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Review

Targeting the MAPK pathway in melanoma: Why some approaches succeed and other fail

Gajanan S. Inamdar [a], SubbaRao V. Madhunapantula [a], Gavin P. Robertson [a,b,c,d,e,f,*]

[a] Departments of Pharmacology, The Pennsylvania State University College of Medicine, Hershey, PA 17033, United States
[b] Departments of Pathology, The Pennsylvania State University College of Medicine, Hershey, PA 17033, United States
[c] Departments of Dermatology, The Pennsylvania State University College of Medicine, Hershey, PA 17033, United States
[d] The Foreman Foundation for Melanoma Research, The Pennsylvania State University College of Medicine, Hershey, PA 17033, United States
[e] Penn State Melanoma Therapeutics Program, The Pennsylvania State University College of Medicine, Hershey, PA 17033, United States
[f] Penn State Cancer Institute, The Pennsylvania State University College of Medicine, Hershey, PA 17033, United States

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ABSTRACT

The Mitogen Activated Protein Kinase (MAPK) pathway plays a key role in melanoma development making it an important therapeutic target. In normal cells, the tightly regulated pathway relays extracellular signals from cell membrane to nucleus via a cascade of phosphorylation events. In melanomas, dysregulation of the MAPK pathway occurs frequently due to activating mutations in the B-RAF and RAS genes or other genetic or epigenetic modifications, leading to increased signaling activity promoting cell proliferation, invasion, metastasis, migration, survival and angiogenesis. However, identification of ideal pathway member to therapeutically target for maximal clinical benefit to melanoma patients remains a challenge. This review provides an overview of the obstacles faced targeting the MAPK pathway and why certain therapeutic approaches succeed while others fail. The review summarizes the roles played by the proteins, therapeutic potential and the drugs available to target each member of the pathway as well as concerns related to each. Potential for targeting multiple points and inhibiting other pathways along with MAPK inhibition for optimal efficacy are discussed along with explanations for development of drug resistance, which includes discussions related to cross-talk between pathways, RAF kinase isoform switching and phosphatase deregulation. Finally, the use of nanotechnology is reviewed as an approach to target the MAPK pathway using both genetic and pharmacological agents simultaneously targeting multiple points in the pathway or in combination with other cascades.

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* Corresponding author at: Department of Pharmacology, Penn State Hershey Cancer Institute, R170, 500 University Drive, Hershey, PA 17033, United States. Tel.: +1 717 531 8098; fax: +1 717 531 0480.
E-mail address: gprobertson@psu.edu (G.P. Robertson).

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1. Introduction

Malignant melanoma is the deadliest form of all skin cancers [1–4]. Incidence and mortality rates for melanoma continue to rise faster than any other cancer type [1,2,5], with 1 American dying from the disease each hour [http://www.skincancer.org/skin-cancer-facts/]. Furthermore, patient survival from metastatic disease is only 15% [5,6], and the prognosis is extremely poor with long-term response rates being marginal [7]. The median survival rate with stage M1c melanoma is 6 months, while the survival time for stages M1a and M1b may be longer except for older patients. No significant difference in survival is based on gender. Though there has been significant progress in understanding the biology of melanoma, there has been no change in practices involved in either therapies or approaches to treat advanced stage disease. Patients diagnosed with early stages of melanoma (stage I and II) have shown fairly good outcome compared to those diagnosed at an advanced stage (stage III and IV), which continue to contribute to high morbidity and mortality rates [7]. Hopes for a break through at present rests heavily on the outcomes of the study on the Plexxikon drug PLX4032 for treating melanoma patients, which have mutated ^{V600E}B-RAF. Initial studies have raised expectation about the clinical efficacy of this inhibitor, however debate remains regarding the explanation for its efficacy and triggering of other cancer [8].

Currently, no FDA approved therapeutic options are available to increase survival or lead to complete tumor regression [7]. Available treatment strategies involve chemotherapies in combination with biotherapies [9,10]. Dacarbazine alone or in combination with IL-2 or IFNα and thymosin α-1 lead to progression free survival of few weeks to few months or with varied marginal response rates [9,10]. Therefore, more effective melanoma treatments are urgently needed, which will require identification of genes deregulated in key pathways; understanding the mechanisms conferring drug resistance; and, discovery of specific, more potent pharmacological agents and delivery systems for these drugs.

Dysregulation of the Mitogen Activated Protein Kinase (MAPK) pathway is common in many human cancers including melanoma, frequently due to mutations in the B-RAF and RAS genes or other genetic or epigenetic events [11–14]. In melanomas, mutation rates for B-RAF is 50–70% and NRAS is 15–30% [11–14]. KRAS and HRAS are mutated in 2 and 1% of patients, respectively [12]. Constitutive activation of the MAPK pathway regulates key processes such as cell proliferation, invasion, metastasis, survival and angiogenesis, which are involved in melanoma development [15–20]. Although the MAPK pathway is activated primarily by mutations in B-RAF and RAS, in melanomas lacking B-RAF or RAS mutations the signaling cascade is triggered by other autocrine mechanisms including C-MET over expression, which is a receptor for hepatocyte growth factor, or through down regulation of MAPK pathway inhibitory proteins such as RAF-1 inhibitory protein or SPRY-2 [12,13,21]. In tumors containing an inactive B-RAF protein, mutant RAS or C-RAF can activate the MAPK cascade thereby inducing melanoma growth and drug resistance [22–24]. This review provides an overview of the therapeutic potential of targeting the MAPK pathway, the functional role played by kinases in this signaling cascade, the clinical utility of pharmacological agents targeting the key members of this pathway, and, recent developments in therapeutic agent delivery systems, with particular emphasis on nanoliposomal encapsulation of drugs and siRNAs to target the MAP-kinase pathway.

2. Overview of the MAPK pathway and its potential therapeutic targets

The classical MAPK pathway consists of RAS, RAF, MEK and ERK, sequentially relaying proliferative signals generated at the cell surface receptors and through cytoplasmic signaling into the nucleus (Fig. 1) [13,15,25–27]. 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 [12,25]. Mechanistically, the phosphorylated SH2 domain (Src Homology 2) of the GRB2 (Growth factor receptor-bound protein 2) adaptor protein brings SOS into close proximity with inactive, membrane localized GDP-bound RAS and converts it into an active GTP-bound RAS. Activated RAS then triggers the formation of the "MAPK complex" with downstream RAF, MEK1/2, ERK1/2 and several scaffolding proteins to initiate the MAPK cascade, and potentiates PI3K-AKT signaling [12,13,28]. The activated RAF proteins trigger dissociation of ERK1/2 from the MAPK complex by phosphorylation, which regulates the expression of several genes involved in cell proliferation, differentiation and survival by phosphorylating nuclear transcription factors such as ETS, ELK-1, MYC or indirectly by targeting intracellular signaling molecules such as p90-RSK [13,27,29]. The 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 [26,29].

2.1. Targeting RAS to inhibit melanoma

The RAS family of small G-proteins consists K-RAS, H-RAS, and N-RAS, which trigger MAPK signaling by activating downstream proteins such as RAF and PI3K [12,13,28]. H-RAS and K-RAS genes were identified as human homologues of the viral proto-oncogenes in the Harvey and Kirsten Rat Sarcoma viruses, respectively [30,31]. RAS proteins function as molecular switches to control cell proliferation and survival [15,25,32]. In human tumors, RAS is activated by mutation, loss of the RAS-GAP NF-1 or by upstream activation of cell surface receptors [12,13,30]. Oncogenic mutations in RAS family members have been reported in 1/3 of all human cancers [12,13,30]. In melanomas, substitution of leucine for glutamine at residue 61 is the most common aberration observed in N-RAS [30,33]. Mutant RAS lacks GTPase activity and

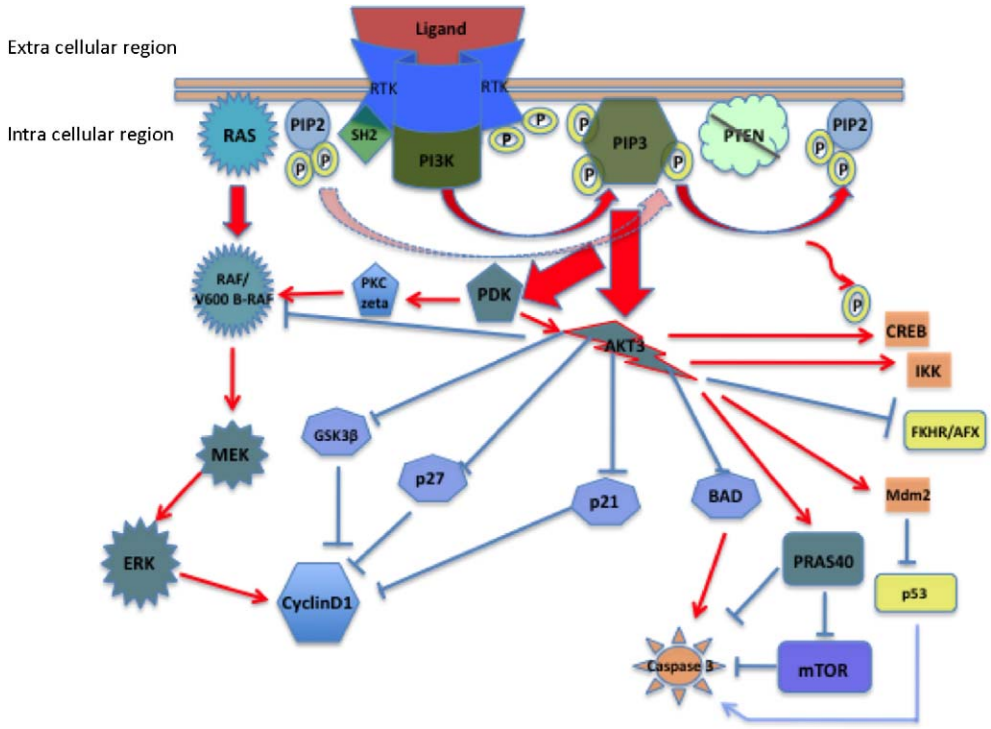

Fig. 1. The MAP and PI3 kinase pathways in melanoma. Cell surface receptor tyrosine kinases are activated by mitogens, which in turn activate phosphoinositide 3-kinase (PI3K) (effectively controlled by the PTEN tumor suppressor gene). PI3K phosphorylates AKT3 thus activating many oncogenic pathways such as CREB, IKK, Cyclin D1 and PRAS40. Activation of receptor tyrosine kinases simultaneously stimulates RAS/RAF/MEK/ERK which can also activate Cyclin D1 and NFkB. All these cascades essentially promote proliferation, cell survival, angiogenesis, and metastasis.

remains active leading to uncontrolled cell proliferation and a transformed phenotype [30]. In melanomas, introduction of activated RAS into melanocytes can lead to melanomas tumor formation in mice [34,35]. Furthermore, expression of RAS can suppress the tumor-suppressors $p16^{INK4A}$, p53, and $p14^{ARF}$ [36,37] and knockdown of H-RAS expression using siRNAs can lead to melanoma regression in an inducible melanoma tumor model [38]. Therefore, RAS is a potentially important target in melanomas.

2.2. Is therapeutically targeting RAS in melanoma working?

Efforts to pharmacologically inhibit RAS or its regulatory components for cancer therapy have so far met with minimal success. Since the activation of RAS requires farnesylation of the carboxy-terminal cysteine residues by farnesyl transferase (FT), it has been thought that targeting FT using farnesyl transferase inhibitors (FTI) or farnesyl cysteine mimetics such as farnesyl thiosalicylic acid (FTS) derivatives might effectively prevent the growth of melanomas [39,40]. However, these agents failed in clinical trials due to non-specific responses, since FTs farnesylate many proteins other than RAS; other mechanisms by which RAS proteins become activated thereby developing resistance to the inhibitors; and, the presence of other active oncogenes and proteins [39]. For example, N-RAS has been shown to be geranylated by geranyl-geranyl transferase (GGT) [41]. Targeting FTs and GGT together, to completely inhibit all forms of RAS activation proved to be toxic as they inhibit the activation of a large number of other protein along with RAS. In a Phase-II study with 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 despite being an effective FT inhibitor (Fig. 2) [41–44].

Another potent FT inhibitor, SCH66336, has been shown to induce G1-phase cell cycle arrest and retinoblastoma protein inactivation to kill melanoma cells [40]. Furthermore, the combination of farnesyl thiosalicylic acid and SCH66336 markedly enhanced cisplatin-induced apoptosis indicating the chemosensitizing activity of FTIs [40,45]. Another farnesyl transferase inhibitor called lonafarnib alone or in combination with chemotherapeutic agents (temozolomide/cisplatin, or MAPK inhibitors sorafenib/U0126/PD98059, or AKT inhibitors LY294002/wortmannin/rapamycin) was tested as regulators of invasion of melanoma cells, proliferation and survival. Lonafarnib was neither able to inhibit the growth of metastatic melanoma cells nor sensitize them to the chemotherapeutic agents tested [46]. In contrast, lonafarnib significantly augmented the growth inhibitory effects of the multikinase inhibitor sorafenib in eight different cultured metastatic melanoma cell lines tested [46]. Moreover, lonafarnib combined with sorafenib was able to trigger apoptosis and abrogate the invasive potential of melanoma cells [46].

In addition to FTIs, pharmacological agents directly targeting RAS have also been developed and evaluated in preclinical studies as well as in clinical trials to inhibit melanoma. BMS-214662 and L-778123, potent non-peptide inhibitors of H-RAS and K-RAS respectively, were tested against melanoma [47–51]. In a phase I study, patients with solid tumors receiving oral BMS-214662 (given once or twice daily for 2 weeks in a 3-week cycle), experienced dose-limiting toxicity manifested as nausea, diarrhea, vomiting, abdominal cramping, anorexia, fatigue and fever. Of the 23 patients treated, all but 1 had disease progression [52]. Although, the pharmacokinetics of the agent suggested favorable oral bioavailability, the oral form was later abandoned due to gastrointestinal intolerance [52]. In another phase I trial, BMS-214662 was initially administered over a 1 h period weekly in 30 patients. A minor response was reported in one patient with chemotherapy-refractory breast cancer [53]. L-778123 has also been evaluated clinically through 5-day continuous infusion of the agent alone or in combination with radiation and paclitaxel for treatment of NSCLC as well as head and neck carcinomas [54–56]. Despite a good clinical response, studies were discontinued due to

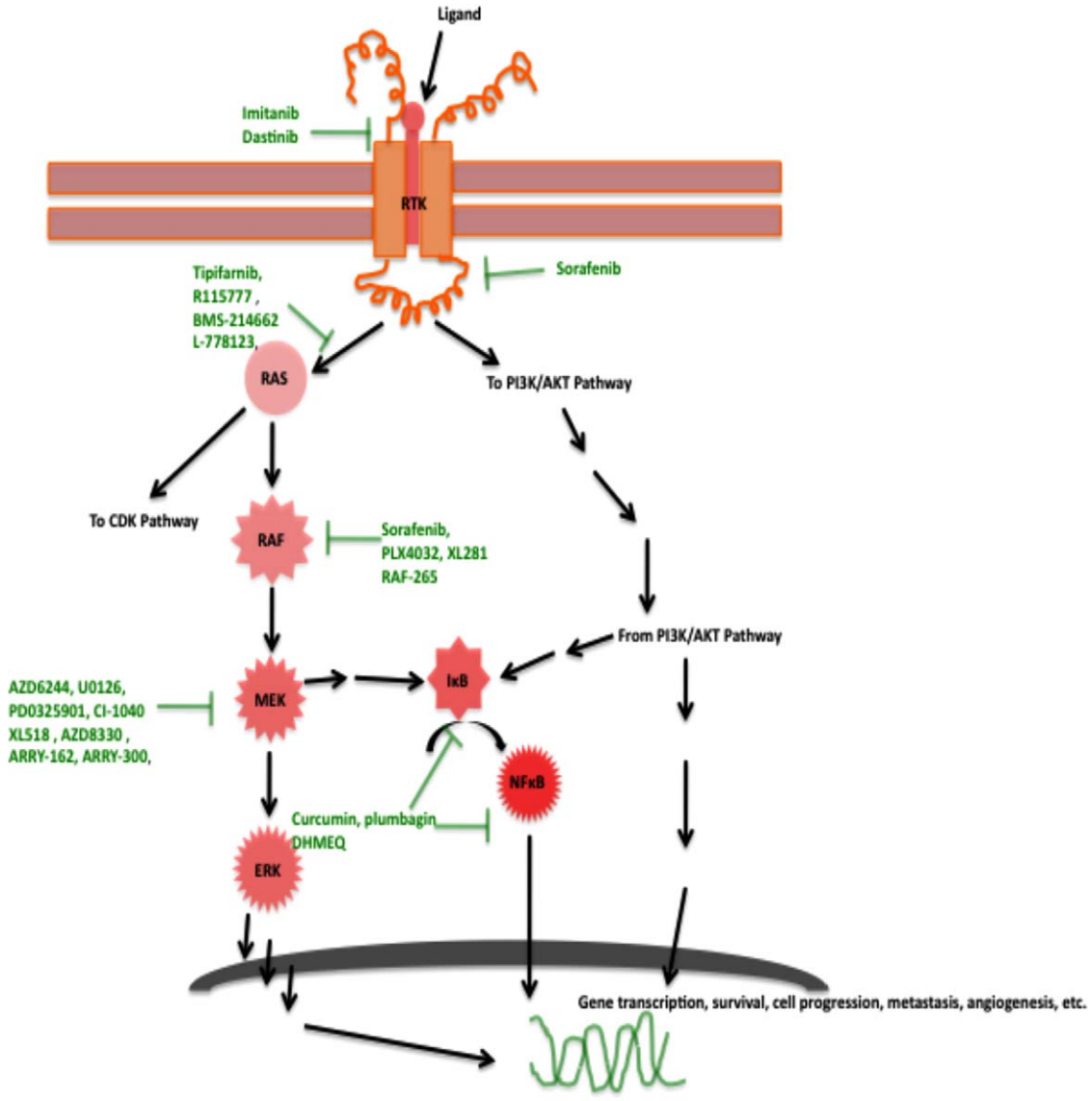

Fig. 2. Inhibitors of the MAPK pathway. Schematic representation of key targets and pharmacological agents that inhibit MAPK signaling proteins. Imitanib, dastinib and sorafenib inhibit receptor tyrosine kinases thereby preventing activation of mitogen dependent activation in the MAPK pathway. Tipifarnib, R115777, BMS-214662 and L-778123 are selective to RAS while PLX4032, RAF-265 and XL281 specifically target activated B-RAF and are at various stages of clinical development. AZD6244, U0126, PD0325901, CI-1040, XL518, AZD8330, ARRY-162 and ARRY-300 inhibit MEK 1/2 while curcumin, plumbagin and DHMEQ target IKKβ and NFκB.

evidence of cardiac related concerns, manifested as a prolongation of the QTc interval [54–57]. Unfortunately, both compounds were ineffective in melanoma, as the majority of harbor N-RAS and not H-RAS or K-RAS mutations. RAS inhibitors in combination with radiation therapy or cytotoxic drugs have also been tested in preclinical studies as well as in clinical trials and found to be ineffective [45]. Thus, therapeutically targeting RAS in melanoma is relatively ineffective suggesting that other points in the MAPK pathway might be more promising targets.

2.3. Targeting B-RAF to inhibit melanoma

B-RAF is one of three members of the RAF family, which includes A-RAF, B-RAF, and C-RAF (or RAF-1), and is a downstream effector of RAS [12,58]. All three mammalian RAF isoforms while sharing three conserved regions (CR1, CR2, CR3) also exhibit considerable differences in variable sequences (Fig. 3). The CR1 (131 amino acids length) contains a RAS binding domain and a cysteine-rich domain [13,59]. The CR2 (16 amino acids length) domain contains serine and threonine residues, playing a role in regulating the activity of B-RAF upon phosphorylation. The CR3 (293 amino acids length) contains the kinase domain and key phosphorylation sites that regulate enzymatic activity (Fig. 3) [13]. The activation of normal non-mutated RAF proteins is a complex process, which 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 [13,60,61].

B-RAF is the most mutated gene in the MAPK signaling cascade in melanomas, with >60% of advanced tumors expressing constitutively active mutant protein [11,12,20]. Activating B-RAF mutations are acquired, somatic, post-zygotic events and are not inherited in families [20,27]. While over 65 different mutations occur in more than 30 B-RAF codons, a single-base missense T to A substitution (at nucleotide 1799), which changes valine to glutamic acid at codon 600 (V600E) in exon 15 is prevalent in 90% of melanoma tumors [11,12,27,62]. Mutated ^{V600E}B-RAF is

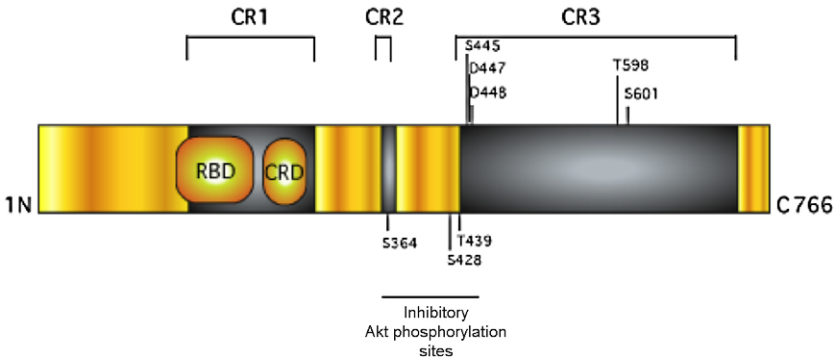

Fig. 3. Schematic depiction of B-RAF showing the various domains of the protein. B-RAF is one of the three RAF kinase isoforms. Structurally, B-RAF contains three highly conserved regions called, CR1, CR2 and CR3. B-RAF activity is regulated primarily by phosphorylation of key serine and threonine residues. Whereas phosphorylation of threonine 598 and serine 601 at the C-terminal kinase domain triggers kinase activity, the phosphorylation of serine 364, serine 428 and threonine 439 by AKT3 inhibits the enzymatic activity.

10.7-fold more active than wild type protein and does not require RAS-mediated membrane translocation to exhibit enzymatic activity [11]. Activation occurs as a result of a conformational change in protein structure, where glutamic acid acts as a phosphomimetic between the Thr^{598} and Ser^{601} phosphorylation sites [12,58,63]. Mutant ^{V600E}B-RAF protein circumvents the requirement of RAS-GTP binding, N-region charge and binding of 14-3-3 to S729 for exhibiting its activity [13,64]. It also confers resistance to negative feed back regulation by Sprouty proteins and the S579A mutation of B-RAF [13]. Thus, B-RAF is an important therapeutic target in melanomas.

2.4. Multiple roles of ^{V600E}B-RAF in melanoma

As a proliferation inducer, ^{V600E}B-RAF leads to hyperactivation of the MAPK pathway, which in turn triggers cell division and survival pathways to promote tumor development [13,63,65,66]. However, only moderate levels of MAPK pathway activation are required for transformation and immortalization of mouse melanocytes, increased in vitro colony formation, and elevation of ERK1/2 activities [25,27,65,67]. ^{V600E}B-RAF also induces formation of new blood vessels by promoting secretion of vascular endothelial growth factors and macrophage inhibitory cytokine-1 [17,19]. Recent studies have shown that ^{V600E}B-RAF regulates expression of IL-8, a pro-inflammatory chemokine and autocrine factor, to promote tumor growth and angiogenesis [68]. ^{V600E}B-RAF also controls metastasis development by triggering invasive cellular behavior as well as by promoting IL-8 mediated anchoring of melanoma cells to the vascular endothelium to aid extravasation and development of lung metastases [18,68,153].

^{V600E}B-RAF can also induce senescence by activating the MAPK pathway to levels that inhibit cellular growth in a wide variety of normal and early melanocytic lesions cells [69–71]. Mutant ^{V600E}B-RAF has been shown to initially stimulate melanocyte proliferation, indicating that it contributes to melanogenesis and development of nevi [62,63,69]. This is followed by subsequent growth inhibition associated with senescence, indicated by proliferative arrest due to increases in $p16^{INK4A}$ and β-Gal activity [62,63,69]. Senescence induction is due to increased cyclin-dependent kinase inhibitors, such as $p21^{Cip1}$, $p16^{INK4A}$, and $p27^{Kip1}$, acting as a putative defense mechanism of normal cells to overcome oncogene activation [70–72]. A recent study has also shown that senescence and apoptosis induction triggered by ^{V600E}B-RAF can be mediated by insulin growth factor binding protein-7 secretion in transformed melanocytes [73].

^{V600E}B-RAF can promote nevi development but the resulting high, intense activation of MAPK pathway triggers senescence thereby inhibiting further tumor progression [27,69,70]. Therefore, additional genetic change such as loss of $p16^{INK4A}$, PTEN or elevation in AKT3 activity through overexpression is required for the quiescent melanocytic cells to overcome the ^{V600E}B-RAF induced senescence in order to reenter the cell cycle [69,74,75]. In one study, zebrafish expressing ^{V600E}B-RAF protein were shown to develop fish-nevi only and when expressed in p53-deficient zebrafish did the melanocytic lesions develop that rapidly progressed into invasive melanomas, resembling those occurring in human tumors [76]. This result provided direct evidence linking functionally interaction between the p53 and ^{V600E}B-RAF pathways and melanoma development [77]. ^{V600E}B-RAF has also been shown to occur with $p16^{INK4A}$ loss in ~60% of melanomas [74]. Furthermore, siRNA targeting B-RAF and expression of INK4A were found to more effectively inhibit melanoma development by up regulating BIM and down-regulating BCL2 proteins [74]. However, a recent study using patients who underwent isolated limb infusion with cytotoxic drugs melphalan and actinomycin-D for metastatic melanoma showed that $p16^{INK4A}$ expression and absence of activated B-RAF are independent predictors of chemosensitivity in melanoma tumors [78].

Recently, AKT3 has been shown to phosphorylate ^{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 by releasing cells from ^{V600E}B-RAF-mediated senescence [69]. Genetically altered mice harboring conditional melanocytes expressing ^{V600E}B-RAF, developed benign melanocytic hyperplasia but failed to develop melanoma. Only following PTEN loss did melanoma develop, which metastasized to lymph nodes and lungs [75]. Use of rapamycin or PD325901 prevented melanoma development, which did form upon cessation of the treatment. A combination therapy using both agents led to shrinkage of established melanomas in this model. These results strongly indicates that PTEN loss or AKT3 activation together with ^{V600E}B-RAF are key to melanoma development [75].

Concurrent mutation of B-RAF and loss or reduced PTEN expression have been reported to occur in 20% patient tumors, with altered MAP and PI3 kinase pathway activity [79]. Occurrence of B-RAF mutation is likely an early event, with the alteration of the PTEN/AKT pathway occurring later in tumor progression [79]. Therefore, it is very likely that a successful targeted therapy would need to simultaneously target both pathways.

2.5. Is therapeutically targeting B-RAF in melanoma working?

^{V600E}B-RAF plays an important role in MAPK pathway activation and is therefore a key target in this signaling cascade. Therapies

targeting ^{V600E}B-RAF have significant potential to halt the progression of malignant tumors by inhibiting growth, preventing angiogenesis, decreasing invasion and metastasis, inducing tumor cell death, or promoting tumor differentiation [12]. Evidence from preclinical studies has shown that ^{V600E}B-RAF has significant potential to be an important target to treat melanoma [21,27,80]. Proof-of-principle studies using siRNA to inhibit expression of wild type or ^{V600E}B-RAF delayed tumor development and reduced metastasis formation in mice [17,18,63,65]. Pharmacological agents inhibiting ^{V600E}B-RAF activity also retarded melanoma tumor development in mice [81,82]. Oral or intraperitoneal administration of sorafenib (BAY 43-9006) reduced the growth of subcutaneous melanoma tumors by inhibiting cell proliferation and vascular development [17,83]. Administration of 50 mg/kg sorafenib retarded tumor growth by ~55%; however, complete regression was not achieved. Sorafenib was more effective than siRNA at blocking B-RAF signaling in melanoma cells suggesting that the effect might be due to the inhibition of other kinases (FGFR1, c-Kit, p38 MAPK) or angiogenic factors (VEGFR1, VEGFR2, VEGFR3, and PDGF), rather than solely due to inhibition of ^{V600E}B-RAF [27,83–85]. Several independent groups have come to same conclusion regarding sorafenib [83,86].

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 ~11 weeks [87]. 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 [87]. Concerns regarding the failure of sorafenib in the clinic has led to first, the development of more effective as well as specific inhibitors targeting ^{V600E}B-RAF. Second, undertaking of preclinical studies evaluating whether targeting ^{V600E}B-RAF alone would be sufficient or whether other members of the MAPK pathway need to be targeted in combination for effective melanoma inhibition. Third, siRNA-mediated targeting of ^{V600E}B-RAF, MEK1/2, ERK1/2, or cyclin-D1 to determine which member of the MAPK pathway to target to most effectively inhibit melanoma development, which showed MEK1/2 inhibition most effective at reducing melanoma lung metastases development [18]. Fourth, the discovery that melanomas containing mutated B-RAF are more responsive to agents targeting MEK in the MAPK pathway than tumors with wild-type B-RAF or harboring a RAS mutation [88]. Fifth, combining sorafenib with other agents to improve clinical efficacy.

Studies combining sorafenib with carboplatin and paclitaxel showed fair clinical efficacy (complete response in one and partial response in 9 of 39 patients enrolled) with an overall response of 26% and myelosuppressive toxicities likely due to combining carboplatin and paclitaxel [27,89,90]. A Phase-II clinical trial evaluating the efficacy of the alkylating agent dacarbazine or temozolomide in combination with sorafenib in advanced-stage melanoma patients showed a statistically significant median progression free survival of 21.1 weeks when combining sorafenib with dacarbazine versus 11.7 weeks for placebo plus dacarbazine. Unfortunately, no improvement in overall survival was achieved using this combination [91]. A phase III trial combining sorafenib with carboplatin and paclitaxel as a second line treatment in patients with unresectable stage III or stage IV melanomas was less promising with a 12% response rate and 17.9 months progression-free survival with placebo plus carboplatin versus 17.4 months progression-free survival with a combination of sorafenib plus carboplatin [89]. Thus, clinical studies using sorafenib lead to the conclusion that targeting B-RAF might be more effective in combination with other chemotherapeutics rather than targeting it alone.

Several new compounds have been developed for targeting B-RAF that have improved pharmacological properties compared to sorafenib, which are being evaluated in clinical trials. These include RAF-265 (Novartis Pharmaceuticals) and PLX4032 (Plexxikon Pharmaceuticals) (Figs. 2 and 4) [92,93]. RAF-265 is a broad-spectrum inhibitor of VEGF receptor 2 and the MAP kinase pathway. It inhibits proliferation of melanoma cell lines harboring B-RAF mutations and to a lesser extent N-RAS mutation, with essentially no activity against cells lacking these mutations. RAF-265 completely inhibits ERK phosphorylation and is capable of causing regression of melanomas containing mutant B-RAF in animal models [93,94].

PLX4032 is a bio-available RAF kinase inhibitor having ten-fold greater activity against ^{V600E}B-RAF compared to wild-type protein [92]. PLX4032 is claimed to have fewer off target effects than sorafenib; however, this remains an area of some controversy. PLX4032 inhibited ERK phosphorylation and proliferation of cancer cell lines that harbor B-RAF mutations but not those cells containing wild type protein [92]. Similarly, PLX4032 inhibited development of xenografted melanoma tumors containing mutant B-RAF with evidence of tumor regression and prolonged delay of tumor growth after ending drug dosing [92].

The clinical efficacy of PLX4032 has been evaluated in a Phase I trial involving 16 melanoma patients harboring ^{V600E}B-RAF by administering the drug twice daily at or above 240 mg [8]. Result showed that PLX4032 was well tolerated even at very high doses (with 960 mg twice a day under evaluation as the maximum tolerated dose). In a phase I extension trial, which included only mutation-positive patients, 15 of 31 had tumor regression of more than 50% and 18 patients partially responded showing greater than 30% tumor regression [8]. Additionally, minor responses were observed in 6 patients showing tumor regression >10% but <30% with disease control lasting up to 14 months with continuous therapy [8]. Preliminary median progression-free survival of at least 6 months has been reported, with many responding patients still receiving treatment. Based on these encouraging Phase-I data, Plexxikon has completed a Phase-II clinical trial with 100 patients, which began in 2009 September and from January 2010, has begun evaluating the compound in a randomized Phase-III trial with 700 patients.

The most frequently observed side effects of PLX4032 have been rashes, fatigue, photosensitivity and joint pains, which have been reported at 1120 mg administered twice a day but these were found to be mild and transient [8]. Analysis of the Phase-I trials results showed development of squamous cell carcinomas or keratoacanthomas in 23% of patients, which might be a serious side-effect of the drug [8]. A recent study has also showed that PLX4032 activates ERK, and enhances cell proliferation as well as migration in melanoma cells containing wild type B-RAF [95]. Although PLX4032 is claimed to be a selective ^{V600E}B-RAF inhibitor, it remains controversial as to whether its clinical efficacy is because of its selective inhibition of ^{V600E}B-RAF or whether it is due to inhibition of targets other than ^{V600E}B-RAF [92]. PLX-4032 might be inducing non-melanoma skin cancer through activation of ERK in normal cells [96].

Concern regarding PLX4032 is further complicated by reports stating that C-RAF suppresses ^{V600E}B-RAF through the formation of ^{V600E}B-RAF and C-RAF dimers, which impairs the activation of MEK/ERK. C-RAF mediated inhibition might be due to restriction of ^{V600E}B-RAF from entering a dynamic state due to physically interacting with C-RAF, which does not occur with A-RAF or wild type B-RAF [97]. Prior reports had shown that C-RAF increases B-RAF activity and MEK phosphorylation in fibroblasts, suggesting C-RAF has potential to negatively modulate MAPK signaling under certain conditions. C-RAF expression is reduced relative to B- RAF in early stage human melanoma cells expressing ^{V600E}B-RAF. In

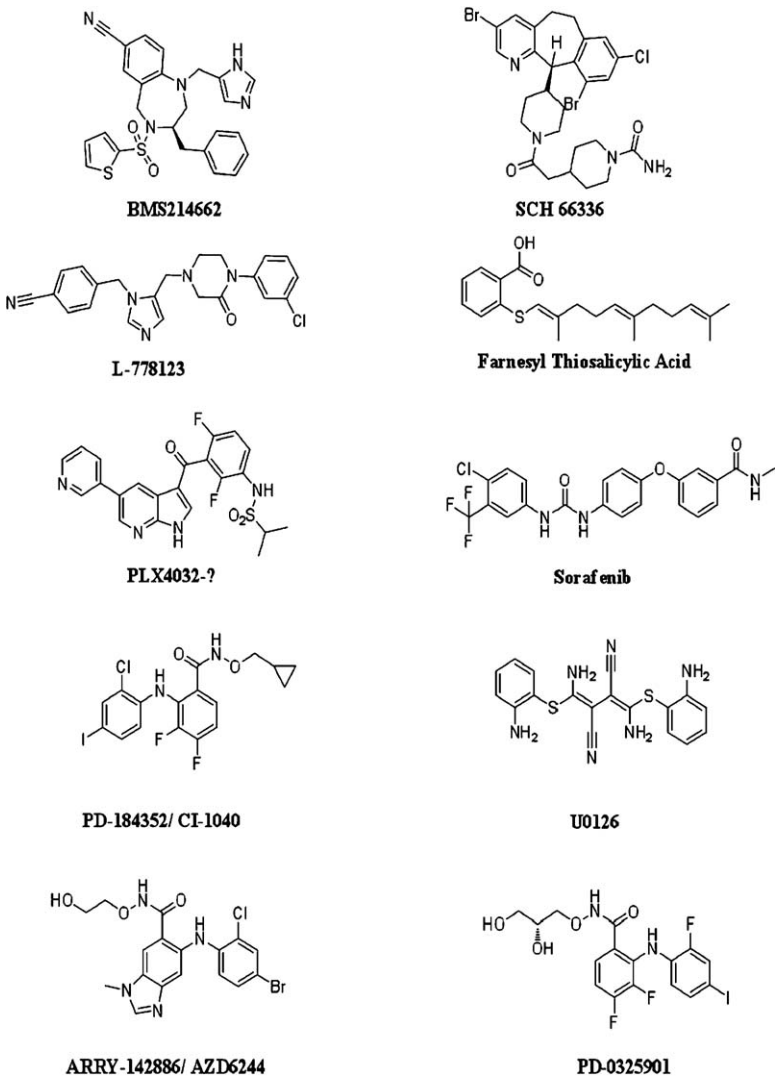

Fig. 4. Structures of selected drugs used for targeting MAPK pathway in melanoma. BMS214662, SCH 66336, L-778123 and farnesyl thiosalicylic acid inhibit Ras activity; Sorafenib (RAF kinase inhibitor) and PLX4032 (a selective ^{V600E}B-RAF inhibitor); MEK inhibitors U0126, CI-1040 (the first MEK inhibitor in clinical trials); ARRY-142886 (AZD6244) and PD-0325901 (highly selective MEK inhibitor) are shown.

contrast, metastatic cell lines have increased B-RAF protein levels and therefore a reduced C- RAF:B-RAF ratio, which could alleviate suppression of ^{V600E}B-RAF [97]. Therefore this important experimental observation would raise some important concerns regarding the clinical safety of PLX4032, where it might lead to high and uncontrolled expression of MEK1/2 and ERK1/2 in N-RAS mutated melanoma and even normal cells, by provoking them to acquire cancerous properties leading to other cancers.

In ^{V600E}B-RAF tumors, RAS is not activated, thus trans-activation is minimal and ERK signaling is inhibited in cells exposed to RAF inhibitors. RAF inhibitors such as PLX-4032 can be effective in tumors in which B-RAF is mutated, because it does not inhibit ERK signaling in normal cells. Therefore PLX4032 has a higher therapeutic index and greater anti-tumor activity than MEK inhibitors, which are suspected of causing toxicity due to MEK/ERK activation in normal cells [98].

In K-RAS mutant and RAS/RAF wild-type tumors, RAF inhibitors are known to activate the MAP kinase pathway in a RAS-dependent manner, leading to enhanced tumor growth in some xenograft models [96]. Inhibitor binding activates wild-type RAF isoforms by inducing dimerization, membrane localization and interaction with RAS-GTP. These events occur independently of kinase inhibition and are linked to direct conformational effects of inhibitors on the RAF kinase domain [96].

XL281 (Exelixis Inc., South San Francisco, CA) is another potent and specific inhibitor of all three RAF kinases (Figs. 2 and 4) [99]. Whereas genotyping and patients selection is required prior to the treatment with PLX4032, XL281 does not require patient selection. A recent Phase-I clinical trial tested the efficacy of XL281 in seven colorectal, five thyroid cancer and five melanoma patients [99]. The results were disappointing as this drug induced squamous cell carcinomas and caused systemic toxicity [http://www.exelixis.-com/pipeline_xl281.shtml].

Although progress has been made in the development of drugs that target RAF, the clinical outcome regarding long-term usage, mechanism of action, specificity, therapeutic efficacy and drug-related toxicity needs further evaluation. Furthermore, while developing RAF inhibitors, it is also important to consider the results of recent studies, which shows that B-RAF inhibition might promote tumor development in cells that harbor RAS mutations. RAF inhibitors might activate the MAPK signaling cascade and promote growth in tumors harboring mutant K-RAS as well as wild type RAS. A recent study has shown that inhibiting ^{V600E}B-RAF to retard melanoma development might induce development of

metastatic melanoma from early stage lesions; hence requiring combinatorial approaches to treat this disease [100]. In this study ^{V600E}B-RAF was shown to activate the neuronal differentiation marker microtubule associated protein (MAP) in melanoma cells by triggering promoter demethylation as well as by down-regulating the transcription repressor HES1 [100]. Ectopic expression of MAP2, a key indicator of tumor progression, inhibited cell cycle progression, caused mitotic spindle defects, which culminated in growth inhibition and apoptosis.

2.6. Targeting MEK to inhibit melanoma

MEK-1 and MEK-2 are dual-specificity tyrosine/threonine protein kinases found to be active in ~30% of all human cancers with activated MAPK signaling [12]. These proteins lie downstream of B-RAF and share ~80% structural homology [12]. ERK is the only known substrate of MEK-1 and MEK-2 kinases [12]. Therefore, MEK-1/2 continue to be popular therapeutic targets in the MAPK signaling cascade [8]. Several studies have demonstrated that targeting these proteins, using siRNA or pharmacological agents is highly specific to the MAPK pathway, and depends on RAS mutational status [101–103]. Tumors that harbor ^{V600E}B-RAF are sensitive to MEK inhibition but not those that harbor mutant RAS [88]. Therefore, B-RAF mutational status is a critical factor needing consideration when selecting MEK inhibitors for melanoma therapy [88]. However, 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 [104]. 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 [104]. Not only did these cells respond to AZD6244 but were sensitive to MEK inhibition by CI-1040 [104]. Furthermore, a recent study has showed that co-targeting mutant B-RAF and MEK1/2 might be more effective than inhibiting either of the proteins alone [105]. Thus MEK is a promising target in melanomas.

2.7. Is therapeutically targeting MEK in melanoma working?

MEK inhibitors CI-1040, PD0325901 and AZD6244 have been developed and tested in preclinical animal models as well as in melanoma patients (Fig. 4) [99,101,103]. These inhibitors have been shown to decrease MEK activity at low nanomolar concentrations with high selectivity and inhibited tumor development in animal models. Although CI-1040 appeared promising in Phase-I trials, the clinical development of this drug has been abandoned due to poor bioavailability and drug metabolism, which required administration of very high doses at frequent intervals [101]. PD0325901 is a second-generation MEK inhibitor with significantly improved pharmaceutical properties (Figs. 2 and 4) [99,106]. PD0325901 is 50-fold more potent against MEK and has improved bioavailability and plasma stability, resulting in longer inhibition of MEK, compared to CI-1040 [99,106]. Even though it is bioavailable and metabolically stable, toxicity was more severe than CI-1040 in Phase-I clinical trials, which has halted further clinical development [107]. Similarly, AZD6244, an analog of PD0325901, produced encouraging results in Phase-I trials but no significant differences were observed when compared to temozolomide from a Phase-II trial [108–112].

Other MEK1/2 inhibitors that are in clinical trials include ARRY-162, ARRY-300, AZD6244 (ARRY-886) and AZD8330 (ARRY-704). ARRY-162 is a novel, non-ATP-competitive, potent and selective orally bioavailable, MEK 1/2 inhibitor that has the potential to treat a range of malignant diseases [http://www.arraybiopharma.com/ProductPipeline/Cancer/MEK.asp]. XL518 (Exelixis Inc., South San Francisco, CA) is yet another selective inhibitor of MEK kinases. Preclinical data using XL518 showed anti-tumor activity in melanoma xenograft studies but no clinical data is available yet [99].

Anti-metastatic and anti-tumorigenic efficacy of U0126, another MEK inhibitor, has been tested in vitro and in vivo using human melanoma cell lines [18,113]. In cultured cells, U0126 treatment reduced invasion more effectively than PD98059 [113]. Mechanistically, U0126 inhibited phosphorylation of MEK1/2, decreased urokinase plasminogen activator, matrix metalloproteinases-9 and c-Jun [113]. Furthermore, intraperitoneal administration of U0126 reduced lung metastasis development in lung metastasis models [18]. However, due to poor bioavailability and lack of therapeutic efficacy in early clinical trials this compound has not been evaluated for clinical applications. Thus, MEK 1/2 remain promising therapeutic targets in the MAP-kinase pathway. However, better agents or more specific delivery systems that would decrease toxicity related issues are needed.

2.8. Targeting ERK to inhibit melanoma

Immunohistochemical studies using antibodies recognizing ERK1/2 and phosphorylated ERK (pERK) have been undertaken on 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). ERK1/2 was found expressed to varying degrees in all cases, ranging from 40% to 100% and was observed in both the cytoplasm and nucleus. Only low levels of ERK1/2 were detected in melanocytes present in normal skin [114]. Patients with metastatic melanoma showed higher levels of pERK in subcutaneous metastases compared to lymph node metastases or compound nevi. pERK was not detected in melanocytes present in normal skin. N-RAS and B-RAF mutations are more frequent in cutaneous or soft tissue melanoma metastases, which could partially account for the differences in pERK levels in subcutaneous metastases and lymph node metastases. There was a non-significant relationship between the depths of melanoma to pERK expression [114]. Higher percentage of pERK-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 [114].

Currently, MEK1/2 inhibitors are employed as inhibitors of its downstream effector ERK1/2, as MEK1/2 is known to activate ERK1/2 selectively [115]. Elevated ERK activity is frequently observed in proliferating metastatic melanoma cell lines as well as in human tumors and is a good indicator of tumor progression [116,117]. Sustained activation of ERK in melanoma cells has been shown to confer resistance to various therapeutic agents. Growth factors can activate ERK in melanomas 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) [15]. In melanocytes, ERK activity can also be stimulated by mitogens such as bFGF and endothelin-1 [118]. However, the degree of contribution of each pathway to the overall stimulation of ERK in melanomas remains to be determined. Activated ERK regulates expression of MITF splice variants, MITF 6a/b, and thereby controls melanoma cell survival, differentiation, proliferation and migration [119]. 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 p16^{INK4A} expression [27,69,72,74]. Thus, ERK has potential to be a significant target in melanomas.

2.9. Is targeting ERK working in melanoma?

Studies using experimental metastasis models have shown that targeting ERK1/2 using siRNAs effectively reduced lung metastasis development and sensitized tumor cells to chemotherapeutic agents such as cisplatin [18,117]. Similarly, low pERK1/2 levels were reported in patients treated with B-RAF inhibitors sorafenib and PLX4032 indicating pERK1/2 is a reasonable biomarker for tumor progression and evaluation of the efficacy of therapeutic agents [8,120]. In contrast to this widely accepted belief in pERK1/2 being a good biomarker, recent reports have shown that pERK level is in fact a poor indicator of B-RAF or N-RAS mutation status, and not a good marker of reduced growth when compared to Ki67 levels [121,122]. Furthermore, a recent study has shown that inhibition of ERK by RAF kinase inhibitors depends on B-RAF mutational status [95]. Therefore, it is important to know the mutational status of B-RAF before assessing the efficacy of pharmacological agents and tumor progression. Currently, to target ERK, either B-RAF or MEK1/2 are inhibited that effectively decreases phosphorylated ERK1/2 levels, but tumors containing wild type RAF showed an increase in pERK level upon treatment with PLX4032 [95]. In these cases, targeting an inhibitor of ERK such as SPRY2 might be a better option for those melanomas containing wild type B-RAF [123]. SiRNA mediated knockdown of SPRY2 in melanocytes increased ERK in melanoma cells containing wild type B-RAF, but not in those containing ^{V600E}B-RAF. SPRY2 and SPRY4 directly bind wild type B-RAF but not mutant B-RAF suggesting that loss of SPRY might enhance levels of active ERK, enabling growth of melanoma cells containing wild type B-RAF [123].

Treatment of melanoma cells with the B-RAF inhibitor AZ628 led to development of clones having high pERK levels, which occurred due to activation of C-RAF leading to continued proliferation in the presence of drug. Hence, combining B-RAF with MEK1/2 and/or C-RAF inhibitors might be the most effective approach to target ERK [124]. Some melanomas with ^{V600E}B-RAF mutations may be intrinsically resistant to inhibitors of B-RAF as a result of cyclin D1 amplification [125]. Thus, there remains a need to develop small molecules inhibitors specific for ERK1/2.

3. Targeting other pathways in combination with MAPK pathway inhibition

Although ^{V600E}B-RAF is key to melanoma development, pharmacological agents inhibiting members of the MAP-kinase signaling cascade either lack therapeutic efficacy or cells rapidly develop resistance to them. Sorafenib, U0126, or PD98059 are ineffective as single agents for treating patients with advanced melanoma [12,90,107,126]. Therefore, it is reasonable to hypothesize that multiple signaling proteins might need to be targeted for better melanoma inhibition [16]. This possibility is supported in studies using siRNA, inhibiting AKT3 and ^{V600E}B-RAF, which showed that simultaneously targeting both proteins synergistically inhibited melanoma tumor cell growth in culture and in xenografted tumors [69,127]. Similarly, combining nanoliposomes containing ceramide (a lipid based AKT inhibitor) with sorafenib (a RAF kinase inhibitor); co-treating with MEK inhibitors U0126, PD98059 and PD325901 or mTORC1 (using rapamycin) more effectively reduced melanoma cells growth compared to any of these individual agents [128–131]. In a separate study, topical application of LY-294002 (a PI3K inhibitor) and U0126 (a MEK inhibitor) in combination more effectively reduced melanoma tumor incidence and delayed tumor development [131,132]. Additionally, PI3K and MEK inhibitors are effective only when used in combination. MEK inhibitors were shown to block the growth of melanoma cell lines through the induction of cell cycle arrest and up-regulation of p27 in cultures, but were readily reversed after inhibitor washout. However, when the PI3 kinase and MEK inhibitors were combined, growth and invasion of metastatic melanoma was blocked [16]. Thus, aggressive melanomas are resistant to strategies targeting one signaling pathway, therefore, multiple signaling pathways may need to be targeted for maximal therapeutic efficacy.

4. Pathway "Cross-talk" that can affect therapeutics targeting the MAPK pathway

Interaction between various signaling pathways has potential to regulate the efficacy of drugs. Cross-talk or interaction of this type has potential to make some compounds more effective, render others useless and has potential to promote drug resistance.

4.1. Inhibitory Cross-talk between AKT3 and MAPK pathway

Numerous studies report cross-talk between the MAPK and PI3K signaling cascades in melanoma [25]. Stimulation of B-RAF activity by epidermal growth factor can be inhibited by co-expression of AKT [133,134]. Furthermore, B-RAF activity rises after LY294002 (a PI3K/AKT inhibitor) treatment, indicating that AKT down-regulates B-RAF activity in melanoma cells [133,134]. B-RAF contains three AKT consensus phoshorylation sites located within its amino-terminal regulatory domain, which regulate the activity of the protein (Fig. 3) [69,129]. AKT phosphorylates B-RAF at Ser^{364} and Ser^{428} to down-regulates its catalytic activity [69,129]. This has been validated in melanoma through ectopic expression of active AKT3 in early melanoma cells, which promoted anchorage-independent growth by inhibiting ^{V600E}B-RAF to lower MAPK pathway activity to levels eliminating senescence and promoting tumor progression. Mechanistically, AKT3 was shown to directly phosphorylated B-RAF on Ser^{364} and Ser^{428}, which decreased MAPK activity and promoted melanocyte transformation [69,129]. Simultaneously inhibiting both proteins was also found to synergistically inhibit tumor development when siRNA was introduced via nucleofection or when using nanoliposomes [69,127]. This was due to inhibition of AKT3 signaling in the PI3K pathways and increased MAPK activity promoting senescence. Studies have also shown that adenosine A_3 receptors might prevent the proliferative activation of ERK1/2 by antagonizing B-RAF via AKT activation and PI3K stimulation [133]. In a spontaneous mouse melanoma model, loss of PTEN has been shown to be required for progression of ^{V600E}B-RAF nevi into melanomas [75]. Thus, cross-talk between MAPK and PI3K pathways can be used to more effectively treat melanoma by inhibiting AKT3 to promote apoptosis and eliminate inhibition of ^{V600E}B-RAF to promote senescence. Targeting both would lead to synergistically acting tumor inhibition.

4.2. Targeting MEK and B-RAF to overcome resistance to MEK inhibitors

Targeting MEK1/2 using siRNA or pharmacological agents, CI1040, U0126, AZD6244 or PD98059 can inhibit growth, invasive potential and sensitize melanoma cells to chemotherapeutic agents. Mechanistically, inhibition of MEK using U0126 or siRNA sensitized human melanoma cells to endoplasmic reticulum stress-induced apoptosis by triggering caspase-4, caspase-9 and caspase-3 [135]. However, chemosensitizing and growth inhibitory properties of MEK1/2 inhibition are not observed universally in all melanoma cells. MEK1/2 inhibitors are more effective in cells harboring mutant B-RAF compared to those with wild-type protein or containing mutant RAS [88,136]. Selectivity is likely due to the "addiction" of melanoma cells to mutant B-RAF [88,136].

Certain melanoma cells are resistant to MEK1/2 inhibitors, protecting these cells from chemotherapeutic agents [137]. For example, treatment of human melanoma cell line C8161 with the MEK1 inhibitor PD98059 sensitized cells to cisplatin-induced apoptosis [137]. However, in three other human melanoma cell lines, PD98059 did not trigger cisplatin-induced apoptosis; and in one cell line, protected the cells [137]. Therefore, blocking MEK1/2 is cell line dependent and cannot be considered as a general approach either to inhibit melanoma tumor growth or sensitize cells to chemotherapeutic agents.

While the mechanism leading to MEK1/2 inhibitor resistance remains uncertain, a recent study sequenced resistant clones generated from a MEK1 random mutagenesis screen, as well as tumors obtained from relapsed patients following treatment with allosteric MEK inhibitor, AZD6244 [105]. Mutations were identified conferring resistance to MEK inhibitors by disrupting the allosteric drug binding pocket or alpha-helix C, which led to an ~100-fold increase in resistance to MEK inhibition [105]. Mutations in MEK1, P124L and Q56P 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 to PLX4720.

Cells from patients that showed transient disease stabilization followed by relapse on AZD6244 were subjected to PLX4720 treatment [105]. AZD6244-resistant melanoma cells were resistant to PLX4720, with a GI_{50} value of >10 μM compared to 5–10 nM in treatment-naïve cells. Mechanistically the resistance developed due to mutations in MEK [105]. P124L and P124S mutations conferred two- to three-fold more resistance compared to wild-type MEK1, while the Q56P mutation conferred robust resistance of >50-fold to PLX4720, comparable to the MEK(DD) allele. pMEK levels following PLX4720 treatment showed comparable reduction across all MEK1 resistance alleles, strongly suggesting that clinically relevant MEK1 resistant mutations may confer cross-resistance to B-RAF inhibition [105].

Preventing MEK mediated resistance will likely require targeting multiple points in the MAPK pathway. Simultaneously exposing melanoma cells containing mutant B-RAF to AZD6244 (a MEK inhibitor) and PLX4720 (a ^{V600E}B-RAF inhibitor) prevented emergence of resistant clones, indicating the potential of targeting multiple points in this signaling cascade to kill melanoma cells in order to prevent the development of resistance, which could have important clinical implications [105]. Therefore, combined inhibition of RAF and MEK might circumvent acquired resistance to targeted therapeutics directed against the MAP kinase pathway.

4.3. Switching from B-RAF to C-RAF

C-RAF is usually not required for signaling to MEK and ERK in melanoma cells when B-RAF is mutated to a constitutively active form. But, it is possible that a switch in RAF isoform occurs dependent on whether B-RAF or RAS is mutated. In melanocytes or melanomas in which B-RAF is mutated, B-RAF is primarily responsible for signaling to MEK and ERK [138]. However, when RAS is mutated, the cells switch to C-RAF [138]. When cAMP signaling was blocked, S43 and S233 of B-RAF become dephosphorylated and conditions favoring melanocyte dedifferentiation existed, switching from B-RAF to C-RAF enabling activation by growth factors [138]. Agents that activate cAMP production did not block proliferation of melanocytes expressing C-RAF mutants, suggesting that C-RAF is the primary growth-regulatory target of PKA and its activity must be suppressed in order to mask its oncogenic activity [138].

Elevated C-RAF protein levels have been shown to promote resistance to AZ628 (a RAF kinase inhibitor), which was associated with a switch from B-RAF to C-RAF dependency in tumor cells [99,124]. Elevated C-RAF protein levels may similarly contribute to RAF inhibitor resistance in a subset of B-RAF mutant tumor cells [99,124]. AZ628-resistant cells were found to be sensitive to the HSP90 inhibitor geldanamycin [99,124]. Geldanamycin promotes the degradation of C-RAF, thereby revealing a potential therapeutic strategy to overcome resistance to B-RAF inhibitors in a subset melanomas switching to C-RAF [99,124]. Induction of apoptosis can be triggered in melanoma cells by blocking C-RAF in tumors lacking ^{V600E}B-RAF and having low-activity B-RAF mutations relying on C-RAF-mediated survival activity [22]. Furthermore, it has been reported that either B-RAF and C-RAF or B-RAF and PI3K need to be targeted together in order to effectively inhibit melanoma and other cancers with mutated N-RAS [24]. Thus, targeting C-RAF and B-RAF might be an important strategy to overcome cellular resistance to B-RAF inhibitors, which co-express mutated N-RAS. Furthermore, inhibition of C-RAF, might be effective for melanomas having activating N-RAS mutations, with low or no mutations in B-RAF that are reliant on C-RAF, or those that become B-RAF inhibitor resistant [22].

4.4. Phosphatase-deregulation promoting resistance to MAPK pathway inhibitors

The members of the MAPK signaling pathway are regulated by phosphatases that dephosphorylate key residues rendering the proteins inactive [139]. Reversible phosphorylation of MAPK proteins emphasizes the importance of balance between the phosphorylating kinases and dephosphorylating phosphatases in regulating these pathways [139]. All members of the MAPK signaling can be regulated by protein phosphatases [139]. In non-transformed cells, phosphorylated MEK1/2 is continuously dephosphorylated by protein phosphatase 2A. The constitutive activity of protein phosphatase 2A is stimulated by at least two kinases: p38 MAPK and casein kinase 2 [139]. Inhibition of p38 MAP-kinase results in the accumulation of phosphorylated MEK-1/2 and ERK-1/2, and rendering the cells resistant to stress-induced MEK-1/2 dephosphorylation [139]. Blockade of p38 signaling was shown to prevent the functional outcome of ERK pathway inhibition, namely stress-induced apoptosis (and muscle differentiation). Expression of p38 increases the physical association of endogenous protein phosphatase 2A with the MEK-1/2-ERK-1/2 complex and protein phosphatase 2A activity required for p38-mediated de-phosphorylation of MEK-1/2 [139]. But, p38/protein phosphatase 2A-mediated MEK1/2 inhibition is an evolutionary conserved process. Casein kinase 2 directly binds protein phosphatase 2A and stimulates protein phosphatase 2A activity toward MEK1 in cultured cells [139]. p38 MAPK has been shown to activate casein kinase 2 and plausibly p38 and casein kinase 2-mediated protein phosphatase 2A activation and MEK-1/2 de-phosphorylation are at least partly the same phenomenon [139]. In keratinocytes, endogenous p38δ and ERK-1/2 were isolated as a complex, and activation of p38δ was associated with inhibition of ERK-1/2 phosphorylation [139]. Mitogen-activated protein kinase kinase 6 prevented ERK-1/2 phosphorylation only after 24–48 h transfection and therefore, it is possible that ERK-1/2 dephosphorylation is mediated by inducing expression of a phosphatase or by some other indirect means [139]. The possible explanation to this phenomenon is MAPK phosphatase 1 expression through the p38-ATF2 pathway [139]. But as to how will this be affecting the physiology of melanoma cells remains to be studied.

5. Targeting two or more proteins in the MAP-kinase pathway or targeting additional signaling cascades

Most clinicians and researcher in the melanoma field believe that multiple signaling cascades needs to be targeted simultaneously to

effectively inhibit melanoma development. Many pathways are deregulated in melanoma cells promoting a highly metastatic phenotype and resistance to chemotherapeutics [27]. As a result, Dacarbazine or the derivative temozolomide is only effective in 15–20% of patients [140,141]. Therefore, combined targeting of the members of MAPK cascade or oncogenic proteins from different signaling pathways will be required to achieve better clinical efficacy [16].

Preclinical studies have shown that targeting PI3K and MAPK signaling pathways using siRNA or pharmacological agents can synergistically inhibit melanoma development and sensitize cells to chemotherapeutic agents [69,127]. For example, treatment of melanoma cells with temozolomide or cisplatin in combination with LY294002 or rapamycin effectively reduced melanoma cell growth and survival [142]. Similarly, co-targeting RAF and mTOR using sorafenib and rapamycin, respectively, more effectively inhibited melanoma cell proliferation, induced cell death and inhibited melanoma cell invasion [142]. Likewise, simultaneous inhibition of MEK and CDK4 kinases using pharmacological inhibitors PD98059 and 219476, respectively, significantly increased apoptosis compared to single agents alone [143]. 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 [131].

Targeting oncogenes while expressing tumor suppressors is another approach for inhibiting melanoma development that is emerging. Recent studies have shown that targeting ^{V600E}B-RAF using siRNA and expressing the tumor suppressor INK4A cDNA induced massive apoptosis in melanomas compared to either of these events alone [74]. Thus, targeting multiple members of a single pathways 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 particular gene or genetic pathway activated, and available approaches to target them.

6. Use of nanotechnology to target MAPK signaling

While targets in the MAPK pathway are better understood today than ever before, no effective treatment options are available to treat patients suffering from advanced disease by inhibiting these proteins [25]. The major hurdles towards solving this problem include the lack of clinically effective pharmacological agents and delivery vehicles to get the drug into the melanoma cells [144]. Therefore, therapies or delivery systems that carry one or more therapeutic agents with minimal toxicity are urgently needed for patients. To solve this problem, nanotechnology can be used for encapsulating one or more therapeutic agent as a single drug in order to evaluate its efficacy in clinical trials [144–146]. Among a wide variety of nanotechnology delivery systems that have been developed for treating tumors are polymeric nanoparticles, silicon and gold nanoshells, dendrimers, carbon-based nanostructures and liposomes [147]. Many nanotechnologies have been shown to improve circulation time, enhance drug uptake into tumors, avoid the reticulo-endothelial system, and minimize toxicity [144].

Liposomes containing chemotherapeutic agents, siRNA, antisense-ODNs, DNA, or radioactive particles that could target the MAPK pathway are at various stages of development [144,146]. For example, liposomes loaded with siRNAs targeting AKT3 and ^{V600E}B-RAF synergistically inhibited melanoma tumor growth in mice [127,144]. Similarly, ceramide-containing liposomes in combination with sorafenib synergistically inhibited melanoma development in animals [128]. Likewise, a Phase-I study has shown that liposomal cisplatin can enhance drug delivery up to 200-fold in tumors [148].

Nanoparticles other than liposomes have also been tested in melanoma animal models. One such study showed that unique hexadentate-poly$_{D,L}$-lactic acid-co-glycolic acid polymer chemically conjugated to PD98059 inhibited melanoma cell proliferation, induced apoptosis in vitro and retarded tumor growth in vivo [145]. Furthermore, these nanoparticles have also been shown to enhance the antitumor efficacy of cisplatins [145]. Thus, nanoparticle delivery systems provide one technology to load multiple drugs, which could be genetic or pharmacological, into a single vehicle and to specifically target these agents to melanoma cells through antibodies or peptides conjugated to the surface [144].

Use of RNAi technology to target the MAPK pathways is emerging as a potential approach. siRNA can specifically inhibit target genes in the MAPK pathway; however rapid degradation in animals has been a major obstacle [145,149,150]. Liposomes protect RNAi from being "detected" by RNAses, and if coupled to specific antibodies or ligands can deliver the particles specifically into melanoma cells. In a recent report researchers at Alnylam Pharmaceuticals Inc. and the Massachusetts Institute of Technology listed ~1200 different class of lipid-like barriers called "lipidoids" that are about 100 times more efficient at delivering small interfering RNA than the earlier reported lipid-based barriers [151]. With this technology one can use a loading ranging from 3 to 30 μg/kg of siRNA compared to commonly used regular lipid barriers that require at least 1 mg/kg to get more than 50% gene silencing [151]. Clinical efficacy of this approach for targeting the MAP kinase pathways remains to be demonstrated [152].

7. Conclusions

In melanomas, targeting the MAPK pathway will be a component of any therapeutic cocktail of drugs to treat this disease. The challenge remains to identify the optimal members of the signaling cascade to target and drugs that are bioavailable with negligible toxicity-related side effects. Although targeting B-RAF or MEK seems to be the best approach, combined inhibition of key members of other signaling cascades regulating melanoma growth also might be required to prevent the development of this disease. Therefore, pharmacological agents selectively inhibiting B-RAF, MEK and key members of other signaling cascades are urgently needed. However, key to success of agents targeting MAP kinase members will be deciphering the mechanistic basis for clinical efficacy. It is now clear that, 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. Therefore, it is possible that B-RAF and MEK might have to be targeted together or in combination with other pathways such as the AKT3 in the PI3K signaling cascades for optimal clinical efficacy. Finally a better understanding of molecular mechanisms leading to the development of resistance to chemotherapeutic is needed and strategies developed to overcome resistance. The use of nanotechnology might be able to overcome certain 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.

8. Key unanswered questions

It is widely accepted that the MAPK pathways is an important therapeutic target in melanoma but it remains uncertain as to which optimal pathway member to therapeutically target for maximal clinical benefit. Therefore, an expanding number of

important questions remain to be answered. For example, which member or members of the MAPK pathway need to be targeted? Why does PLX4032 have clinical efficacy while sorafenib failed in patients? Why does PLX4032 trigger other skin cancers and what is the mechanism? What pathways need to be inhibited in combination with MAPK inhibition to synergistically inhibit melanoma development? How can bioavailability issues related to MAPK pathway inhibitors be overcome? If combination therapies were required, what other kinases would synergize with the MAPK pathway in melanomas? Will targeting B-RAF, MEK or other MAPK pathway members promote melanoma invasiveness or metastasis? What combination of drugs can be loaded into nanoliposomes to synergistically inhibit melanoma development and prevent development of drug resistance? Addressing these aspects might provide better understanding of the MAPK pathway and thereby aid development of novel therapeutics to more effectively target this important signaling cascade.

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