Chapter 5

Possibilities for the Therapy of Melanoma: Current Knowledge and Future Directions

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Additional information is available at the end of the chapter

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Abstract

This chapter presents an overview of possibilities for the therapy of melanoma, current knowledge and future direction. Skin cancer is one of the most frequent types of cancers. Melanoma is much less common than basal cell and squamous cell skin cancers, but it is far more dangerous. Detailed knowledge of melanoma at the molecular level allows to develop new treatment alternatives and to design effective new drugs. There are two approaches in therapy of melanoma in the present based on immunotherapy and targeted therapy or their combination. Immunotherapy includes immune checkpoint blockades, whereas targeted therapy is represented by protein kinase inhibitors, such as BRAF inhibitors, MEK inhibitors, and NRAS inhibitors. Detailed knowledge of protein structure and the understanding of its role in key signaling pathways in melanoma development lead to the designation of new protein kinase inhibitors in targeted therapy.

Keywords: melanoma, chemotherapy, immunotherapy, targeted therapy, protein kinase inhibitors

1. Introduction

The incidence of melanoma is increasing worldwide. Melanomas represent 3% of all skin cancers but 65% of skin cancer deaths [1]. Melanoma is currently the fifth and sixth most common solid malignancy diagnosed in men and women, respectively [2]. The rates of melanoma have been rising for at least 30 years [3]. Although melanoma is no longer considered just 'one disease', pathologists will continue to have important role in identifying and describing tumor subtypes [4]. More detailed understanding of melanoma allows the development

of new specific treatment alternatives, which are targeted at specific receptors or the genes of tumor cells. In 2011, new molecules were discovered and designed on the basis of new knowledge in the molecular biology of melanoma. These new facts have resulted in the existence of two new approaches to therapy: immunotherapy and targeted therapy of melanoma.

2. Genesis of melanoma

Melanoma is derived from melanocytes—normal pigment cells of the skin. Most commonly, melanoma arises from epidermal skin melanocytes, but primary tumors can also be found lining the choroidal layer of the eye (uveal melanoma) or the mucosal surfaces of the respiratory, genitourinary, and gastrointestinal surfaces [5]. Melanoma is much less common than basal cell and squamous cell skin cancers, but it is far more dangerous. Like basal cell and squamous cell cancers, melanoma is almost always curable in its early stages. However, it is much more likely to spread to other parts of the body than basal or squamous cell cancer if not caught early. Melanocytes that produce melanin are usually uniformly localized at the interface of the dermis and epidermis of the skin. If the melanocytes are found in denser groups, they create different forms of birthmarks—nevus. Later, they may be the cause of developing benign skin tumors—dysplastic nevus [6].

The term "dysplastic nevus" implies that this nevus exists as a distinct and defined entity of potential detriment to its host. Rosendahl et al. examine the current data, which suggest that this entity exists as histologically and possibly genetically different from common nevus, with some overlapping features. Studies show that a melanoma associated with a nevus is just as likely to arise in a common nevus as in dysplastic nevus [7].

Human nevi are benign tumors of melanocytes that are frequently associated with oncogenic mutations predominantly in BRAF V600E. However, nevi typically remain in a growth-arrested state for decades and only rarely progress into malignant melanoma. Very important features of nevus include oncogene-induced senescence [8] and oncogene-induced trans-lineage differentiation [9], which prevent benign nevi from malignant transformation.

In recent years, researchers have learned a great deal about how certain changes in DNA can make normal cells become cancerous. Cancers can be caused by DNA changes that turn on oncogenes or turn off tumor suppressor genes. Changes in several different genes are usually needed for a cell to become cancerous. Damage of DNA may be in the form of inherited genetic mutation, but in most cases it occurs gradually over the life due to the influence of environmental factors, such as UV rays from the sun [10–12].

There are two melanin pigments synthesized in the melanocytes: eumelanin, a dark brown-black insoluble polymer, and pheomelanin, light red-yellow sulfur containing soluble polymer [13]. Pheomelanin has a weak shielding capacity against ultraviolet radiation compared to eumelanin, and has been shown to amplify ultraviolet-A-induced reactive oxygen species. Mitra et al. suggested that the pheomelanin pigment pathway produces ultraviolet-radiation independent carcinogenic contributions to melanomagenesis by a mechanism of oxidative damage [14].

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The incidence of melanoma is increasing at one of the highest rates of any form of cancer in fair-skinned populations around the world. The exposure to sunlight during the past 50 years is an important factor for the increasing incidence of melanoma. Mortality rates of melanoma show stabilization in Australia, in North America, and also in European countries. Prevention campaigns aim on reducing incidence and achieving earlier diagnosis, which resulted in an ongoing trend toward thin melanoma since the last two decades. However, the impact of primary prevention measures on incidence rates of melanoma is unlikely to be seen in the near future; rather, increasing incidence rates to 40–50/100,000 inhabitants/year should be expected in Europe in the next decades [15].

3. The possible signs and symptoms of melanoma

The possible signs and symptoms of melanoma are new moles or spots on the surface of skin that are changing in size, shape and color. Another important sign is a spot that looks different from all of the other spots on skin. There are the **ABCDE** criteria for these signs, which guide to the usual signs of melanoma:

A – Asymmetry; one half of a nevi or birthmark does not match the other.

B – Border; the edges are irregular, jagged, or blurred.

C – Color; the color is not the same all over and may include shades of brown or black, or sometimes with patches of pink, red, white, or blue.

D – Diameter; the spot is larger than 6 mm across, although melanomas can sometimes be smaller than this.

E – Evolving; the nevi are changing in size, shape, or color.

4. Melanoma classification and staging

The classification schemes **Breslow's thickness (depth)** and **Clark's level** have been developed based on either the vertical thickness of the lesion in millimeters or the anatomic level of invasion of the layers of skin. Breslow's depth is considered significant factor in predicting the progression of the melanoma. Increased tumor thickness is correlated with metastasis and poorer prognosis. Tumors are classified into four categories based on the depth: thickness of 0.75 mm or less, thickness of 0.76–1.5 mm, thickness of 1.51–4 mm and thickness greater than 4 mm.

Clark's level of invasion has far less importance and is used only in the staging of thin melanomas (<1 mm). Tumors are classified into five levels:

Level I – melanoma involves only epidermis (melanoma *in situ*);

Level II – melanoma invades papillary dermis but not papillary-reticular dermal interface;

Level III – melanoma invades and expands papillary dermis up to the interface with, but not into, reticular dermis;

Level IV – melanoma invades reticular dermis but not into subcutaneous tissue;

Level V – penetration of melanoma into the subcutaneous tissue.

Cancer staging system, called the TNM (Tumor, Node, Metastasis) system by the American Joint Committee on Cancer (AJCC) is used for clinical staging [16]. The stage of melanoma refers to the thickness, depth of penetration, and the degree to which the melanoma has spread. The staging is used to determine treatment. There are five stages of melanoma: stage 0 and stages I–IV.

Stage 0 refers to melanoma *in situ*, which means melanoma cells are found only in the outer layer of skin or epidermis. This stage of melanoma is very unlikely to spread to other sites of the body. **Stage I** the primary melanoma is still only in the skin and is very thin. Stage I is divided into stages IA and IB, depending on the thickness of the melanoma and the mitotic rate. **Stage II** melanoma is thicker than stage I melanoma, extending through the epidermis and further into the dermis, the dense inner layer of the skin. It has a higher chance of spreading. Stage II is divided into IIA, IIB and IIC depending on thickness the melanoma and ulceration. **Stage III** melanoma has spread through the lymphatic system, either to a regional lymph node located near where the cancer started or to a skin site on the way to a lymph node. Stage III is also divided into IIIA, IIIB and IIIC depending on the size and number of lymph nodes involved with melanoma and whether the primary tumor appears ulcerated under a microscope. In **stage IV**, melanoma has spread through the bloodstream to other places of the body, such as lung, liver, brain, bone, soft tissue, or gastrointestinal tract. Stage IV is further divided into M1a, it means the cancer has only spread to distant skin and/or soft tissue sites; M1b involves metastasis to the lung; and M1c describes distant metastasis at any other location or an elevated serum lactate dehydrogenase [17].

5. Current possibilities for the therapy of melanoma

Similar to other tumors the progressive stage of melanoma is predictive for therapeutic success. Early stage melanomas (thin tumors) result in a 97% 5-year survival rate of the patients, after surgical removal [18].

6. Surgery and chemotherapy

The treatment of cutaneous melanoma has historically been essentially surgical. Much progress has been made in this area, and the resection margins have been established based on tumor depth. Candidates are also identified for lymphadenectomy, avoiding the morbidity of the procedure in patients who do not require it.

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Topical formulations are examined and, where available, skin penetration properties of the various drugs are detailed. New strategies for targeted drug delivery to skin cancers are considered with an emphasis on studies conducted *in vitro* with porcine or human tissue, or in patients.

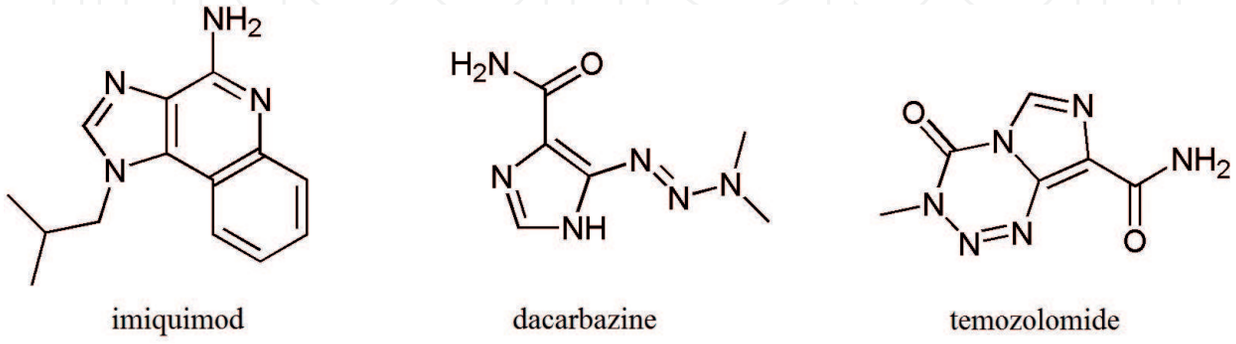
Imiquimod cream may be used to stimulate the local immune response in early stage melanoma patients (**Figure 1**) [19, 20].

The decision to treat melanoma by adjuvant therapy has the opposing arguments: the risk of recurrence, progression and high toxicity, and price of treatment.

The risk of recurrence and death after complete surgical resection of clinically detectable primary cutaneous melanoma ranges from low, intermediate to high risk depending on the stage of disease at diagnosis. This is determined by the depth, ulceration status and mitotic rate of the primary tumor, the presence of regional nodal disease or distant metastasis. For high-risk melanoma, adjuvant therapy is aimed at eradicating melanoma micrometastases in the patients that carry an unacceptable risk of mortality from melanoma recurrence. The ultimate goal of adjuvant therapy is to provide a potential cure before progression of melanoma into advanced inoperable stages [21].

A little progress has been made in systemic treatment since the 1970s when the use of dacarbazine was introduced for the treatment of patients with tumor progression or distant metastasis, with disappointing results.

Dacarbazine and *temozolomide* (**Figure 1**) belong to the group of alkylating agents. These triazene compounds have excellent pharmacokinetic properties and limited toxicity. The active moiety of these drugs is represented by the triazenyl group of three adjacent nitrogen atoms, which are responsible for the physico-chemical and antitumor properties of the molecule. Mechanism of action of both compounds is mainly related to the methylation of O^6-guanine, mediated by methyldiazonium ion, a highly reactive derivative. O^6-methylguanine is responsible for incorrect base pairing and damaging of DNA [22]. Dacarbazine is a prodrug structurally related to purines activated by liver microsomes. This chemotherapeutic agent was approved by the Food and Drug Administration (FDA) for the treatment of melanoma, and often regarded as the standard treatment for advanced melanoma. However, therapy with



imiquimod dacarbazine temozolomide

Figure 1. Chemical structures of imiquimod, dacarbazine, and temozolomide.

dacarbazine is characterized with low overall response rates (approximately 10–15%) and there is no valid evidence of survival benefit [23]. Temozolomide is a monofunctional alkylating agent of the imidotetrazine class. It is stable at the acid pH of the stomach and administered orally with 100% bioavailability [22].

7. New approaches

For years, the cornerstones of cancer treatment have been surgery, chemotherapy, and radiation therapy. Significant changes occurred in antitumor therapy for disseminated melanoma during the last decade. Detailed knowledge in the molecular biology of melanoma and immune response lead to the two directions: immunotherapy and targeted therapy. Before 2011, two approved drugs were used to treat patients with metastatic melanoma in the USA: dacarbazine and recombinant human interleukin-2 (IL-2) [24]. The treatment landscape for advanced stage melanoma was revolutionized in 2011 with the approval of ipilimumab and vemurafenib, both of which improved overall survival in phase III clinical trials. More recently, the targeted inhibitors dabrafenib and trametinib have demonstrated similar therapeutic profiles [25]. The latest approved (PD-1)-blocking antibody pembrolizumab is indicated for the treatment of patients with unresectable or metastatic melanoma [26].

8. Immunotherapy

The immune system recruitment may represent a powerful and innovative strategy in cancer therapy. Genetic mutations and alterations in regulatory processes of cancer cells lead to expression of various tumor-related antigens that can be presented to cytotoxic T-lymphocytes by antigen-presenting cells. A major understanding of immune activation, especially T-lymphocyte activation, has identified multiple co-stimulatory and co-inhibitory pathways regulating this process. The two most important targets of immunotherapy are co-inhibitory receptors, such as CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4) and programmed cell death-1 (PD-1) receptor, expressed on the T-lymphocyte surface [26].

A molecule of IL-2 was first approved by the US FDA for immunotherapy of melanoma. It is of limited use due to the serious toxic side effect of this treatment [27]. The first approved checkpoint blocking antibody was ipilimumab.

Ipilimumab (Yervoy®) is a human monoclonal IgG1 antibody that binds the human antigen CTLA-4 located on the surface of T-lymphocytes and blocks its interaction with molecule on the surface of antigen presenting cells. CTLA-4 is a key negative regulator of adaptive immune response and works as a brake on the immune response. Blocking immune response to anticancer leads to a longer and stronger activation of T-lymphocytes and, ideally, to an attack and destruction of the tumor tissue, resulting in long term remission for 15–20% of patients [28]. Although its effectiveness is tested with many carcinomas, the best results were achieved just in the treatment of melanoma [29]. Randomized clinical studies show that the treatment

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with ipilimumab leads to a significant extension of the survival of patients with metastatic melanoma. Side effects of ipilimumab are related to the mechanism of its action. Typical side effects are accompanied by diarrhea, skin rash, pruritus, enteritis, vitiligo, endocrinopathies and hepatotoxicity. Ipilimumab is approved in the USA for the treatment of patients with advanced melanoma and in Europe for patients with previously treated advanced melanoma.

Tremelimumab, another drug of this group, is human therapeutic monoclonal antibody IgG2, with the same mechanism of action as ipilimumab. This antibody is currently in progress in phase II/III clinical study [30].

Ipilimumab, in combination with high dose IL-2, and tremelimumab, in combination with interferon alfa provide increased overall response rate, progression-free survival, or higher percentage of complete responses. *Interferon alfa* is FDA approved in adjuvant treatment for patients with high-risk melanoma and it has significant immunomodulatory effects [31–33]. Interferon alfa monotherapy has limited utility in the treatment of stage IV melanoma; therefore, its antitumor activity has led to profound investigation of its use in combination with other therapies [34].

Cancer immunotherapy can be achieved by inhibition of the PD-1/PD-Ll axes, which affect the overall survival in an important fraction of patients. PD-1 is an inhibitory receptor that is upregulated on activated lymphocytes. PD-1 has two known ligands, PD-L1 and PD-L2, which can be expressed on tumor and stromal cells; PD-L1 expression can be induced by cytokines produced by tumor-infiltrating lymphocytes [35].

Pembrolizumab (Keytruda®, Merck & Co) is the first anti-PD-1 immunotherapeutic agent approved by FDA. Keytruda® was granted FDA approval on September 4, 2014 for the treatment of patients with unresectable or metastatic melanoma. This molecule is a potent and highly selective humanized monoclonal antibody of IgG4-kappa isotype, designed to directly block the interaction between PD-1 receptor, expressed on T-cells, and its ligands, PD-L1 and PD-L2, without antibody-dependent cell-mediated or complement-dependent cytotoxicity. In practice, blocking PD-1 activity is believed to prevent inhibition of T-cell immune surveillance of tumors and, in some models, has resulted in decreased tumor growth [26]. The recommended dose of pembrolizumab is 2 mg/kg administered as an intravenous infusion over 30 min every 3 weeks until disease progression or unacceptable toxicity. Most common adverse reactions (reported in ≥20% of patients) included fatigue, cough, nausea, pruritus, rash, decreased appetite, constipation, arthralgia, and diarrhea [36].

Another approach, which has already been tested, is to combine anti-PD-1 and anti-CTLA-4 treatment and is represented by *nivolumab* (Opdivo®, Bristol-Meyers Squibb). Nivolumab is used alone or in combination with ipilimumab [37, 38]. Combination therapy with anti-CTLA-4 and anti-PD-1 monoclonal antibodies has recently led to remarkable antitumor effects, long-term survival and potential cures [39].

BRAF-mutant and BRAF-wild type patients, who progressed after ipilimumab therapy, were included into the Phase III study. One group of patients received nivolumab (3 mg/kg every 2 weeks), and in comparator group patients were treated with chemotherapy. Patients treated with nivolumab demonstrated higher response rate compared to the chemotherapy group—32%

vs. 11% [40]. There was no statistical difference in two contemporary oncology median overall survivals between the study arm with nivolumab, 15.7 months, vs. the comparator group with chemotherapy, 14.4 months. The limitation of the clinical benefit of nivolumab could be related to the fact that control group patients (40%) received pembrolizumab, when progressed during chemotherapy. Furthermore, the number of patients with elevated lactate dehydrogenase levels and brain metastases was imbalanced, favoring the chemotherapy [41]. Adverse events are less frequent in patients treated with nivolumab than in those treated with ipilimumab or chemotherapy [4, 10]. The most frequently observed adverse events included fatigue, pruritus, diarrhea, rash, and nausea. The most commonly observed immune-related adverse events were pruritus, rash, diarrhea, vitiligo, hypothyroidism, and elevated aminotransferase activities [42].

In the study of Pyo and Kang the effects of various immunotherapeutic agents and chemotherapy for unresected or metastatic melanomas were compared. They performed a network meta-analysis using a Bayesian statistical model to compare objective response rate of various immunotherapies from 12 randomized controlled studies. The estimated overall response rates of immunotherapy and chemotherapy were 0.224 and 0.108, respectively. The overall response rates of immunotherapy in untreated and pretreated patients were 0.279 and 0.176, respectively. In network meta-analysis, the odds ratios for overall response rate of nivolumab (1 mg/kg)/ipilmumab (3 mg/kg), pembrolizumab (10 mg/kg) and nivolumab (3 mg/kg) were 8.54, 5.39 and 4.35, respectively, compared with chemotherapy alone. Their results showed that various immunotherapies had higher overall response rates rather than chemotherapy alone [43].

Except immunological checkpoint blockades the approach of *adoptive T cell therapy* seems to be a highly promising in use against cancer including melanoma. The ability of T cells to specifically lyse tumor cells and secrete cytokines to recruit and support immunity against cancer make them an attractive proposition for therapy. Since the first idea in 1989 to genetically redirect T cells, a lot of experiments have been performed. Recent methods of generating tumor-specific T cells include the genetic modification of patient's lymphocytes with receptors to endow them with tumor specificity. These T cells are then expanded *in vitro* followed by infusion of the patient in adoptive cell transfer protocols. Genes used to modify T cells include those encoding T-cell receptors and chimeric antigen receptors. Several trials with gene-modified T cells are ongoing and some remarkable responses have been reported [44]. In fact, current adoptive T cell therapy response rates are 80–90% for hematological malignancies and 30% for metastatic melanoma refractory to multiple lines of therapy. Although these results are encouraging, there is still much to be done to fulfill potential of adoptive T cell therapy, specifically with regard to improving clinical efficacy, expanding clinical indications and reducing toxicity [45].

9. Cancer vaccines

Melanoma vaccines have the goal to induce long lasting immunity against melanoma to prevent the development of metastases. However, melanoma cells express many different tumor-associated antigens. Ideally, vaccines need to contain all these different tumor-associated antigens for antigen-presenting-cells (APC) to induce an adequate immune response [46].

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Vaccines specific for cancer antigens exert antitumor effects by inducing cytotoxic T lymphocytes (CTLs) that recognize and attack antigenic cancer-derived peptides comprising 8–10 amino acid residues presented on major histocompatibility complex molecules on the cancer cell surface. Peptides, proteins, mRNA, DNA, and viral vectors can be used as cancer vaccines. Various peptides derived from Wilms' tumor gene-1, glycoprotein 100 (gp100), and melanoma-associated antigen 3 (MAGE-A3) have been used as vaccines against melanoma [47].

Tumor antigen encoded by genes of the MAGE-A family, MAGE-A3 is of importance because they are expressed in a wide array of malignancies including melanoma, brain, breast, lung and ovarian cancer. Its ability to elicit spontaneous humoral and cellular immune responses has been shown in cancer patients. As antigen-specific immune responses can be stimulated by immunization with MAGE-A3, several clinical trials have used MAGE-A3 vaccines to observe clinical responses. The frequent expressions of this antigen in various tumors and its immunogenicity in cancer patients have led to application of this antigen in cancer immunotherapy. Indeed, the initial trials performed with MAGE-A3 peptides showed no significant toxicity [48]. Vaccination with a tumor-specific MAGE-A3 peptide, even without adjuvant, has been shown to induce a CTL response in a melanoma patient followed by reduction in tumor mass. MAGE-A3 protein produced by recombinant technology is more popular in clinical trials as a result of its potential to activate a wide range of T-cell responses as well as its potential application in a larger population of patients with MAGE-A3 expressing tumors [49]. As MAGE-A3 specific therapies have not reached their final goals to cause a significant improvement in the survival of patients. For future perspective of MAGE-A3 therapy, studies are needed to find the most effective vaccine formulations, the most immunogenic adjuvants as well as the most applicable criteria for selection of patients.

10. Oncolytic virus therapy

Over the past several years, oncolytic viruses for treating various cancers have been investigated. Oncolytic viruses play a role in cancer vaccination because antigen-specific immunity is effectively evoked against components released by destroyed cancer cells due to virus-induced production of type I interferon. The effects of oncolytic virus therapy are mediated not only by direct cell disruption, but also by indirect induction of cancer-specific immune responses [48].

Oncolytic viruses selectively replicate within and lyse cancer cells without damaging normal cells. On October 27, the FDA approved the first oncolytic virus therapy, *talimogene laherparepvec (Imlygic™, T-VEC)*. The agency approved T-VEC for the treatment of some patients with unresectable cutaneous, subcutaneous, and nodal lesions in melanoma recurrent after initial surgery [50]. T-VEC is a modified herpes simplex virus, type 1 (HSV-1) that has undergone genetic modifications to promote selective tumor cell replication, while reducing viral pathogenicity and promoting immunogenicity. T-VEC improves overall response rate and durable response rate as a single agent, shows promise in combination therapy with immunotherapy, and is well tolerated. Ongoing trials will determine if T-VEC has a role in early

treatment or in combination therapy for melanoma or other malignancies, such as hepatocellular carcinoma, metastatic liver tumors, advanced non-central nervous system tumors, breast cancer, pancreatic cancer, Merkel cell carcinoma, head and neck cancer, sarcoma, lymphomas. In a Phase I study, T-VEC has been combined with ipilimumab or pembrolizumab, with promising results without overlapping toxicities. The results of larger studies are awaited to further delineate T-VEC's place with combination therapy [51].

11. Targeted therapy

Selected somatic changes such as BRAF mutations have been described, and then applied to the targeted treatments. BRAF gene is located in chromosomal region 7q34; it consists of 18 exons and transcribed mRNA length was 2478 bp. Targeted therapy is based on the knowledge of the molecular biology of the gene encoding the BRAF kinase, belonging to the RAF kinase family. It is a serine/threonine kinase that takes part in the Mitogen Activated

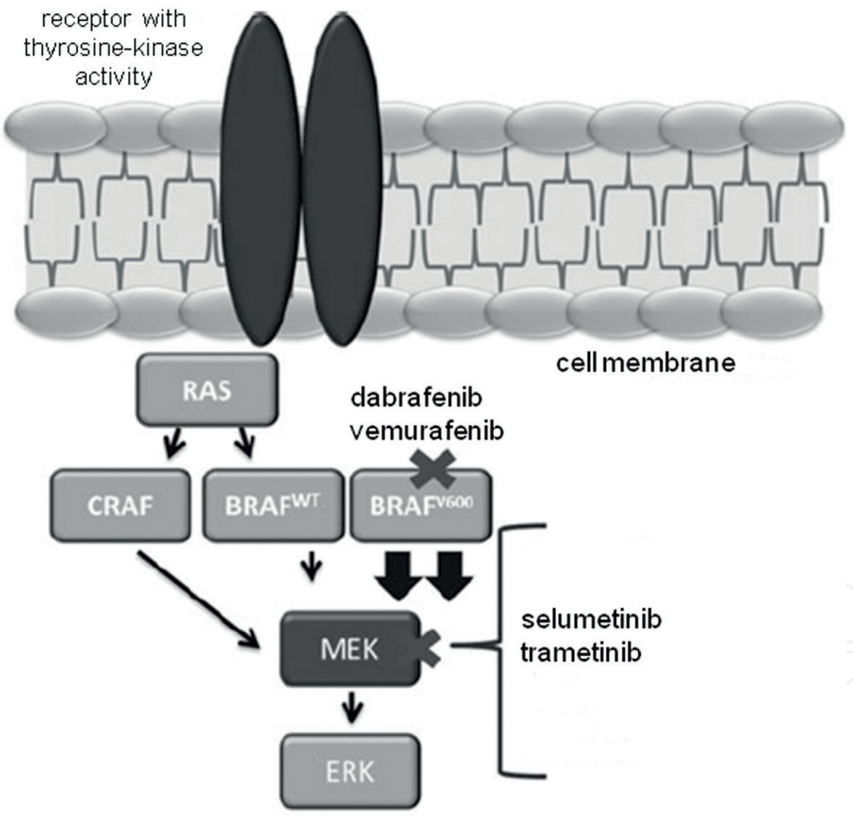

Figure 2. Mechanism of action of kinase inhibitors. This figure shows a schema of signaling pathways triggered by binding of growth factors to tyrosine kinase receptor that triggers RAS, RAF, MEK and ERK pathways leading to cell growth and proliferation. Mutations in BRAF (V600E) can lead to accelerated cell growth and cancer formation of melanoma cells. Inhibition of mutant BRAF by dabrafenib, vemurafenib in the melanoma cells shuts down the signaling pathway causing tumor regression following cell apoptosis, tumor antigen expression and decreased release of cytokines and VEGF. MEK is a member of the MAPK signaling cascade that is activated in melanoma. Inhibition of MEK by selumetinib, trametinib blocks cell proliferation and induces apoptosis (controlled cell death). MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; VEGF, vascular endothelial growth factor.

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Protein Kinase (MAPK) cascade, which modulates cell growth and proliferation. This pathway is activated by binding of the extracellular physiological growth factor to its receptor. Conformational change of the receptor leads to the activation of RAS protein (GTP-binding), which activates RAF protein, which activates other kinases MEK and ERK. This pathway may be activated by mutation of specific proteins, including BRAF [52]. It is reported that 40–60% of melanomas have a mutation of the gene leading to the pathological-activated signaling pathways and to uncontrolled growth of malignant transformed cells [53]. The most common gene mutations are V600E or V600K known as an amino acid substitution at position 600 in BRAF, from a valine (V) to a glutamic acid (E) or to a lysine (K), respectively. In the structure of protein kinases there is a DFG motif, which is a highly specific site for interaction with kinase inhibitors. It contains Asp (D), Phe (F) and Gly (G) and exists in a conformational active or inactive state. Just the knowledge in this field has led to the development and screening of new selective inhibitors of BRAF and MEK (**Figure 2**) [54]. Targeted therapy is associated with improved clinical benefit; however, the mechanism of resistance often varies and includes activation of alternative signaling pathways [55].

12. BRAF inhibitors

Vemurafenib (Zelboraf® tablets, Roche) is the first selective inhibitor of BRAF developed by Plexxikon and approved by FDA in 2011 (**Figure 3**). It leads to a rapid, and sometimes the complete remission of the disease in patients with a mutated BRAF V600E. A clinical study on 675 respondents treated with vemurafenib, 960 mg twice daily, demonstrated survival of 6 months in 84% of patients versus 64% of patients treated with dacarbazine. Despite significant benefit in the treatment, there were new challenges identified – the development of resistance to reactivation of MAPK signaling and growth of keratoacanthomas and squamous cells. The most common adverse events were headache, joint pain, fatigue, skin hyperkeratosis and 6% of the patients experienced a squamous cell carcinoma [56].

Dabrafenib (Tafinlar® capsules), developed by GlaxoSmithKline (**Figure 3**), selectively inhibits BRAF ValGlu [57]. It is a thiazole derivative, which binds to the ATP binding site of BRAF kinase. It has a shorter half-life than vemurafenib (5.2 h versus 50 h). In 2009, first clinical studies in

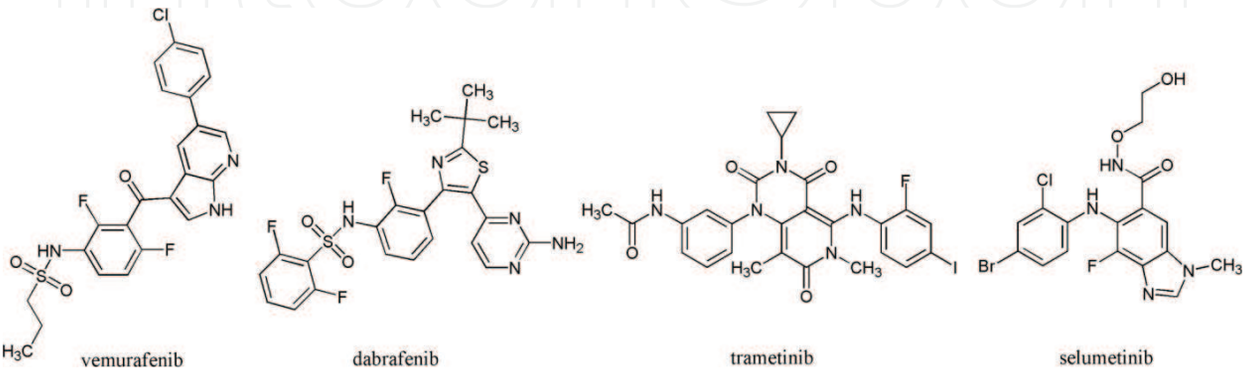

Figure 3. Chemical structures of vemurafenib, dabrafenib, trametinib, and selumetinib.

Phase I/II began. In Phase III clinical trials, the dosing regimen was 150 mg of dabrafenib twice daily, which significantly extended the survival to 5.1 months versus 2.7 months with dacarbazine. Hyperkeratosis, headache and joint pain, fatigue, heartburn have been reported as adverse events [24].

13. MEK inhibitors

Trametinib (Mekinist® tablets, GlaxoSmithKline) is the first selective allosteric inhibitor of MEK1 and MEK2 (**Figure 3**). In May 2013, it was approved by the FDA as a single agent for the treatment of patients with V600E mutated metastatic melanoma [58]. The recommended daily dose of trametinib is 2 mg orally daily. It has a long half-life, i.e. 4 days at the previously mentioned dosing. In Phase III clinical study, trametinib was well tolerated by patients who most commonly experienced side effects such as diarrhea, asthenia, rash, nausea and vomiting [59]. Development of squamous cell carcinoma as a side effect did not occur at all unlike in treatment with BRAF inhibitors [24].

Selumetinib, licensed by Array BioPharma Inc. to AstraZeneca in 2003, inhibits the MEK enzyme in the RAS/RAF/MEK/ERK pathway in cancer cells to prevent the tumor from growing (**Figure 3**). In April 2015, selumetinib was granted Orphan Drug Designation by the U.S. FDA in recognition of the need for new, safe and effective therapies for the uveal melanoma [60]. Uveal melanoma is a rare disease in which cancer cells form in the tissues of the eye. It is the most common primary intraocular malignancy in adults and comprises 5% of all melanomas [61]. In July 2015, AstraZeneca announced that the Phase III clinical SUMIT study of selumetinib in combination with dacarbazine for the treatment of patients with metastatic uveal melanoma did not meet its primary endpoint of progression-free survival. This combination therapy showed an adverse event profile generally consistent with current knowledge of the safety profiles of dacarbazine and selumetinib [62].

Currently, there are being conducted ongoing studies in the elimination of resistance of the MAPK cascade by concomitant administration of inhibitors of MEK and BRAF [52]. This combination of BRAF and MEK inhibitors may prolong progression-free survival, and consequently increase the overall survival of patients. Therapy reactions or responses in patients may be different; the anti-CTLA-4 immunotherapy may lead to long-term response, but not in all patients, whereas targeted drugs may cause responses in most patients, though almost all of them eventually experience relapses due to pre-existing or acquired resistance.

A wide range of mutations are known to prevent effective treatment with chemotherapeutic drugs. Hence, approaches with biopharmaceuticals including proteins, like antibodies or cytokines, are applied [5]. Modern therapeutic approaches in melanoma provide profound and long lasting effects and can even cure some patients. Rational consecutive and combined application of current methods, proper diagnostic and management of related adverse events can prolong life span of patients and meaningfully increase their quality of life [63].

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Cobimetinib (Cotellic™) was granted FDA approval on November 10, 2015 in combination with vemurafenib (BRAF inhibitor) for the treatment of patients with metastatic melanoma. The approval was based on the effectiveness of cobimetinib plus vemurafenib in a randomized Phase III clinical trial of 495 patients whose tumors had specific mutations in the BRAF gene and who were not candidates for surgery. Patients who received vemurafenib plus cobimetinib had a median progression-free of 12.3 months, compared with 7.2 months in patients who received vemurafenib plus placebo. At 17 months after initiating treatment, about 65% of patients who received the two-drug combination were still alive, compared with 50% of those who received vemurafenib alone [64].

14. NRAS inhibitors

NRAS-mutant melanoma is a common subtype of this disease with a poor prognosis. NRAS is a low-molecular plasma-membrane–associated GTP-binding protein that constitutively activates intracellular signaling through a variety of pathways, most notably the RAS–RAF-MAPK and PI3K-AKT pathways. NRAS mutations activate MAPK signaling to a similar degree as BRAF mutations and rarely co-occur with mutations in the PI3K-AKT pathways, suggesting that mutant NRAS drives this pathway, too. NRAS mutation occurs in approximately 15–20% of melanomas, and it is the second most common oncogenic mutation in this disease [65].

Farnesyltransferase inhibitors (FTIs) showed the most promising therapy targeting the NRAS mutant. FTIs alter post-translational NRAS modification to prevent insertion into the plasma membrane. A Phase II study of FTI R115777 was performed in advanced melanoma and its results showed no evidence of clinical activity despite potent inhibition of FTIs in tumor tissue. The effect of these inhibitors has resulted in a stage of toxicity involving myelosuppression, nausea/vomiting and anorexia. Gajewski et al. concluded that FTIs were originally developed as RAS inhibitors and they affect several signaling pathways with potential outcomes for out-of-target toxicity. Multiple farnesylated proteins are involved in signal transduction in cancer. FTIs have been developed as a strategy to inhibit the function of these proteins. FTIs inhibit proliferation of melanoma cell lines. Farnesylated proteins are also important for T cell activation and measurement of effects on T cell function was also pursued [66].

Alternative strategies for a directly targeted NRAS mutant include the use of either antisense oligonucleotides or small interfering RNAs (siRNAs, small interfering RNAs) on the mutant NRAS gene. However, this has proved to be technically very demanding and requires advances in siRNA technology before considering use under clinical conditions. Therefore, NRAS mutant melanomas are currently being treated by MEK inhibitors or by newer types of immunotherapy that are not found in the presence of oncogenic mutation.

The first agent to show robust activity specifically in NRAS-mutant melanoma has been an allosteric inhibitor of MEK1 and MEK2 *binimetinib* **(MEK162, ARRY-162)** developed by Array BioPharma. As part of a combined study of BRAF and NRAS mutant melanoma patients, 30 metastatic melanoma patients whose tumors harbored a NRAS mutation were enrolled

and treated with binimetinib. The objective response rate was 21% and the progression-free survival was 3.7 months. Further study in this patient population will be necessary to confirm its clinical activity in comparison to other standard therapies. While prospective data with trametinib in NRAS mutant melanoma patients is not available, early retrospective data from ongoing clinical studies suggests that trametinib may have activity in a subset of NRAS mutant melanoma patients [67].

15. BRAF plus MEK inhibitors

Therapy with a MEK inhibitor in combination with a BRAF inhibitor is more effective and less toxic than treatment with a BRAF inhibitor alone, and has become the standard of care for patients with BRAF-mutated melanoma. Trametinib, the first MEK inhibitor was approved for the treatment of BRAF-mutated metastatic melanoma not previously treated with BRAF inhibitors, and is also approved in combination with the BRAF inhibitor dabrafenib [68].

The clinical study about combination dabrafenib and trametinib versus dabrafenib monotherapy in BRAF V600E/K–mutant metastatic melanoma demonstrated improved progression-free survival and overall survival. Phase III clinical study enrolled previously untreated patients with BRAF V600E/K–mutant unresectable stage IIIC or stage IV melanoma. Patients were randomized to receive dabrafenib, 150 mg twice daily, plus trametinib, 2 mg once daily, or dabrafenib plus placebo. The primary endpoint was progression-free survival; secondary endpoints were overall response, duration of response, pharmacokinetics and safety. Results showed that 423 of 947 screened patients were randomly assigned to receive dabrafenib plus trametinib (n = 211) or dabrafenib monotherapy (n = 212). At data cutoff, outcomes remained superior with the combination: 3-year progression-free was 22% with dabrafenib plus trametinib versus 12% with monotherapy, and 3-year overall response was 44 versus 32%, respectively. Twenty-five patients receiving monotherapy crossed over to combination therapy, with continued follow-up under the monotherapy arm. Of combination-arm patients alive at 3 years, 58% remained on dabrafenib plus trametinib. Three-year overall response with the combination reached 62% in the most favorable subgroup (normal lactate dehydrogenase) versus only 25% in the unfavorable subgroup (elevated lactate dehydrogenase). The dabrafenib plus trametinib safety profile was consistent with previous clinical trial observations, and no new safety signals were detected with long-term use. These data demonstrate that durable survival is achievable with dabrafenib plus trametinib in patients with BRAF V600–mutant metastatic melanoma [69].

The optimal timing and sequence of combination therapy (in particular targeted therapy in combination with immunotherapy) is currently in progress and cannot be precisely predicted for all patients with melanoma. Due to the existence of many potential targets in the immune system many critical questions arise, e.g. which therapy combinations should move forward in development and which patients will benefit from these treatments [70].

16. Combination immunotherapy and targeted therapy

Studies about combinations of anti-PD-1/PD-L1 agents with other immunotherapeutic agents are currently conducted in treatment of multiple tumor types. Targeting immune checkpoints such as PD-1, PDL-1 and CTLA-4 has achieved remarkable benefit in multiple cancers by blocking immunoinhibitory signals and enabling patients to produce an effective antitumor response. Inhibitors of CTLA-4, PD-1 or PDL-1 administered as single agents have resulted in durable tumor regression in some patients, and combinations of PD-1 and CTLA-4 inhibitors may even enhance antitumor benefit [70]. The combination of ipilimumab and nivolumab was studied in a phase I trial of 86 patients with pretreated malignant melanoma and demonstrated a 40% objective response rate [71]. In Phase II [72] and III studies [73] of this combination used in the treatment of advanced melanoma response rates were quite impressive, but toxicity was notably increased. Almost 83–89% of patients required either topical or oral immunosuppressive therapy for immune-related adverse events (irAE), which led to treatment discontinuation in 36–47% of all patients [72, 73]. However, almost all of the patients (80–100%) treated with immunosuppressive agents had their irAE completely resolved [74].

Recent study by Kim et al. suggests that the addition of MEK inhibitors to targeted and immunotherapy combinations may be associated with increased toxicity; several patients treated by dabrafenib (BRAF inhibitor), trametinib (MEK inhibitor), and ipilimumab (CTLA-4 inhibitor) developed adverse events related to colonic perforation. This condition found in several patients increases the need to further understand the immunomodulatory effects of trametinib [75].

Promising results have been presented in a Phase I study in BRAF-mutant advanced melanoma patients receiving atezolizumab (anti PD-L1) combined with vemurafenib (BRAF inhibitor) and cobimetinib (MEK inhibitor), with a response rate of 83%; currently a Phase III study is on-going [76].

Atezolizumab (Tecentriq, Genentech Oncology) is PD-L1 blocking antibody that previously received FDA accelerated approval for the treatment of locally advanced or metastatic urothelial carcinoma that has progressed after platinum-containing chemotherapy. Atezolizumab was granted FDA approval on October 18, 2016 for the treatment of patients with metastatic non-small cell lung cancer whose disease progressed during or following platinum-containing chemotherapy. Also the combination of atezolizumab with trametinib in patients with BRAF-wild type melanoma demonstrated encouraging results in an early phase study—a Phase III study is planned [77]. Patients with advanced melanoma and high serum lactate dehydrogenase activity present very poor prognosis, regardless of the systemic treatment used [78]. Current research should be focused on understanding the relationship between high serum lactate dehydrogenase activity and the lack of treatment efficacy with immunotherapy and targeted therapy. Probably novel treatment strategies should be developed in this patient population [42].

17. Future direction in targeted therapy

Despite extensive new approaches in the treatment of advanced stage melanoma, i.e. chemotherapy, targeted therapy and immunotherapy, response rate is rarely higher than 20%. Especially in the treatment with BRAF inhibitors the drug resistance is very common [79]. Due to this reason there is an urgent need to invent other alternatives and targeted therapies. Preclinical studies looking at least this main drug association strategies seems to be very promising: targeting of either MEK or phosphatidylinositol-3 kinase (PI3K)/mammalian target of rapamycin (mTOR); strategies aimed at blocking anti-apoptotic proteins belonging to B-cell lymphoma (BCL-2) or inhibitors of apoptosis (IAP) families associated with MEK/BRAF/p38 inhibition; co-inhibition of other molecules important for survival (proteasome, histone deacetylase and signal transducers and activators of transcription) [80]. *PI3K-AKT-mammalian target* of rapamycin signaling pathway is important for melanoma initiation and progression so the preclinical investigation of a novel and highly potent PI3K-mTOR dual inhibitor *VS-5584* was realized. VS-5584 induced caspase-dependent apoptotic death in melanoma cells, and its cytotoxicity was alleviated by the caspase inhibitors [81]. Whereas the main aim of inhibiting MAPK signaling pathway is to prevent cancer cell proliferation, apoptosis is controlled by the availability of anti-apoptotic *BCL-2 proteins* (e.g. BCL-2), which reside at the outer mitochondrial membrane. BCL-2 supports neoplastic growth by blocking cell death and this target may be future direction in the treatment of various types of cancers [82]. Development of small molecule inhibitors specific for antiapoptotic BCL-2 proteins is a novel approach not only for therapy of chronic lymphocytic leukemia [83] but is very promising in therapy of advanced melanoma [84]. This new targeted approach could by more successful when the combination with retinoid derivative is used [85]. *Venetoclax (ABT-199)* (**Figure 4**) is the first orally bioavailable selective inhibitor of BCL-2 protein often over-expressed in chronic lymphotic leukemia (CLL) and other types of B-cell related cancers developed by AbbVie in partnership with Roche. It is currently being evaluated in Phase II and Phase III studies for CLL and in Phase I and II studies for several other blood cancers and can be one of the next molecules used in the treatment of melanoma in the near future [86].

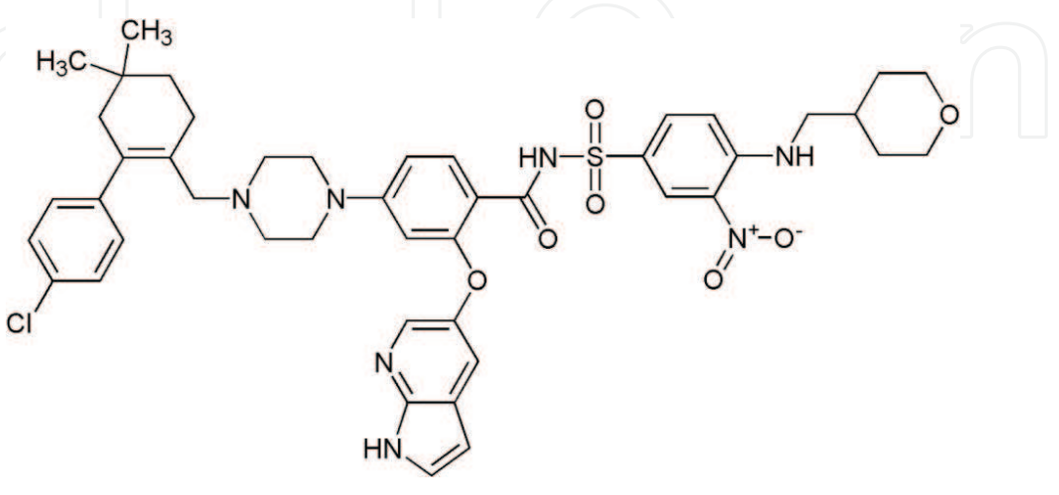

Figure 4. Chemical structure of venetoclax.

In the field of other genetic abnormalities such as *CDKN2A* also known as cyclin-dependent kinase inhibitor 2A, EGF (epidermal growth factor, which plays a role in skin cell growth), *Fas gene*, tumor suppressor gene *PTEN* (phosphatase and tensin homolog), there is a challenge in the research of new therapeutic targets and development of new anti-melanoma drugs in the future that can eventually lead to therapeutic benefit. Recent study by Hodis et al. describes six novel melanoma genes (PPP6C, RAC1, SNX31, TACC1, STK19, and ARID2), three of which RAC1, PPP6C, and STK19 harbored recurrent and potentially targetable mutations [87]. The prevalence of BRAFV600 and KIT mutations were significantly associated with melanoma subtypes and BRAFV600 and TP53 mutations were significantly associated with cutaneous primary tumor location. These results enrich understanding of the patterns and clinical associations of oncogenic mutations in melanoma, which could be the goal of future direction of melanoma therapy [88].

18. Conclusion

The development of drugs in the treatment of melanoma has never been as intense as at present. Single-agent chemotherapy is considered to have rather palliative effect on patients with melanoma; it is usually well tolerated but is associated with lower response rate. Detailed knowledge of protein structures and the understanding of their role in key signaling pathways in melanoma development lead to the designation of new targets for treatment of melanoma. Targeted therapy for patients whose tumors harbor the BRAF mutation achieves high response rates and OS benefit with combination BRAF/MEK inhibition. No other therapy in melanoma has shown a better response rate in late-phase clinical trials than combined BRAF and MEK inhibitors. The rapid kinetics of response to BRAF plus MEK targeted therapies represents the ideal frontline treatment for symptomatic, BRAF-mutant advanced melanoma patients. Although the concept of a combination of immunotherapeutic and targeted agents appears to be crucial in the treatment of melanoma, the synergy between these two approaches in melanoma treatment remains controversial due to the potential increased toxicity. Recently enormous progress in cancer therapy has been achieved by the use of immune checkpoint inhibitors. Activating the body's own immune system has added a novel and powerful therapeutic option for the treatment of melanoma. The potential use of immunotherapy is being extensively explored also in other malignancies. In the future, it is necessary to conduct further clinical trials and collect more data about overall survival, response rates, appropriate timing and sequence of combination therapy to manage the complexity of melanoma treatment.

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