not directly involved in melanoma drug resistance to VAs, but it could be a marker of resistance. However to confirm this idea is necessary to perform *in vivo* testing, which is unfortunately very expensive and demanding because of big patients number with different stages of

melanoma and in different phases of treatment with different therapeutic approaches. (38–41).

The institute of Pathology in Berlin (Charité Campus Mitte, Universitätsmedizin Berlin, Germany) was also focused on analysis of gene expression in melanoma cells (human melanoma cell line MeWo and drug-resistant MeWo variants) with acquired resistance against commonly used drugs in MM treatment (cisplatin, etoposide, fotemustine and vindesine). They also used global transcriptomic analysis and qRT-PCR for confirmation of results from DNA Microarray analysis. According their results, about 50% of selected genes were differentially expressed in at least one of the resistant cell lines, the smallest difference can be seen between parental cell line and the vindesine-resistant malignant cell subline-this suggests that resistance against vindesine occurs with minimal changes in gene expression of MeWo cells (46).

Finally, it is difficult to find the role of KIT in malignant melanoma because in human body, there are a lot of different metabolic pathways which are associated together and it will take much research time to fully understand this complicated metabolic tangle and melanoma drug resistance.

8. Conclusion

This diploma thesis should help understand mechanism of melanoma chemoresistance to VAs trough transcriptomal analysis on melanoma CAL1 cell lines. After comparison of expression profile of CAL1–wt cells on one hand, and of CAL1R cells on the other hand, we selected one underexpressed gene in resistant cell lines: the proto-oncogene KIT.

Despite the fact that underexpression of KIT in resistant CAL1R–VAs cell lines in comparison with parental CAL1–wt cell line was confirmed, functional analysis did not show any significant effect on melanoma drug resistance. Although KIT gene is not directly involved in melanoma acquired resistance, its underexpression is really interesting for another testing as biomarker of resistance using biopsies analysis. But also other genes are studied and they maybe could work together with KIT in resistance mechanism; this must be investigated in following studies.

To clarify the mechanisms of melanoma drug resistance and KIT role, it is necessary to continue with another *in vivo* testing which is, however, very expensive and demanding. The role of biomarkers is very interesting and important to explore because they would optimize and individualize treatment as well as they would provide new therapeutic targets.

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