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Current and Future Trials of Targeted Therapies in Cutaneous Melanoma

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Abstract

In order to effectively treat melanoma, targeted inhibition of key mechanistic events regulating melanoma development such as cell proliferation, survival, angiogenesis and invasion or metastasis needs to be accomplished. The Mitogen Activated Protein Kinase (MAPK) pathway has been identified as a key player in melanoma development making this cascade an important therapeutic target. However, identification of the ideal pathway member to therapeutically target for maximal clinical benefit remains a challenge. In normal cells, the MAPK pathway relays extracellular signals from the cell membrane to the nucleus via a cascade of phosphorylation events, which promote cancer development. Dysregulation of the MAPK pathway occurs frequently in many human cancers including melanoma. Mutations in the B-RAF and RAS genes, genetic or epigenetic modifications are the key aberrations observed in this signaling cascade. Constitutive activation of this pathway causes oncogenic transformation of cells by promoting cell proliferation, invasion, metastasis, migration, survival and angiogenesis. This review provides an overview of (a) key members of MAPK signaling regulating melanoma development; (b) key proteins which can serve as biomarkers to assess disease progression; (c) the clinical efficacy of various pharmacological agents targeting MAPK pathway; (d) current clinical trials evaluating

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downstream targets of the MAPK pathway; (e) issues associated with pharmacological agents such as drug resistance, induction of cancers; and finally (e) various strategies overcoming drug resistance.

Keywords

AZD6244; Drug resistance; MAPK signaling; Melanoma; PLX-4032; ^{V600E}B-Raf

Melanoma Background

Skin cancer is the most common malignancy in the United States [1]. Although melanoma represents a small subset, it is the most deadly cutaneous neoplasm and is an increasingly common malignancy affecting a younger population than most cancers. Melanoma is diagnosed more commonly in whites than non-whites with the lifetime risk of developing invasive melanoma being 2.04% for white men and 1.45% for white women [2]. In other words, about one in 74 Americans will be diagnosed with melanoma with the median age at diagnosis of 57 years. Numerous risk factors for development of melanoma have been identified, including white skin, fair hair, light eyes, sun sensitivity, tendency to freckle, family history of melanoma, dysplastic nevi, increased numbers of typical nevi, large congenital nevi and immunosuppression. Although sun exposure is a risk factor for melanoma, cutaneous melanomas can arise frequently in areas of the body not exposed to the sun. Sun exposure in childhood and having more than one blistering sunburn in childhood are associated with an increased risk of melanoma [3].

There are four major subtypes of invasive cutaneous melanoma including superficial spreading which accounts for approximately 70% of all melanomas, nodular melanoma which accounts for 15–30% of all melanomas, lentigomaligna and acrallentiginous. Most melanomas arise as superficial tumors confined to the epidermis and may remain for several years in a stage known as the horizontal or “radial” growth phase in which they are almost always curable by surgical excision alone. Melanomas that infiltrate into the dermis are considered to be in a “vertical” growth phase and have metastatic potential. Vertical growth phase melanoma is most strongly predicted by measuring the thickness of the tumor (i.e., Breslow depth), in millimeters, from the granular cell layer of the epidermis to the deepest malignant cell in the dermis [4]. Nodular melanomas have no identifiable radial growth or in situ phase, and enter the vertical growth phase almost from their inception. Other histologic factors that affect metastatic potential include ulceration of the tumor, mitotic rate, presence of lymphovascular invasion, microsatellites, regression, perineural invasion, and the presence of lymphocytes infiltrating the tumor.

The primary mode of treatment for localized cutaneous melanoma is surgery. Surgical margins of 5 mm are currently recommended for melanoma in situ, and margins of 1 cm are recommended for melanomas ≤1 mm in depth [5]. For tumors of intermediate thickness (1–4 mm Breslow depth), randomized prospective studies show that 2-cm margins are appropriate, although 1-cm margins have been proven effective for tumors of 1- to 2-mm thickness [6, 7]. Margins of 2 cm are recommended for cutaneous melanomas greater than 4 mm in thickness (high-risk primaries) to prevent potential local recurrence in or around the scar site.

Numerous adjuvant therapies have been investigated for the treatment of localized cutaneous melanoma following complete surgical removal. Adjuvant interferon (IFN) alfa-2b is the only adjuvant therapy approved by the US Food and Drug Administration for high-risk melanoma. However, no overall survival benefit has been demonstrated for adjuvant

chemotherapy, nonspecific (passive) immunotherapy (including interferon), radiation therapy, retinoid therapy, vitamin therapy, or biologic therapy [8]. This makes evaluating for targeted therapies vitally important in treatment of melanoma.

Overview of the MAPK Signaling Pathway

The classical MAPK pathway consists of RAS, RAF, MEK1/2 and ERK1/2, which sequentially relay proliferative signals generated at cell surface receptors through cytoplasmic signaling into the nucleus (Fig. 1) [9–13]. In normal cells, the signaling cascade is stimulated by the binding of mitogens, hormones, or neurotransmitters to receptor tyrosine kinases, which upon dimerization triggers the activation of oncogenic RAS to increase cellular RAS-GTP levels [10, 14]. Activated RAS then triggers the formation of the "MAPK complex" with downstream RAF, MEK1/2, ERK1/2 and several scaffolding proteins initiating the MAPK cascade. The activated RAS activates RAF, which in turn causes the dissociation of ERK1/2 from the MAPK complex.

Activation of MAPK pathway regulates the expression of several genes involved in cell proliferation, differentiation, angiogenesis and survival by phosphorylating nuclear transcription factors such as ETS, ELK-1, MYC or indirectly by targeting intracellular signaling molecules [11, 13, 15]. For instance, activated MAPK pathway induces angiogenesis by increasing the levels of VEGF and MIC-1. MAPK pathway also effects the post-translational phosphorylation of apoptotic regulatory molecules like BAD, BIM, MCL-1, caspase 9 and BCL-2, thereby regulating cellular apoptosis [12, 15]. In melanoma, active mutant ^{V600E}B-Raf induces the expression of proliferation marker Cyclin D1.

Targeting RAS in Melanoma

The RAS family of small G-proteins consists of K-RAS, H-RAS, and N-RAS, which are involved in triggering MAPK signaling by activating downstream proteins such as RAF and PI3K [11, 14, 16]. Structurally in the catalytic domain of the RAS family proteins, the first 80 amino acids are identical and the next 85 amino acids differ only by 5%. In mammalians, all these three RAS genes are universally expressed, even though the expression pattern for each gene is quantitatively different depending on the organ. RAS proteins function as molecular switches regulating cell proliferation and survival [9, 10, 17] and are activated by upstream activation of cell surface receptors, mutation and loss of the RAS-GAP NF-1 [11, 14, 18].

In one third of all human cancers, including melanoma, oncogenic mutations in RAS family members have been reported [11, 14, 18]. Although oncogenic mutations have been frequently reported in codons 12, 13 and 61 of RAS, substitution of leucine for glutamine at residue 61 is the most common aberration observed in N-RAS present in melanomas [18, 19]. Mutant RAS lacks GTPase activity and remains active leading to uncontrolled cell proliferation and a transformed phenotype [18]. Furthermore, expression of RAS can suppress the tumor-suppressors p16INK4A, p53, or p14ARF [20, 21]. Introduction of activated RAS into melanocytes leads to melanoma tumor formation in mice [22, 23] and knockdown of H-RAS expression using siRNAs can cause melanoma regression in an inducible melanoma tumor model [24].

Given the involvement of RAS in tumor growth and control of cell proliferation, it was felt to be a potential drug target for several years.

Clinical Efficacy of Drugs Targeting RAS

Given the fact that the activation of RAS requires farnesylation of the carboxy-terminal cysteine residues by farnesyltransferase (FT), it has been proposed that targeting FT using

farnesyltransferase inhibitors (FTI) or farnesyl cysteine mimetics such as farnesylthiosalicylic acid (FTS) derivatives might prevent growth of melanomas [25, 26]. Unfortunately, efforts to pharmacologically inhibit RAS or its regulatory components for cancer therapy have so far met with minimal success.

A potent FT inhibitor, SCH66336, was noted in preclinical studies to induce G1-phase cell cycle arrest and retinoblastoma protein inactivation to kill melanoma cells [26]. Additionally, a combination of farnesylthiosalicylic acid and SCH66336 markedly enhanced cisplatin-mediated apoptosis demonstrating the chemosensitizing activity of FTIs in melanoma [26, 27]. Lonafarnib, another farnesyltransferase inhibitor, was tested in regards to regulation of proliferation, survival and invasive potential of melanoma cells in monolayer or organotypic culture systems either alone or in combination with chemotherapeutic agents (temozolomide/cisplatin, or MAPK inhibitors sorafenib/U0126/PD98059, or AKT inhibitorsLY294002/wortmannin/rapamycin). In these studies, lonafarnib was neither able to inhibit the growth of metastatic melanoma cells nor sensitize them to the chemotherapeutic agents tested [28]. However, lonafarnib did significantly augment the growth inhibitory effects of the multi-kinase inhibitor sorafenib in eight different cultured metastatic melanoma cell lines [28]. Furthermore, lonafarnib combined with sorafenib was able to trigger apoptosis and prohibit the invasive potential of melanoma cells [28]. Despite FTIs promise in preclinical studies, in a Phase-II study of 14 metastatic melanoma patients, oral administration of FT inhibitor R115777 (300 mg orally twice a day for 21 days) was toxic and lacked therapeutic efficacy [29–32].

In addition to FTIs, direct RAS inhibitors, such as BMS-214662 and L-778123, which are potent non-peptide direct inhibitors of H-RAS and K-RAS respectively, have been evaluated for the treatment in melanoma patients [33–37]. In review of a phase I study of patients with solid tumors receiving oral BMS-214662 (given once or twice daily for 2 weeks in a 3-week cycle), the patients experienced dose-limiting toxicity of nausea and diarrhea with additional toxicities of vomiting, abdominal cramping, anorexia, fatigue and fever. Additionally, of the 23 patients treated, all but 1 had progressive disease [38]. L-778123 has also been evaluated clinically as a 5 day continuous infusion either alone or in combination with radiation and paclitaxel for treatment of NSCLC, as well as head and neck carcinomas. Despite a good clinical response, studies were discontinued due to evidence of cardiac toxicity, manifested as a prolongation of the QTc interval [39–42]. Unfortunately, in addition to limiting toxicity, both compounds have been ineffective in melanoma since these tumors harbor N-RAS and not H-RAS or K-RAS mutations targeted by these agents.

Despite the promise of agents directed towards RAS in preclinical studies, they have failed in clinical trials since FTs farnesylate many proteins other than just RAS, other mechanisms activate RAS proteins promoting development of resistance and deregulation of the pathway by other oncogenes [25]. Thus, therapeutically targeting RAS in melanoma is relatively ineffective suggesting that other points in the MAPK pathway might be more promising to target.

Targeting RAF in Melanoma

The RAF family consists of A-RAF, B-RAF, and C-RAF (or RAF-1), and are downstream effectors of RAS (14, 43). All three mammalian RAF isoforms share three conserved regions (CR1, CR2, CR3) and areas of variable sequences. The CR1 (131 amino acids length) contains a RAS binding domain and a cysteine-rich domain [11, 43]. The CR2 (16 amino acids length) domain contains threonine and serine residues which play a role in regulating the activity of B-RAF upon phosphorylation. The CR3 (293 amino acids length) contains a kinase domain and key phosphorylation sites that regulate enzymatic activity

[11]. A complex process that involves a series of events including membrane translocation; protein dimerization; phosphorylation likely by SRC-family tyrosine kinases; dissociation from RAF kinase inhibitory proteins; and, association with scaffolding proteins is required for the activation of normal non-mutated RAF proteins [11, 44, 45].

Greater than 60% of advanced melanomas express constitutively active mutant B-RAF, which is the most mutated gene in the MAPK signaling cascade [14, 46, 47]. These mutations are acquired, somatic, post-zygotic events and are not inherited in families [13, 46]. Mutated ^{V600E}B-RAF does not require RAS-mediated membrane translocation to exhibit enzymatic activity and is 10.7-fold more active than wild type protein [47]. It also confers resistance to negative feedback regulation by S579A mutation of B-RAF and Sprouty proteins [11]. Even though there are over 65 different mutations that occur in more than 30 B-RAF codons, a single-base missense T to A substitution(at nucleotide 1,799) is prevalent in 90% of melanoma tumors, causing a change of valine to glutamic acid at codon 600 (V600E) in exon 15 [13, 14, 47, 48]. The glutamic acid then acts as a phosphomimetic between the Thr^{598} and Ser^{601} phosphorylation sites which causes a conformational change in protein structure and activation of the protein [14, 49, 50].

^{V600E}B-RAF leads to hyperactivation of the MAPK pathway, which in turn triggers survival pathways and cell division to promote tumor development by inducing proliferation [11, 50–52]. However, only moderate levels of MAPK pathway activation are required for increased in vitro colony formation, elevation of ERK1/2 activities and transformation and immortalization of mouse melanocytes [10, 13, 51, 53]. Recent studies have shown that ^{V600E}B-RAF regulates expression of IL-8, a pro-inflammatory chemokine and autocrine factor, to promote angiogenesis and tumor growth [54]. Additionally, mutant B-RAF can control metastatic development by promoting IL-8 mediated anchoring of melanoma cells to the vascular endothelium to aid extravasation as well as triggering invasive cellular behavior in the development of lung metastases [54, 55]. ^{V600E}B-RAF also induces formation of new blood vessels by promoting macrophage inhibitory cytokine-1 (MIC-1) secretion and vascular endothelial growth factors (VEGF) [56, 57].

^{V600E}B-RAF can also activate the MAPK pathway to levels that inhibit cellular growth and induce senescence in a wide variety of normal and early melanocytic lesion cells [58–60]. However, mutant ^{V600E} B-RAF has been shown to initially stimulate melanocyte proliferation, indicating that it contributes to development of nevi and melanogenesis [48, 50, 58]. This is followed by senescence and subsequent growth inhibition as indicated by proliferative arrest due to increases in β-Gal and $p16^{Ink4a}$ [48, 50, 58]. Increased cyclin-dependent kinase inhibitors, such as $p21^{Cip1}$, $p16^{Ink4a}$, and $p27^{Kip1}$, leads to sensescence induction and acts as a putative defense mechanism to overcome oncogene activation in normal cells [59–61]. A recent study in transformed melanocytes has also shown that senescence and apoptosis induction triggered by ^{V600E}B-RAF can be mediated by insulin growth factor binding protein-7 secretion [62]. Furthermore, additional genetic changes such as loss of p53, $p16^{INK4a}$, PTEN or elevation in AKT3 activity is required for melanoma development to occur in nevi containing ^{V600E}B-RAF and for the quiescent melanocytic cells to overcome the ^{V600E}B-RAF induced senescence in order to reenter the cell cycle [58, 63, 64]. In one study, zebrafish expressing ^{V600E} B-RAF protein developed fish-nevi; however, only when expressed in p53-deficient zebrafish did rapid progression of melanocytic lesions develop into invasive melanomas, resembling those occurring in human tumors [65]. This provided direct evidence that linked melanoma development to an interaction between the ^{V600E}B-RAF and p53 pathways [66]. ^{V600E}B-RAF has also been shown to occur with $p16^{INK4A}$ loss in ~60% of melanomas [63]. A recent study showed that absence of activated B-RAF and $p16^{INK4a}$ expression were independent predictors of melanoma tumor chemosensitivity in a group of patients who underwent isolated limb

infusion with cytotoxic drugs actinomycin-D and melphalan for metastatic melanoma [67]. In regards to PTEN, genetically altered mice harboring conditional melanocytes expressing ^{V600E}B-RAF, developed benign melanocytic hyperplasia but failed to develop melanoma. Only when PTEN was lost did melanoma develop, which metastasized to lymph nodes and lungs [64]. AKT3 has been shown to release cells from ^{V600E} B-RAF-mediated senescence via phosphorylating ^{V600E} B-RAF on S364 and/or S428 in order to reduce its activity to levels that promote rather than inhibit melanoma development from melanocytes [58]. Occurrence of B-RAF mutation is likely an early event, with the alteration of the PTEN/AKT pathway occurring later in tumor progression [68]. Therefore, a successful targeted therapy will likely require targeting both pathways simultaneously.

Clinical Efficacy of Therapies Targeting RAF

Given the importance of B-RAF mutations in melanoma, small molecule inhibitors targeting mutated ^{V600E}B-RAF kinase have shown efficacy in the clinic. Initially, the RAF inhibitor Sorafenib was studied following both oral or intraperitoneal administration. Sorafenib (BAY 43-9006) reduced growth of subcutaneous melanoma tumors by inhibiting cell proliferation and vascular development [57, 69]. However, clinical trials using sorafenib as a monotherapy in advanced melanoma have failed to demonstrate significant anti-tumor activity. Only 19% of patients exhibited stable disease with a progression free survival of 16–37 weeks, while 62% showed progressive disease with progression free survival of about 11 weeks [70]. No relationship between B-RAF mutational status and disease stability was observed raising concerns regarding the clinical utility of targeting B-RAF to treat melanoma [70]. It is felt that failure of Sorafenib clinically is likely due to its inhibition of other kinases (FGFR1, c-Kit, p38 MAPK) or angiogenic factors (VEGFR1, VEGFR2, VEGFR3, and PDGF), rather than solely due to inhibition of RAF [69, 71–73].

Given the concerns raised regarding Sorafenib, other mutant B-RAF kinase inhibitors have been developed. Of these, PLX4032 (Vemurafenib) directly targets ^{V600E}B-Raf. It was first discovered using a scaffold-based drug design approach [74], along with another promising mutant B-RAF kinase inhibitor PLX4720. Initial xenograft studies with PLX4032 revealed dose dependent inhibition of tumor growth in those with B-RAF mutation and no effect on tumors containing wild type protein. Both of these B-RAF inhibitors were chosen for further study over similar compounds because of their consistent pharmokinetics in rodents and PLX4032 was ultimately chosen for clinical trials over PLX4720 because of more favorable outcomes in beagle dogs and cynomolgus monkeys [75]. During Phase I clinical trials, the maximum tolerated dose of reformulated PLX4032 as a micro-precipitated bulk powder was discovered to be 960 mg po bid and an extension of this trial was performed with 32 patients with B-RAF mutant melanomas as detected via PCR analysis. Of those treated, 24 achieved partial remissions, and three achieved complete remission. Respondents had near complete inhibition of ERK signaling, which may be needed for significant tumor response as those patients with tumor regressions showed a greater than 80% inhibition in cytoplasmic ERK phosphorylation. The median progression free survival in this Phase I extension cohort has not been reached, but is estimated to be about 7 months [76].

A randomized Phase III trial comparing vemurafenib (PLX4032) and dacarbazine, a commonly used chemotherapeutic agent in melanoma, was recently published. In this trial, a total of 675 metastatic melanoma patients with the ^{V600E}BRAF mutation that had not been previously treated were randomly assigned to vemurafenib (960 mg po bid) or dacarbazine (1,000 mg per square meter of body surface area IV q 3 weeks). In this trial, overall survival with a 95% confidence interval was 84% in the vemurafenib group and 64% in the dacarbazine group. Vemurafenib was associated with a relative reduction in the risk of death of 63% and 74% in the risk of either death or disease progression compared with dacarbazine ($p < 0.001$) [77].

Additionally, GSK2118436 is another BRAF inhibitor that has been studied and recently is starting Phase III trials. During the ESMO 2010 meeting in Milan, Phase I data was presented and revealed that treatment shrunk the overall size of brain metastases by 20–100% (3 mm or larger before treatment) in nine out of ten treated patients, which was noted to be remarkable as typical treatment responses are 10–15% [78]. Additionally, treatment with GSK2118436 in these trials revealed an impressive 60% response for melanomas outside of the brain. At this point, PLX4032 is likely to reach the market first since results from its Phase III trial as noted above has shown significantly extended survival in metastatic melanoma.

Toxicities and Development of Resistance of Drugs Targeting ^{V600E}B-RAF

Major concerns related to B-RAF inhibitors include development of resistance and to a lesser extent its side effects. Minor side effects included rash, joint pain and fever [76]. Additionally, approximately 23% of patients developed cutaneous squamous cell carcinoma during the first few months of treatment [79]. In the recently published Phase III trial of vemurafenib (PLX4032), initial observations reported side effects that included arthralgia, rash, fatigue, alopecia, photosensitivity, nausea, diarrhea, keratocanthoma or squamous cell carcinoma with 38% of patients requiring a dose modification to lessen these issues [77].

A more serious concern related to patients treated with Vemurafenib has been disease recurrence as early as 3–4 months in those who initially responded to the drug [79]. To characterize the underlying mechanisms leading to development of drug resistance, Nazarian et al. examined three cell lines with the ^{V600E}B-RAF that were very sensitive to growth inhibition from PLX4032. These cell lines were subjected to chronic PLX4032 exposure to develop resistant sublines [80]. Analysis of these cell lines revealed that the ^{V600E}B-RAF did not develop secondary mutations promoting the development of resistance. Resistance developed by the formation of RAF dimers either via RAS activation or increased RAF expression, since binding of these inhibitors to RAF dimers leads to transactivation of the nonbound member of the dimer, bypassing the inhibitory effect [81]. Melanomas with the ^{V600E}B-RAF, do not have Ras levels high enough to promote dimerization of RAF. However, in in vitro cell lines that developed resistance, N-Ras mutations occurred, leading to increased RAS activation and thereby RAF dimerization and development of resistance [80]. Another mechanism leading to resistance was by overexpression of mitogen-activated protein kinase 8 (MAP3K8), which encodes the protein kinase COT that activates ERK through phosphorylation in a RAF-independent manner, leading to resistance to RAF inhibition [82]. In ^{V600E}B-RAF cells, expression of MAP3K8 mRNA levels and its associated COT protein were undetectable. However, treatment with the RAF inhibitor PLX4720 increased COT protein levels in a dose dependent manner. Clinically, two thirds of biopsied samples from ^{V600E} B-RAF melanoma patients treated with PLX4032 showed increased MAP3K8 mRNA expression by quantitative PCR analysis. Resistance can also develop due to over-expression of platelet-derived growth factor receptor (PDGFR), activating a receptor tyrosine kinase dependent survival pathway and through parallel signaling pathways triggering downstream effectors of cell transformation [80, 81]. Clinically, the overexpression of PDGFR was observed in 4/11 patient derived samples from resistant tumors. B-RAF inhibitors, such as PLX4720, also appear to cause hyperactivation of the MEK-ERK 1/2 pathway in mutant N-Ras melanoma cells which can cause pathway hyperactivation leading to apoptotic resistance [83].

Targeting MEK in Melanoma

MEK-1 and MEK-2 are dual-specificity tyrosine/threonine protein kinases that lie downstream of B-RAF and are found to be active in ~30% of all human cancers with activated MAPK signaling [14]. The only known substrate of MEK-1 and MEK-2 kinases is

ERK [14]. Therefore, MEK-1/2 is a popular therapeutic target in the MAPK signaling cascade [84]. It has been shown that tumors that harbor ^{V600E}B-RAF are sensitive to MEK inhibition but not those that harbor mutant RAS [85]. Therefore, when selecting MEK inhibitors for melanoma therapy, B-RAF mutational status is a critical factor needing consideration [85].

Clinical Efficacy of Therapies Targeting MEK

A wide range of different cancer cell lines possessing either K-RAS, N-RAS or B-RAF mutations are sensitive to AZD6244 at <1 μmol/L which is a selective, potent, allosteric inhibitor of MEK [86]. Initial in vitro studies by Davies et al. noted that the majority of cell lines that are sensitive to AZD6244 possess a mutation in the RAF or RAS genes, while none of the resistant lines possessed a B-Raf mutation. Thus, most cell lines containing mutant B-RAF are dependent on MEK activity and therefore sensitive to MEK inhibition. In contrast, presence of K-RAS mutation makes cells less sensitive to MEK inhibition, which might be due to RAS initiating signaling through other signaling pathways implicated in cancer development [86].

Efficacy of AZD6244 was tested in nude mice containing xenografts from cells with B-Raf and K-Ras mutations that were highly sensitive to AZD6244 (Colo-205, Calu-6, and SW620) [86]. Dosing with 25 mg/kg bid resulted in 94% inhibition of Calu-6 tumor growth, 73% inhibition of SW620 tumor growth and stasis of Colo-205 tumors if started when tumors were about 0.2 cm^3 or partial regression if dosing started when tumors were larger at about 0.55 cm^3. Phosphorylated-ERK levels were measured to determine the level of inhibition in each of these xenografts. In Calu-6 xenografts, an acute dose of 25 mg/kg sufficiently inhibited p-ERK by >90% after 1 h as measured by immunohistochemistry or western blotting. Moreover, inhibition of ERK phosphorylation was significant but to a lesser degree in Colo-205 and SW620 xenografts. In the two most sensitive xenografts, Colo-205 and Calu-6, a single dose of AZD6244 was sufficient to trigger apoptosis. A combination of AZD6244 with either irino-tecan or docetaxel has also been shown to significantly inhibit xenografted tumor development in this study [86].

Phase I clinical trials with AZD6244 were published in 2008 with 57 patients enrolled [87]. The maximum tolerated dose in this trial was 100 mg po bid. Pharmacokinetics revealed a median half life of approximately 8 h, supporting twice daily dosing, and pharmacodynamic studies demonstrated dose dependent inhibition of ERK phosphorylation with up to 100% inhibition occurring 1 h after treatment with the first dose. Additionally, Ki-67, a marker of cell proliferation, decreased compared to pretreatment levels in tumor biopsies, but not as consistently as pERK levels did. The most common side effect was a rash followed by gastrointestinal related toxicities including nausea and diarrhea. Stable disease lasted for five or more months in nine of 57 patients enrolled, stable disease at end of cycle 2 (each cycle is 28 days) for 19 of the patients and one patient with uveal melanoma and renal cell carcinoma with stable disease for 22 cycles and another with medullary thyroid cancer that had stable disease for 19 cycles.

A non-ATP competitive MEK 1/2 inhibitor with a unique structure and mechanism of action is R05068760. Daouti et al. published an in vivo characterization of the pharmacokinetics, pharmacodynamic and efficacy of RO5068760 in multiple xenograft tumor models [88]. The estimated EC_{50} in plasma was 1.36 umol/L (880 ng/ml) in the LOX melanoma models and a plasma drug concentration of 0.65 or 5.23 umol/L was needed for tumor growth inhibition (>90%) in ^{V600E}B-Raf or K-ras mutant tumor models.

Development of Resistance to Drugs Targeting MEK 1/2

Certain melanoma cells are resistant to MEK1/2 inhibitors [89]. While mechanisms leading to MEK1/2 inhibitor resistance remains uncertain, a recent study sequenced tumors obtained from relapsed patients following treatment with the allosteric MEK inhibitor AZD6244 and resistant clones generated from a MEK1 random mutagenesis screen [90]. Mutations were identified disrupting the allosteric drug binding pocket or alpha-helix C, which led to an ~100-fold increase in resistance to MEK inhibition [90]. Mutations in MEK1, Q65P and P124L have also been identified in patients treated with the MEK inhibitor AZD6244. These mutations affected MEK1 codons located within or adjacent to the N-terminal negative regulatory helix A and conferred resistance.

Cells from patients that initially showed transient disease stabilization after being treated with AZD6244 followed by relapse on this drug have been subsequently treated with PLX4720 (a BRAF inhibitor described above) [90]. AZD6244-resistant melanoma cells exhibited resistance to PLX4720 with a GI50 value of >10 μM compared to 5–10 nM in treatment-naïve cells. Mechanistically, the cause was P124L and P124S MEK mutations, which conferred two- to threefold more resistance compared to wild-type MEK1. Meanwhile, robust resistance of >50-fold to PLX4720 compared to the MEK (DD) allele was conferred by the Q56P mutation. Clinically relevant MEK1 resistance mutations may confer cross-resistance to B-RAF inhibition as evidenced by pMEK levels following PLX4720 treatment that showed comparable reduction across all MEK1 resistance alleles [90].

Preventing MEK mediated resistance will likely require targeting multiple points in the MAPK pathway. Exposing melanoma cells containing mutant B-RAF simultaneously to PLX4720 (a ^{V600E}B-RAF inhibitor) and AZD6244 (a MEK inhibitor) prevented emergence of resistant clones, which indicates the potential of targeting multiple points in this signaling cascade to prevent development of resistance and to kill melanoma cells [90]. Therefore, combined inhibition of MEK and RAF might bypass acquired resistance to targeted therapeutics directed against the MAP kinase pathway.

Targeting ERK in Melanoma

ERK is the only known downstream substrate for MEK 1/2 [14]. Elevated ERK activity is frequently observed in human tumors as well as proliferating metastatic melanoma cell lines and is a good indicator of tumor progression [91, 92]. Growth factors in melanomas can activate ERK either by the “classical” pathway (utilizing receptor tyrosine kinases such as the c-KIT ligand SCF), or through a pathway that is coupled to G-protein receptors (such as the α-MSH activated melanocortin receptors) [9]. In melanocytes, ERK activity can also be stimulated by mitogens such as bFGF and endothelin-1 [93]. However, the degree of contribution of each pathway to the overall stimulation of ERK in melanomas remains to be determined. Additionally, sustained activation of ERK in melanoma cells has been shown to confer resistance to various therapeutic agents. Although elevated ERK activity has been shown to promote cell proliferation; under certain circumstances, the activation of ERK can inhibit cell cycle by up-regulating p53 and p16INK4a expression [13, 58, 61, 63].

Further evidence of ERK expression in melanomas was performed using immunohistochemical studies with antibodies to ERK 1/2 and phosphorylated ERK (p-ERK). In these cases, ERK was noted to be expressed in varying degrees in formalin fixed sections from 42 primary melanomas, 38 metastases, and 20 nevi (14 of the primary melanomas were in the radial and 28 in the vertical growth phase), either in the cytoplasm and/or nucleus. Only low levels of ERK1/2 were detected in melanocytes and no pERK was detected in normal skin [94]. In patients with metastatic melanoma, higher levels of pERK

were noted in subcutaneous metastases compared to lymph node metastases or compound nevi. Since N-RAS and B-RAF mutations are more frequent in cutaneous or soft tissue melanoma metastases, this could partially account for the differences in pERK levels in these cases. Additionally, there was a non-significant relationship between the depths of melanoma and pERK levels [94]. Higher percentage of p-ERK-positive cells have been reported in nodular melanoma compared with benign nevi and superficial spreading melanoma. Thus ERK activation is directly related to the stage of disease with higher activity occurring in more advanced melanomas [94].

Currently, the MEK1/2 inhibitors as described above are employed as inhibitors of its downstream effector ERK1/2, as MEK1/2 is known to activate ERK1/2 selectively [95] (Table 1).

Targeting Other Downstream Targets

Targeting Aurora Kinases in Melanoma

The aurora kinase family consists of aurora kinase A (AURKA), aurora kinase B (AURKB), and aurora kinase C (AURKC), which are involved in mitotic spindle assembly regulating centrosome duplication and separation, microtubule-kinetochore attachment, spindle-checkpoint, and cytokinesis [96–98]. The family members range in size from 309 to 403 amino acids with AURKA sharing 53% homology with AURKB and 73% homology with AURKC [99–101]. AURKA is involved in mitotic spindle formation and centrosome maturation that are required for chromosome segregation [102]. AURKB is a chromosomal passenger protein regulating early mitotic stage transition of prophase to metaphase [103, 104]. Inhibition halts a crucial spindle checkpoint causing premature exit from mitosis disrupting chromosome segregation and cytokinesis. AURKC is localized to the centrosome and involved in spermatogenesis.

In humans, although three isoforms of Aurora kinases, Aurora-A, -B and -C, were identified, only Aurora-A and -B are expressed at detectable levels in all somatic cells, therefore, have been characterized in greater detail for their involvement in cellular pathways relevant to the development of cancer [105]. Elevated expression of AURKs has been reported in cancers of skin, breast, colon, prostate and ovaries [106]. In addition, genetic variants of AURKs have been found in various clinical biopsies excised from patients suffering from non-melanoma skin cancer, and cancers of breast, prostate and ovaries [107, 108]. For example, a genetic variant of AURKA, STK15 T + 91A, which resulting in the amino acid substitution F31I, has been associated with increased aneuploidy in colon tumors and cell transformation in vitro [109]. Furthermore, meta-analysis of 9,549 cases of breast, colon, ovarian, prostate, lung, esophageal and non-melanoma skin cancers showed an increased risk in T + 91A homozygotes of breast and colorectal cancers. In addition, genomic analysis of cutaneous melanomas showed frequent gains at chromosome 20q that includes Aurora A gene.

Clinical Efficacy of Therapies Directed at Aurora Kinase—Since elevated levels of these kinases have been detected in several cancers, the aurora family of serine/threonine kinases are another target of therapies [110–112]. In vivo studies of 12 cancer cell lines, including melanoma, was performed in female mice that had subcutaneous implantation of tumor cells with the aurora kinase inhibitor, SNS-314 [110]. These assays revealed decreased phosphorylation of histone H3 on serine 10, a marker of activity of aurora kinases, and significant tumor growth inhibition in a dose dependent manner. This trial concluded that SNS-314 is a potent small molecule inhibitor of Aurora kinases and may be a novel therapeutic agent for human cancers, including melanoma [110].

Additionally, a recent study evaluating the effect of inhibiting Aurora kinase-A and Aurora kinase-B activities using isoform specific pharmacological agents VE-465 and ZM447439, respectively, demonstrated that Aurora kinase-A targeting is more effective than Aurora kinase-B inhibition for the induction of melanoma cell death [113]. A phase I trial examining the safety, pharmacokinetics and pharmacodynamics of an oral Aurora kinase-A inhibitor, MLN8054 has been performed in patients with advanced solid tumors. The data showed induction of two dose limiting toxicities when MLN8054 was given QID at a dose of 80 mg [114, 115]. Aurora kinase-A inhibition was evidenced by pharmacodynamic analysis of skin and tumor mitotic indices, mitototic cell chromosome alignment and spindle bipolarity. Recently a more potent second generation Aurora kinase-A inhibitor MLN8237 was synthesized and is currently in early phase clinical trials [116].

Targeting Macrophage Inhibitory Cytokine-1 in Melanoma

MIC-1, also known as PTGF-β, PLAB, GDF15, PDF, and NAG-1, is a member of the transforming growth factor-beta super-family proteins implicated in melanoma development [56, 117]. Expression of MIC-1 is upregulated in 66% of metastatic melanoma cell lines (35/53) and 100% metastatic patient biopsies (16/16) compared to normal melanocyte controls [56]. Another recent study also showed elevated MIC-1 expression in 67% advanced melanomas [118]. In addition, five- to six fold increase in secreted MIC-1 protein was observed in the serum of these patients indicating that MIC-1 can serve as a prognostic marker for identifying melanoma patients [56, 118]. Prior studies also showed elevated expression of MIC-1 in a wide variety of tumors including carcinomas of prostate, large bowel and breast. Expression of MIC-1 is regulated by MAP kinase and PI3 kinase pathways in melanoma [56, 118]. For example, pharmacological agents U0126 and PD098059 inhibiting MEK1/2 activity decreased expression of MIC-1 [56]. Similarly, cells treated with PI3 kinase inhibitor LY294002 also modestly reduced expression. MITF, a key member of MAP kinase pathway regulating the expression of various cell cycle and cell proliferation proteins, has been shown to control MIC-1 protein levels [56]. Factors stimulating MITF activity such as stem cell factor or exposure to PMA elevated expression of MIC-1. A prior study using short-hairpin RNAs demonstrated that MIC-1 inhibition decreased xenografted melanoma tumors development compared to cells transfected with control sh-RNAs [56]. Similarly, another recent study also demonstrated that targeting Mic-1 using siRNAs reduces the xenografted melanoma tumors growth [118]. Similar to VEGF, MIC-1 also stimulated the vessels development, thereby augmented tumor growth.

Although MIC-1 expression has been shown to be upregulated in advanced melanomas, the precise role of MIC-1 in tumor biology is unclear. For example, it is not known whether MIC-1 expression is leading to metastasis development or metastatic tumors are releasing MIC-1 into serum to perform some yet unknown role. Furthermore, the role of MIC-1 in different stages of melanoma development needs to be studied in detail as recent studies demonstrated that the MIC-1 function varies with the stage and extent of the tumor producing it. At present time, there are no apparent trials evaluating direct MIC-1 inhibitors in melanoma.

Targeting Interleukin-8 (IL-8) in Melanoma

IL-8 is an important autocrine multifunctional cytokine implicated in melanoma cell proliferation, angiogenesis, migration and metastasis development [119]. IL-8 is also an indicator of tumor aggressiveness as elevated expression of this cytokine is observed in melanoma tumor biopsies [120]. Furthermore, introduction of IL-8 into non-metastatic melanoma cells lines or cells that are negative for IL-8 expression induced expression and activity of MMP-2, which increased invasion and angiogenesis thereby transforming them in to highly tumorigenic, metastatic cell types [121]. IL-8 expression can be induced by

phosphoglucoseisomerase/autocrine motility factor (AMF) in autocrine manner thereby promoting melanoma cell migration [122]. Targeting IL-8 using siRNAs reduced IL-8 secretion from melanoma cells, which resulted in the down regulation of β2 integrin on neutrophils thereby inhibiting metastasis development [54, 123]. Tumor-derived cytokines IL-6 and IL-8 can act as attractants for circulating tumor cells (CTCs) thereby promoting a process called “tumor self-seeding” [124]. Tumor self-seeding is a process in which tumor cells colonize their tumors of origin. Tumor self-seeding is primarily responsible for local recurrence occurring after complete tumor excision.

Signaling pathways regulating IL-8 expression and secretion in melanoma cells involve MAP kinase pathway signaling [54, 123]. A recent study demonstrated that AMF induced IL-8 production was mediated by ERK1/2 in melanoma cells [122]. Therefore, targeting members of MAP kinase signaling could potentially decrease IL-8 levels thereby inhibiting melanoma tumor and metastasis development. For example, knockdown of mutant (V600E) B-Raf inhibited the constitutive secretion of IL-8 thereby decreasing melanoma cell extravasation and subsequent metastasis development [54, 123]. Pharmacological agents targeting members of MAP kinase pathway also reduced the production of IL-8 in melanoma cells [125, 126]. For example, inhibition of B-RAF using derivatives of diarylimidazoles decreased colony formation in soft agar, reduced proliferation and retarded melanoma tumor growth in animal models. Targeting B-Raf reduced IL-8 in the plasma of animals, suggesting that it could serve as a marker for clinical assessment of B-Raf inhibition [125]. MEK inhibitor PD0325901 has been shown to reduce IL-8 and VEGF levels thereby decreasing melanoma cell proliferation and angiogenesis [126].

Other key regulators of IL-8 production in melanoma cells include STAT3 and PAR-1. For example, whereas introduction of constitutively active STAT3 into WM35 melanoma cells enhanced IL-8 production, targeted inhibition of STAT3 in 1205 Lu cells reduced IL-8 levels [127]. Similarly, systemic delivery of PAR-1 siRNA incorporated into neutral liposomes [1, 2-dioleoyl-sn-glycero-3-phosphatid ylcholine (DOPC)] decreased VEGF and IL-8 production thereby reducing melanoma growth and metastasis in animals [128].

While IL-8 can serve as a biomarker of B-Raf inhibition, some pharmacological agents targeting MAP kinase signaling can induce the production of IL-8. For example, dacarbazine, an FDA approved agent for melanoma, activates the extracellular signal-regulated kinase pathway, and increases expression and secretion of IL-8 and VEGF [129, 130]. In addition, some selenium containing chemotherapeutic agents such as PBISe targeting Akt induce MAP kinase pathway activity [13, 131]. However, it is unknown whether increased MAP kinase activity mediated by PBISe promotes IL-8 secretion. Therefore, clinical trials should consider using IL-8 neutralizing antibodies such as ABX-IL8 while evaluating these agents [132]. In fact, the use of fully human antibodies against IL-8 have been studied thus far in vitro [133]. In this study, fully human IL-8 antibodies reduced the invasion of metastatic melanoma cells. They also appeared to sensitize tested cell lines when treated with dacarbazine and also decreased cell viability in metastatic melanoma cell lines.

Targeting WEE1 in Melanoma

WEE1, another downstream member in the MAPK signaling, is a key protein kinase involved in maintaining G(2)-cell-cycle checkpoint arrest for pre-mitotic DNA repair [134, 135]. WEE1 phosphorylates Tyr-15 of CDC2 thereby inhibiting its activity, which results in G2/M arrest [136]. Elevated expression of WEE1 is observed in glioblastoma and breast cancer [134, 136]. Furthermore, studies have also shown that targeting WEE1 using siRNA or pharmacological agents inhibited cancer cell survival and reduced the development of xenografted tumors demonstrating the therapeutic potential of targeting this key kinase for

cancer therapy [137, 138]. In addition, targeting WEE1 in combination with either radiotherapy or treatment with cytostatic agents enhanced the therapeutic potential. Pharmacological inhibition of WEE1 using MK-1775 selectively sensitized p53 deficient cancer cells to DNA damaging agents such as gemcitabine, cisplatin and carboplatin [137]. PD0166285, a known WEE1 inhibitor, radio-sensitized cells to radiation-induced cell death in a p53 dependent manner [139]. Cells lacking p53 showed higher sensitivity to WEE1 inhibition compared to those harboring p53. Mechanistically, targeting WEE1 induces a mitotic catastrophe due to premature entry into mitosis with unrepaired lethal damaged DNA [139]. Since cancer cells, unlike normal cells that require G1-arrest, largely depend on G2-M arrest for damaged DNA repair, targeting WEE1 in combination with DNA damaging agents is a promising therapy for cancers. While the role of WEE1 is well studied in several other cancer types, a few studies have reported the therapeutic potential of targeting WEE1 in mouse melanoma cells. Targeting WEE1 using PD0166285 reduced cell proliferation by decreasing Cyclin-D levels [140]. Since melanomas are known to contain functionally active p53 protein, it is interesting to determine whether targeted inhibition of WEE1 alone is effective for retarding melanoma development. At present time, there are no apparent trials evaluating direct WEE1 inhibitors in melanoma.

Targeting VEGF in Melanoma

VEGF is another key target in melanomas regulating angiogenesis, which is required for invasive tumor growth and metastasis [141–143]. Immunohistochemical studies have shown that 20–77% of pr human primary melanomas express VEGF (Potti, A. Anticancer Res. 2003. pp 4023–2026). Targeted inhibition of VEGF may be a valuable approach to cancer therapy. Studies have shown that targeting B-Raf inhibits VEGF expression in melanomas [57]. siRNA mediated inhibition of B-Raf reduced endogenous as well as secreted VEGF levels, which in turn decreased blood vessel development thereby retarding xenografted melanoma tumors growth [118]. Furthermore, prior studies have demonstrated that inhibition of Raf, either by the use of MEK inhibitor (PD98059) or by siRNA specific to B-Raf, significantly lowered VEGF-A expression [144]. Treating melanoma cells with sorafenib also decreased MAPK activity and reduced blood vessel density through the inhibition of VEGF [57]. Proof of principle studies using siRNAs targeting VEGF retarded melanoma tumor development indicating VEGF could be a therapeutic target for inhibiting melanoma [57]. Further circulating VEGF (cVEGF) has been correlated with disease progression in melanomas, indicating VEGF could be a biomarker for disease diagnosis as well as a marker for measuring the therapeutic efficacy of various treatment interventions. However, a recent study showed that cVEGF may not be a good indicator of assessing the disease severity and treatment efficacy as the true VEGF levels in cancer patients appears to be very low, except in renal cell carcinoma [145]. High levels of VEGF measured in clinical specimens could be due to the artificial release from activated platelets. Activated platelets in cancers have been found to secrete increased VEGF, primarily during the blood harvest procedure [145]. Therefore care must be taken while considering VEGF as a biomarker for disease prognosis.

Sorafenib, which originally was developed as a BRAF inhibitor, also selectively inhibits VEGFR-2 and -3, was initially studied but did not show evidence to improve standard of care [146]. Axitinib, a potent oral inhibitor of VEGF Receptor Tyrosine Kinase 1, 2, and 3 is currently in development by Pfizer Inc for the potential treatment of a variety of solid tumors. Thus far, preclinical and clinical data is available for axitinib [147]. Phase II studies in many tumor types including malignant melanoma and renal, pancreatic, thyroid, breast, lung and colorectal carcinomas showed that axitinib is well-tolerated [147]. However, in metastatic melanoma, recent Phase II trials revealed an unimpressive overall response rate of 15.6% and median survival of 6.8 months [146]. Additionally, due to frequent side effects

including fatigue, hypertension, diarrhea, hand-foot syndrome and proteinurea, its clinical development has been hampered [147, 148]. Recent ongoing phase III studies in pancreatic and metastatic renal cell carcinoma will ultimately define the therapeutic role of this targeted agent for the treatment of melanoma and other malignancies [147]. Thus far, the failures of these selective tyrosine kinase VEGF inhibitors, along with others including sunitinib, dovitinib and vatalanib are believed to be multifactorial secondary to the chemoresistant nature of metastatic melanoma, the cystostatic rather than cytotoxic nature of tyrosine kinase inhibitors, and that these studies have been primarily for inhibition ability in established metastatic tumors [146].

More recently, monoclonal antibodies directed against VEGF ligand have been reported with bevacizumab (Avastin). A recent phase II trial for first line therapy for malignant melanoma tested carboplatin and paclitaxel chemotherapy with and without bevacizumab and reported that progression free survival had improved to 22% and overall survival improved to 21% in patients treated with bevacizumab [146]. The primary progression free survival endpoint was not met but the gain in overall survival has led to a planned subsequent definitive trial [146]. Larger Phase II trials are needed to further delineate the use of VEGF monoclonal antibodies.

Additionally, afibercept, a fusion protein that incorporates portions of human VEGFR-1 and VEGFR-2 with human IgG1 has been studied. Acting as a soluble decoy VEGF receptor, preclinical studies showed a favorable pro fi le over other VEGF inhibitors [149]. In an interim analysis of a current Phase II study, one of 21 patients with treatment-naïve metastatic melanoma received complete remission [146].

Targeting Cyclin D-1 and B-RAF in Melanoma

D-type cyclins, which are regulated themselves by B-Raf, regulate G1 cell cycle progression by enhancing the expression and activities of cyclin-dependent kinases [150]. In normal cells, levels of cyclins and cyclin-dependent kinase (Cdk) inhibitors are tightly controlled. However, in melanomas, this normal balance is frequently dysregulated. In one study, immunohistochemical analysis of cyclin D1 showed elevated expression in early melanomas [151]. Cyclin D1 positivity increased during tumor progression, but was observed in lower levels in metastases. Survival analysis in this study failed to detect any linkage to shorter or longer survival among patients expressing either cyclin D1 c-Kit, or p-ERK [151]. Additionally, this study found that cyclin D1 expression lacked prognostic potential as low levels of cyclin D1 occurred in metastatic melanomas [151]. In contradiction to this report, another found that cyclin D1 expression may be related to malignant phenotype and might be associated with high proliferation rates in metastatic melanomas. Analysis of formalin-fixed paraffin-embedded material from 21 common melanocytic nevi, 42 dysplastic nevi, and 17 primary cutaneous metastatic melanomas showed elevated Cyclin D1 expression in advanced compared to earlier stage lesions [152].

Currently, a Phase II Study to evaluate the safety and efficacy of P276-00, a cyclin D1 inhibitor, has finished recruiting and is currently ongoing with results pending at this time. Previously, P276-00 was studied in vitro and in vivo [153]. In this study, P276-00 was tested for its antiproliferative potential in a panel of 16 cisplatin-resistant and cisplastin sensitive cell lines and noted to have a ~30-fold higher effect than cisplatin. Furthermore, 22 human xenografts in a clonogenic assay showed tumor sensitivity to P276-00 was ~26-fold more potent than cisplatin and also effective against cisplatin resistant lines in melanomas, CNS, renal and prostate cancer. In flow cytometry testing, an asynchronous population of human prostate cancer and human promyelocytic leukemia cells showed arrest of prostate cancer cells in G2-M with no significant apoptosis and significant apoptosis in faster growing promyelocytic leukemia cells. P276-00 in synchronized human non-small cell lung cancer

showed arrest of cells in G1 followed by apoptosis if exposed for 48 h. Further testing was performed with P276-00 in vivo with murine tumor and human xenograft models showing significant growth inhibition in murine colon cancer when administered i.p. at 50 mg/kg for 20 treatments and in murine lung cancer models when administered i.p. at 60 mg/kg every alternate day for seven treatments. In human xenograft models, P276-00 showed significant inhibition in human colon carcinoma HCT-116 xenografts at a dose of 35 mg/kg i.p for 10 days and human non-small cell lung carcinoma H-460 xenograft at a dose of 50 mg/kg daily or 30 mg/kg twice daily i.p. for 20 treatments [153]. Cyclin-D1 remains an interesting potential target for therapies in melanoma and results from the noted Phase II trial are pending.

Targeting Members of Other Pathways

Agents currently used in treatment of melanoma, such as Dacarbazine or the derivative temozolomide, are only effective in 15–20% of patients [154, 155], partly secondary to deregulation of many pathways in melanoma cells that promote highly metastatic phenotypes and resistance to chemotherapeutics [13]. As such, most clinicians and researchers in the melanoma field believe that multiple signaling cascades will need to be targeted simultaneously to effectively inhibit melanoma development. Therefore targeting of the members of MAPK cascade or other oncogenic proteins from different signaling pathways combined with these therapies will be required to achieve better clinical efficacy [156].

As alluded to in the previous sections, preclinical studies have shown that targeting PI3K and MAPK signaling pathways using siRNA or pharmacological agents can sensitize cells to chemotherapeutic agents and synergistically inhibit melanoma development [58, 157]. For instance, co-targeting RAF and mTOR using sorafenib and rapamycin, respectively, more effectively inhibited melanoma cell proliferation, inhibited melanoma cell invasion and induced cell death [158]. Likewise, treatment of melanoma cells with cisplatin or temozolomide in combination with LY294002 or rapamycin effectively reduced melanoma cell growth and survival [158]. Similarly, simultaneous inhibition of CDK 4 kinases and MEK using pharmacological inhibitors PD98059 and 219476, respectively, significantly increased apoptosis compared to single agents alone [159]. Another independent study combined MAPK and PI3K signaling pathway inhibition to show that the anti-proliferative and pro-apoptotic effects of inhibitors alone were disappointing compared to using a panel of pharmacological inhibitors (BAY 43-9006, PD98059, U0126, wortmannin, LY294002) which significantly inhibited growth and enhanced apoptosis in monolayer culture [160].

Targeting oncogenes while expressing tumor suppressors is another alternative approach for inhibiting melanoma development. For instance, massive apoptosis in melanomas was observed when ^{V600E}B-RAF was targeted using siRNA while expressing the tumor suppressor INK4A cDNA compared to either of these events alone [63]. Building on this approach, targeting multiple members of a single pathway or members of different pathways is an approach to more effectively treat melanomas that will continue to evolve in the next decade. However, the combination would need to be selected based on the genetic pathway activated and available approaches to target them.

Impact of Tumor Microenvironment and Cancer Stem Cells—The Future of Melanoma Targeted Therapy?

Melanomas, like many other cancer types, depend on interactions with microenvironment for tumor growth as well as metastasis formation [161]. Therefore, tissue microenvironment does play a critical role in cell survival and growth and likely contributes to cell

transformation and tumor development [162]. Cellular interactions with the stroma and with other cells provide key signals that control cellular arrest or division, survival or death, and entrance or exit from a quiescent state [161]. For example, tumor cell adhesion to blood vessel endothelial cells (EC) followed by trans-endothelial migration is critical event responsible for the metastasis development [163–165].

Recent studies have shown the involvement of polymorphonuclear neutrophils (PMNs) for facilitating melanoma cell adhesion to the endothelium as well as subsequent extravasation under flow conditions [166, 167]. Experiments demonstrating the involvement of neutrophils in the development of melanoma metastasis showed enhanced metastatic tumors when neutrophils were injected immediately after melanoma cells injection [123]. Mechanistically, entrapped melanoma cells produced interleukin-8 thereby attracting neutrophils. In addition, IL-8 also increased the beta [2] integrin expression by 75–100% leading to the anchoring of melanoma cells to endothelial cells via interaction with ICAM-1 on melanoma cells. Targeted inhibition of IL-8 secretion from melanoma cells decreased Beta-2-integrin on neutrophils by 50%, which in turn reduced neutrophil-mediated extravasation, and resulting in 50% fewer melanomas in lungs. Several studies have shown direct regulation of IL-8 expression and ^{V600E}B-Raf activity in melanomas. Therefore targeted inhibition of V600EB-Raf might be inhibiting metastasis development through reducing the IL-8 mediated melanoma cells-neutrophil interactions, further demonstrating the involvement of extracellular matrix in the melanoma metastasis formation [123, 168].

Melanoma cells actively interact with the tumor microenvironment, through molecular signals, to promote tumor formation [161]. For example, collagen, a key extracellular matrix component regulate the development of melanomas [169]. Melanoma cells containing tumor suppressor KLF6 when grown in collagen rich media failed to develop tumors [170]. However, when similar cells were grown in polyHEMA coated plates or plastic plates they grew with the proliferation rates similar to KLF6 null cells, indicating the involvement of tumor microenvironment in the tumor development. Mechanistically, KLF6 inhibited pErk1/2 as well as cyclin D1 levels thereby reduced melanoma cell proliferation in a collagen rich environment. Therefore, loss of KLF6 promotes melanoma tumor development by upregulating MAPK pathway [170].

Several studies have reported elevated COX-2 levels in human melanomas [171–173]. In addition, studies have also shown that COX-2 expression is regulated by MAPK pathway, and inhibiting ^{V600E} B-Raf in melanomas effectively reduces COX-2 expression without altering COX-1 levels [174]. Elevated COX-2 triggers cell proliferation, invasion and metastatic abilities of melanoma cells thereby promote metastasis formation in distant organs [175–177]. For example, a recent study showed that inhibition of COX-2 decreases systemic and bone metastasis of melanomas [176]. Furthermore, inhibition of COX-2 using celecoxib reduced melanoma bone metastasis incidence as well as tumor volume in mice models. Since COX-2 inhibition retarded melanoma metastasis and tumor formation, several derivatives of COX-2 have been prepared and tested for efficacy for inhibiting melanoma.

Furthermore, the concept of cancer stem cells (CSCs) has been first established for human myeloid leukemia in the 1960s [178]. Recent studies found CSCs in many other solid tumors including cancers of breast, brain and skin [179]. A study isolated morphologically heterogeneous populations of cells, as demonstrated by the coexistence of multiple genetic sub-clones, in melanomas and showed their involvement in tumor recurrence as well as drug resistance [180–182]. A recent study also showed that melanoma stem cells are also responsible for tumor initiation, development, growth as well as metastasis [183]. However, it is presently unclear which role a sufficiently characterized population of melanoma stem cells plays in cancer promotion and progression [181].

Melanoma stem cells have been isolated from about 20% of the metastatic melanomas cultured in growth medium suitable for human embryonic stem cells and their properties studied [184]. It has been observed that multipotent melanoma stem cells possess self-renewal ability and persisted after serial cloning in vitro and transplantation in vivo. In vivo studies also showed the enhanced tumorigenic potential of melanoma stem cells compared to melanoma cells, suggesting targeting this sub-population might help to eliminate melanomas more effectively. Furthermore, dedifferentiated melanoma cells have been found to be more resistant to various treatments compared to melanoma cells and formed tumors more quickly [185].

Strategies to Overcome Drug Delivery Issues Using Nanotechnology Based Therapeutic Agents

A continued barrier in the availability of effective treatment options and drugs in melanoma that target the MAPK pathway, despite our knowledge of this pathway to date, continues to be the lack of clinically effective pharmacological agents and delivery vehicles to get the drug into the melanoma cells [186]. Nanotechnology, which is capable of encapsulating one or more therapeutic agents as a single drug in order to evaluate its efficacy in clinical trials, may be part of the possible solution to this problem [186–188]. Additionally, many nanotechnologies are shown to improve circulation time, enhanced drug uptake into tumors, avoid the reticulo-endothelial system, and minimize toxicity [186]. There are currently a wide variety of nanotechnology delivery systems that have been developed for treating tumor including silicon and gold nanoshells, polymeric nanoparticles, carbon-based nanostructures, dendrimers, and liposomes [189].

Currently in various stages of development are liposomes that contain chemo-therapeutic agents, antisense-ODNs, siRNA, DNA, or radioactive particles that could target the MAPK pathway [186, 188]. For instance, liposomes loaded with siRNAs targeting ^{V600E}B-RAF and AKT3 synergistically inhibited melanoma tumor growth in mice [157, 186]. Similarly, sorafenib in combination with ceramide-containing liposomes synergistically inhibited melanoma development in animals [190]. Additionally, a Phase-I study has shown that liposomal cisplatin can enhance drug delivery up to 200 fold in tumors [191]. Another study showed the use of other nanoparticle technology, such as the unique hexadentate-poly D,L-lactic acid-co-glycolic acid polymer chemically conjugated to PD98059 (MEK1 inhibitor), which induced apoptosis in vitro, retarded tumor growth in vivo and inhibited melanoma cell proliferation [187]. Furthermore, the antitumor efficacy of cisplatins have also been enhanced by use of nanoparticles [187]. Thus, nanoparticle delivery systems provide one technology to load multiple drugs, which could be genetic or pharmacological, into a single vehicle and to target to the melanoma cells.

Another potential approach that is currently being evaluated is the use of RNAi technology to target the MAPK pathways. siRNA can specifically inhibit target genes in the MAPK pathway; however rapid degradation in animals has been a major obstacle [187, 192, 193]. Liposomes can protect RNAi from being “detected” by RNAses, and if coupled to specific antibodies or ligands can deliver the particles specifically into melanoma cells. Approximately 1,200 different classes of “lipidoids”, which are lipid-like barriers, were noted to be about 100 times more efficient at delivering small interfering RNA than the earlier reported lipid-based barriers in a recent report from the Massachusetts Institute of Technology and Alnylam Pharmaceuticals Inc. [194]. Clinical efficacy of this approach for targeting the MAP kinase pathways remains to be demonstrated [195].

Conclusion

In order to effectively treat melanomas, targeted inhibition of key mechanistic events regulating melanoma development such as cell proliferation, survival, angiogenesis and invasion or metastasis is required to prevent the tumor growth. A targeted approach, particularly targeting the MAPK pathway, will likely be a component of any therapeutic regimen for cutaneous melanomas. As this review demonstrates, targeting B-RAF or MEK may be the best approach for clinical efficacy and combining inhibition of key members of this signaling cascade and its downstream targets that regulate melanoma growth may be required to prevent the progression of this disease and development of resistance. Furthermore, understanding the molecular mechanisms that lead to the development of resistance to chemotherapeutic agents, as well as strategies to overcome resistance is needed. The use of nanotechnology might prove to be a potential avenue to overcome some of these issues by providing a single platform in which multiple genetic or pharmacological agents can be loaded to synergistically inhibit melanoma development and overcome the occurrence of resistance. The challenge remains in identifying the optimal targets in addition to discovery of drugs that have negligible toxicity-related side effects and are bioavailable.

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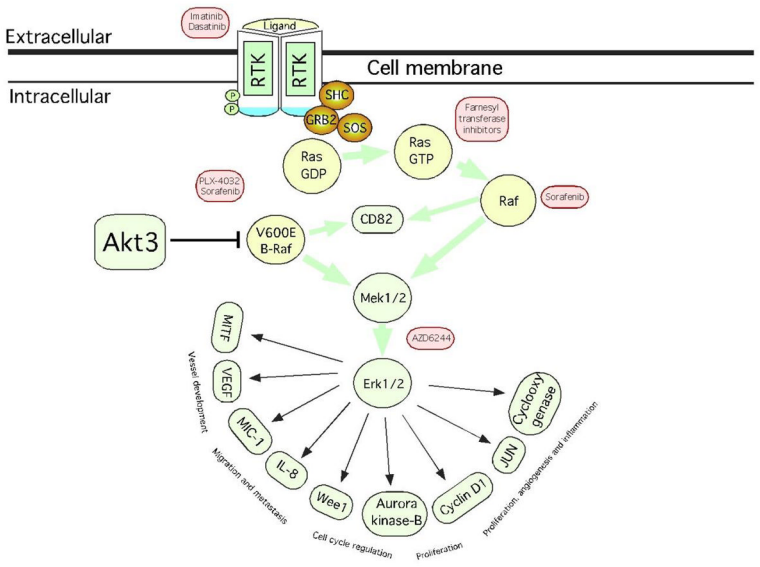

Fig. 1.

MAPK signaling cascade: diagram depicts initial ligand binding to receptor tyrosine kinases (RTK), leading to activation of RAS, then RAF, then MEK 1/2, then Erk 1/2 followed by several downstream targets. In pink are therapies directed at these targets, which are discussed in this review

Table 1

Classes of therapies and associated resistance patterns in targeted therapies most extensively studied to date

Target	Drug	Specificity of the drug	Development of Resistance	Overcoming of Resistance
Raf Inhibitors	PLX-4032 (vemurafenib)	100-fold selective to mutant ^{V600E} B-Raf over wild-type B-Raf.	Insensitivity to drug secondary to promotion of RAF dimer formation via: • Overexpression of RAF1 • RAS activation secondary to RAS mutation	Requires further study Add an inhibitor to RAS (studies have failed to find an inhibitor to RAS)
	Sorafenib	Inhibits both wild type and mutant B-Raf activities Inhibits both wild type and mutant B-Raf activities		
	XL281 GSK2118436	100-fold selective to mutant ^{V600E}B-Raf over wild-type B-Raf	Overexpression of MAP3K8 (COT)	Add an inhibitor of MAP3K8
			Overexpression of PDGFRβ	Add an inhibitor of PDGFRβ
			Hyperactivation of MEK-ERK 1/2 signaling	Add a MEK 1/2 inhibitor
MEK 1/2 inhibitors	AZD6244 R05068760	Inhibits MEK phosphorylation of ERK	Unclear at this time. Further clinical trials are needed	
Aurora Kinase Inhibitors	SNS-314 VX680 MLN8054	Inhibits phosphorylation of serine 10 of aurora kinases	Unclear at this time. Further clinical trials are needed	