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NON-THEMATIC REVIEW

Targeting MAPK pathway in melanoma therapy

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Abstract New drugs targeting the mitogen-activated protein kinase (MAPK) pathway have generated striking clinical response in melanoma therapy. From the discovery of *BRAF* mutation in melanoma in 2002, to the approval of first BRAF inhibitor vemurafenib for melanoma treatment by the US Food and Drug Administration in 2011, therapies targeting the MAPK pathway have been proven effective in less than a decade. The success of vemurafenib stimulated more intensive investigation of the molecular mechanisms of melanoma pathogenesis and development of new treatment strategies targeting specific molecules in MAPK pathway. Although selective BRAF inhibitors and MEK inhibitors demonstrated improved overall survival of metastatic melanoma patients, limited duration or development of resistance to BRAF inhibitors have been reported. Patients with metastatic melanoma still face very poor prognosis and lack of clarified therapies. Studies and multiple clinical trials on more potent and selective small molecule inhibitory compounds to further improve the clinical effects and overcome drug resistance are underway. In this review, we analyzed the therapeutic potentials of each member of the MAPK signaling pathway, summarized important MAPK-inhibiting drugs, and discussed the promising combination treatment targeting multiple targets in melanoma therapy, which may overcome the drawbacks of current drugs treatment.

Keywords BRAF · MAPK · MEK · Melanoma · RAS

1 Introduction

Malignant melanoma is the most deadly form of skin cancer, and the resistance to traditional chemo- and radiotherapy make it a stubborn and notorious malignancy [1]. Melanoma arises from abnormal proliferation of melanocytes and occurs within any anatomic territory occupied by melanocytes [2]. The major risk factors for malignant melanoma are personal or family history of melanoma, exposure to intense and intermittent ultraviolet irradiation, phenotypic characteristics (fair skin or red hair), and multiple nevi [3]. Although melanoma can be completely cured by surgical excision if an early diagnosis could be made, patients with metastatic melanoma have to face a very poor prognosis with less than 15 % patients surviving for 3 years [4]. Until 2011, no US Food and Drug Administration (FDA)-approved therapy was able to increase the overall survival of metastatic melanoma patient. Dacarbazine (DITC), which is a DNA-alkylating agent, has long been used as the first choice of chemotherapeutic drug in patients with unresectable or metastatic melanoma, although the response rate was marginal (10–20 %) and patient survival benefit has never been reported [5, 6]. The immune-based therapies, including interferon (IFN)-α and high-dose interleukin 2, generated similar response rate (6–10 %) with severe adverse effects, and also failed to improve the overall survival for metastatic melanoma patients [7]. More efficient and safe therapies for metastatic melanoma are desperately needed.

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Advances of basic research and clinical investigation lead to breakthrough recently. Within 1 year, FDA-approved two novel drugs for metastatic melanoma treatment: an immune stimulatory agent, iplilimumab (Yervoy), and a BRAF V600E inhibitor, vemurafenib (Zelboraf) [8]. Vemurafenib is the first BRAF inhibitor that has been approved for treatment of human cancer harboring BRAF V600E mutation, indicating that the oncogene targeting strategy could be a very promising and important option in future cancer therapy. The recognition of key molecular mutation, BRAF V600E mutation, provided the new therapeutic opportunities in the first place and facilitated the development of promising small molecule inhibitory compounds later on. Therefore, identification of genes abnormally regulated in key signalling pathway is critical for antitumor drug development.

In melanoma, mitogen-activated protein kinase (MAPK) signalling cascades are critical and constitutively activated by a variety of mechanisms, making it a fascinating target for pathway targeting therapies. MAPK signalling pathway is one of the most important cellular mechanisms responsible for melanoma metastasis by promoting cell proliferation, survival, invasion, and tumor angiogensis [9, 10]. This pathway consists of four well-studied MAPKs, and these cascades sequentially transfer proliferative signals from the cell surface receptors into the nucleus via a series of phosphorylation events. Each MAPK cascade includes three protein kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK [11]. Extracellular signal-regulated kinase 1 and 2 (ERK1/2), the c-Jun amino-terminal kinase (JNK 12/3), p38 kinase, and ERK5 are the four alternative MAPKs activated by distinct stimuli [1]. Among these four cascades, RAS-RAF-MEK-ERK1/2 pathway attracted most attentions and research interests due to its critical role in regulating cell proliferation and survival. In response to binding of various activators, including growth factors, mitogen, or hormones, receptor tyrosine kinases (RTKs) form a dimer, triggers the activation of Ras to GTP-bound state, and leads to recruitment of Raf from the cytosol to cell membrane where it turns to activated form. The adaptor protein growth factor receptor-bound protein 2, which contains Src homology 2, facilitates SOS binding thereby converting inactivated, GDP-bound RAS into an active, GTP-bound RAS [12, 13]. Activated Raf then phosphorylates mitogen-activated protein (MAP) kinase extracellular signal regulated kinase 1 and 2 (MEK1/MEK2), which in turn causes the phosphorylation and activation of p44 ERK1 and p42 ERK2 at Thr and Tyr residues [14, 15]. Phosphorylated ERK1/2 enter nucleus and activate several transcription factors and cell cycle regulatory proteins, such as ELK-1, Myc, CREB, Fos, and so on, to control multiple cellular processes [16–20].

Dysregulation of the MAPK pathway is common in multiple types of human cancers, including melanoma. Up to 90 % melanoma showed constitutively activated ERK, due to multiple mechanisms, including mutations in neuroblastoma oncogene homolog (NRAS) and BRAF, stimulation of growth factor receptors, such as c-MET (hepatocyte growth factor receptor, also known as HGFR) and fibroblast growth factor receptor 1 (FGFR1), activation by integrin $\alpha v\beta_3$, or increase of notch signals [21–24]. Previous studies showed that MAPK pathway activation increases cyclin D1 expression and reduces the cell cycle inhibitor $p27^{KIP1}$, so as to drive melanoma cell growth and proliferation. It also controls melanoma cell survival and apoptosis through regulating the function and expression of a set of proteins, such as BIM, BAD, BCL-2, caspase-9, and MCL-1 [25, 26]. Melanoma migration and invasion behaviors are also, at least partially, driven by constitutive activation of MAPK signal cascades through crosstalk between MAPK pathway and Rho/Rock/LIMK/Cofilin pathways [27, 28]. Several studies demonstrated that MAPK pathway activation is required for melanoma metastasis because the signal cascades facilitate melanoma cells pass into the vascular system, survive during blood flow, penetrate the endothelial lining of vessels into the tissue, and finally proliferate in the new tissue environment [9, 29, 30]. Govindarajan et al. found that introduction of constitutively active MAPKK into immortalized melanocytes leads to tumorigenesis in nude mice [31]. The activation of MAP kinase results in the activator protein 1 activation and subsequent transcription thus increases the production of proangiogenic factor, vascular endothelial growth factor (VEGF), and matrix metalloproteinases [31]. It has also been recently reported that *BRAF V600E* mutation-induced MAPK pathway activation contributes to immune escape of melanomas by suppression of melanocyte differentiation antigens (MDA) [32, 33].

To inhibit MAPK signalling pathway in melanoma patients, various small molecule compounds targeting the member(s) of MAPK pathway were developed and tested (Fig. 1). In this article, we will overview and discuss the MAPK pathway inhibiting therapies and major inhibitors that have been studied, the advances and limitation of each small molecule compound, and also the combination therapy strategies with MAPK inhibitors in the treatment of melanoma.

2 Targeting RAS

2.1 Overview

The Ras proteins are founding members of Ras superfamily, which are small GTPases (guanosine triphosphatases) superfamily. Based on the structure, sequence, and function similarities, the Ras protein subfamily can be divided into

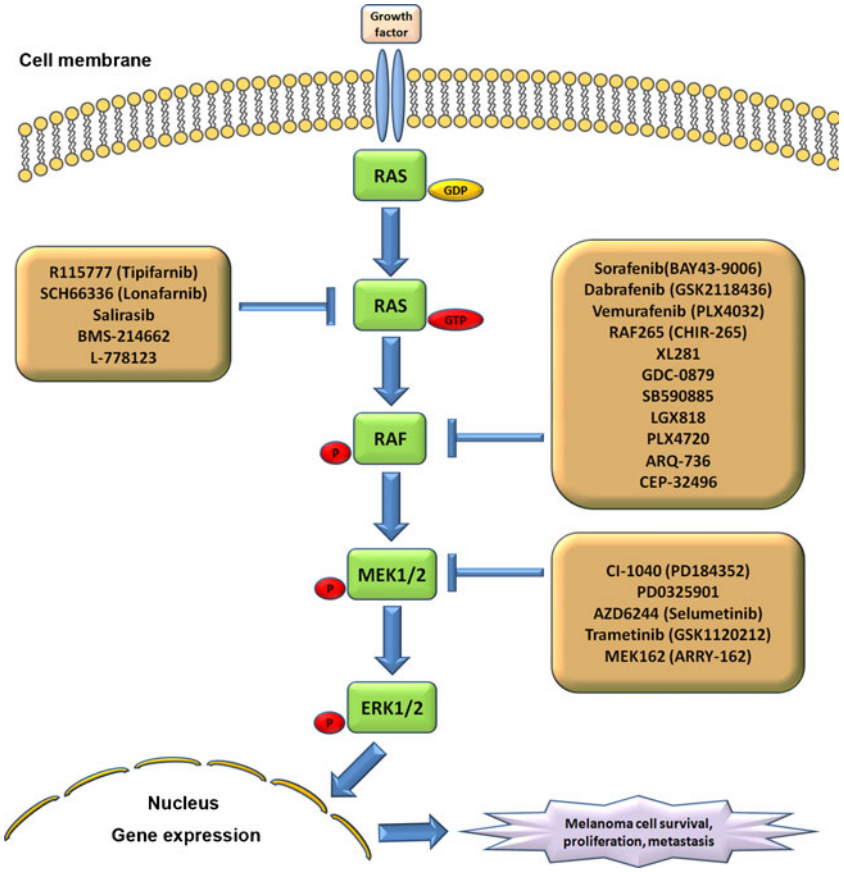

Fig. 1 Schematic diagram of the MAPK-ERK signalling pathway and selected inhibitors

nine main families: Ras, Rad, Rab, Rap, Ran, Rho, Rheb, Rit, and Arf [34]. The Ras subfamily itself consists of at least 30 members; among them, prototypic Kirsten murine sarcoma viral oncogene homolog (KRAS), NRAS, and Harvey rat sarcoma viral oncogene homolog (HRAS) are the clinically most notable members [35]. Mutations for *RAS* are very common, being found in 20–30 % of all human cancers [35] and 15–20 % in melanoma specifically [36] (Fig. 2). Mutations of *KRAS* are most commonly found, while *NRAS* mutations are less and *HRAS* mutation occur rarely [37]. Ras protein mutation type maybe associated with tumor type. Interestingly, except K-Ras, N-Ras, and H-Ras, no other Ras protein member has been found mutated in human cancers [37, 38]. The contribution of other family members to cancer development is poorly understood and remains to be demonstrated.

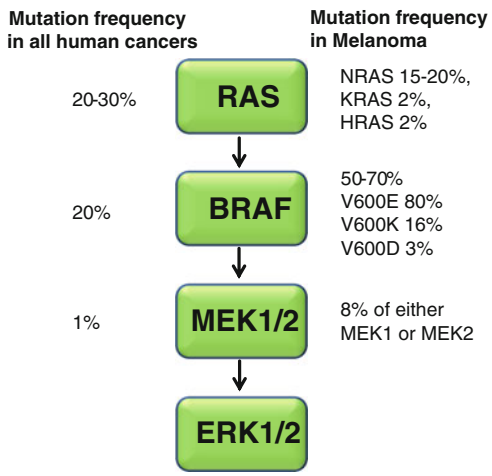

Fig. 2 Mutation rate of members of MAPK pathway

The Ras proteins have highly conserved structures across mammalian species indicating that they have specific functions. The Ras proteins are a set of key molecular switches for multiple cell signalling transduction pathways that controls important cellular processes such as cytoskeletal integrity, cell proliferation, adhesion, apoptosis, and migration [39, 40]. Mutated Ras proteins are oncogenic because they are capable of activating downstream effector pathways without any upstream stimulation. Downstream effector pathways of Ras include RAF kinases, class I phosphatidylinositol 3-OH kinase (PI3K), RALGEFs, T-cell lymphoma invasion and metastasis 1, and phospholipase Cε. Therefore, efforts have been focused on pharmacological inhibiting Ras upstream regulator and Ras proteins themselves; however, the results are relatively ineffective and unfavorable.

In melanoma, activating mutations in *NRAS* have been identified in approximately 20 % of clinical samples [41–43]. Among them, leucine substitution for glutamine (Q61L) in exon 2 at codon 61 is the most frequent mutation occurs, while Q61R and Q61H mutations and other amino acid change in exon 1 at codon 12 and 13 were also observed [25]. A recent meta-analysis indicated that NRAS mutations occurs more in nodular melanomas and melanomas caused by chronic sun damage [44]. Moreover, *NRAS* mutations demonstrated a worse clinical outcome compared to patients with *BRAF V600E* mutations or without *NRAS* or *BRAF* mutations [45]. *KRAS* and *HRAS* mutations are much less than *NRAS* mutation in melanoma patient accounting for ~2 % of each [46] (Fig. 2).

2.2 Farnesyl transferase inhibitor

Ras protein is synthesized in the cytoplasm as a biologically inactive Pro-Ras and then undergoes a series of posttranslational modifications to the carboxy terminus. Farnesylation is the most important process, which is catalyzed by Farnesyl transferase (FTase) to add a 15-carbon isoprenoid farnesyl group to a CAAX motif, thereby increasing Ras hydrophobicity [47]. After the C-terminal cleavage, Ras then localizes to plasma inner membrane, in which Ras starts the GDP/GTP-bound cycle. In GTP-bound state, the Ras switch is on and activates several downstream signalling cascades [48, 49]. Since FTase is essential for activation of Ras, FTase inhibitors have been used for the anti tumor effects by targeting FTase and consequently inhibiting Ras. The action mode of typical farnesyl transferase inhibitors (FTIs) is to inhibit prenylation or farnesylation of the peptide substrate by competing for the CAAX binding site [50]. In the laboratory setting, FTIs have been shown to be effective to inhibit growth of a wide range of human tumor cell lines, as well as in xenograft and transgenic models [51]. Here, we listed some FTIs that have been tested in melanoma specifically (Table 1).

2.2.1 R115777

R115777 (Tipifarnib, Zarnestra) is an imidazole-containing methylquinolone which has been shown to be a potent and selective inhibitor of FTase [52]. In a preclinical experiment, approximately 75 % of total 53 human tumor cell lines showed growth inhibition by R115777. This study also revealed that *RAS* status of tumor cell lines, as well as the heterogeneity of tumor was associated with the response to R115777 [50]. Marked antitumor effects of R115777 on melanoma cells led to clinical exploration in patients with advanced melanoma. Following phase II study with 14 metastatic melanoma patients showed that R115777 was able to inhibit FT activity by 85–98 %; however, no clinical activity to metastatic melanoma was observed (NCT00060125) [53]. Side-effects, like myelosuppression, neurotoxicity, gastrointestinal toxicities, and fatigue, were reported in several clinical trials [54, 55]. Another randomized phase II trial comparing BRAF inhibitor sorafenib in combination with either the mTOR inhibitor temsirolimus or R115777 in 102 melanoma patients also did not show significant clinical response (NCT00281957) [56]. These findings suggest that inhibition of FT alone is not sufficient to achieve clinical response and inhibition of tumor growth and tumor cell proliferation by R115777 might not be relevant to RAS family proteins, since other subsets of molecules also undergo post-translational modification through farnesylation. Although R115777 has limited antitumor efficacy, it provides an important option for elderly patients who have low tolerance to aggressive chemotherapy because of its relatively low toxicity.

2.2.2 SCH66336

SCH66336 (Lonafarnib, Sarasar) is another strong FT inhibitor that has drawn great attention in cancer treatment. SCH66336 is a tricyclic halogenated CAAX-competitive FT inhibitor, which is orally active [57] and is capable of blocking protein farnesylation in cell lines [58, 59]. Previous studies showed that SCH66336 was able to inhibit both human and mouse melanoma cell growth by inducing G1-phase cell cycle arrest and inhibiting retinoblastoma protein activation [60]. Another study demonstrated that SCH66336 alone could not inhibit melanoma cell growth; however, enforced RAF inhibitor sorafenib-induced apoptosis of melanoma cell through mTOR signaling, instead of MAPK pathway, and this combination completely suppressed tumor invasion both in monolayer and organotypic cultures [61]. These findings suggest that SCH66336 may sensitize melanoma cells to sorafenib-induced apoptosis and achieve better clinical responses in combination therapies. Due to limited response in melanoma, SCH66336 is currently tested in other malignancies other than melanoma.

2.3 Farnesylthiosalicylic acid

2.3.1 Salirasib

Salirasib (Strans, trans farnesylthiosalicylic acid, FTS) is another RAS antagonist, which mimics the C-terminal farnesylcysteine, thereby competing with the active, GTP-bound form of RAS proteins for specific binding sites on the cellular membrane [62, 63]. It has also been reported that salirasib disrupted the binding of RAS-GTP to the β-galactoside-binding protein, galectin 1, so as to interrupt the localization of RAS proteins to cytoplasmic membrane [64]. Preliminary studies showed the efficacy of salirasib in

Table 1 Summary of drugs targeting MAPK pathway in melanoma

Target molecule	Agent	Direct target(s)	IC_{50} (nM)	Phase	Sponsor	Status	Note	References
RAS	R115777 (Tipifarnib)	Farnesyl transferase (FT)	0.6	II	National Cancer Institute	Completed	Limited anti-tumor activity	[53, 56]
	SCH66336 (Lonafarnib)	Farnesyl transferase (FT)	1–2	I, II, III	Schering-Plough	Ongoing	Focus on combination therapy in melanoma	[60, 61]
	Salirasib	RAS	–	I, II	Concordia	Ongoing	Need validation in melanoma	[67, 68]
	BMS-214662	HRAS/KRAS	1.3–8.4	I	Multiple	Completed	Not effective in melanoma	[69]
	L-778123	HRAS/KRAS	–	I	Memorial Sloan-Kettering	Closed	Cardiac effects found	[70]
RAF	Sorafenib (BAY43-9006)	RAF-1/BRAF	6/22	I, II, III	National Cancer Institute	Ongoing	Limited activity in melanoma	[103–107, 109]
	Dabrafenib (GSK2118436)	$BRAF/BRAF^{V600E}$/CRAF	3.2/0.8/5.0	I, II	GlaxoSmithKline	Ongoing	Under evaluation	[111–113]
	Vemurafenib (PLX4032)	$BRAF^{V600E}$	31	I, II, III	Hoffmann-La Roche	Ongoing	First drug approved by FDA for BRAF-mutant cancer	[118–120]
	RAF265 (CHIR-265)	BRAF/VEGFR2	3–60/30	I, II	Novartis	Ongoing	Combined with MEK162	[123, 124]
	XL281	ARAF/BRAF/CRAF	–	I	Exelixis	Completed	Caused squamous cell carcinomas systemic toxicity	[126]
	GDC-0879	$BRAF^{V600E}$	0.13	None	–	–	Preclinical studies underway	[127]
	SB590885	BRAF	0.16	None	–	–	Preclinical studies underway	[128]
	LGX818	BRAF	–	I	Novartis	Ongoing	-	[129]
	PLX4720	$BRAF^{V600E}$/CRAF-1	13/6.7	None	–	–	Preclinical studies underway	[130]
	ARQ-736	BRAF	–	I	ArQule	Ongoing	-	-
	CEP-32496	$BRAF/BRAF^{V600E}$/CRAF	14/36/39	–	Teva	–	Preparing for clinical trial	[131]
MEK	CI-1040 (PD184352)	MEK1/2	17	I, II	Pfizer	Closed	Well tolerated, but with poor biavailability, and insufficient anti-tumor activity	[140, 141]
	PD0325901	MEK1/2	0.33	I, II	Pfizer	Closed	Unexpected musculoskeletal and neurological toxicity and visual disturbance	[142]
	AZD6244 (Selumetinib)	MEK1	14	I, II	Array BioPharma	Ongoing	Under evaluation	[146–149]
	Trametinib (GSK1120212)	MEK1/2	0.7–14.9	I, II, III	GlaxoSmithKline	Ongoing	Significant improved survival than chemotherapy drugs	[150–154]
	MEK 162 (ARRY-162)	MEK1/2 and pERK	12/10	I, II, III	Novartis	Ongoing	First time shows clinical activities in NRAS mutated melanoma	[155–157]

inhibiting melanoma growth *in vitro* and in xenograft models [65, 66]. Recent clinical trials with lung and pancreatic cancer patients demonstrated the ability of salirasib to suppress RAS function and some possible survival benefits [67, 68]. However, the clinical effects of salirasib on melanoma patients still need validation.

2.4 RAS inhibitors and interfering RNAs

Two agents directly targeting RAS have been proposed for the treatment of melanoma. BMS-214662 and L-778123 are able to directly target HRAS and KRAS, respectively [69, 70]. However, several preclinical and clinical studies showed both compounds are ineffective in melanoma, due to the major *RAS* mutations occur in melanoma are *NRAS* mutation, rather than *HRAS* and *KRAS* [1]. Moreover, toxicities like nausea, diarrhea, vomiting, abdominal cramping, anorexia, fatigue, and fever have been observed [71]. Although L778123 showed a good response in clinical trial, the studies on this drug was stopped because of the cardiac-related concern [1]. Another approach to directly target RAS is blocking mRNA expression with antisense oligonucleotides or small interfering RNAs (siRNAs). One study showed that suppression of NRAS in melanoma cell lines harboring NRAS mutation by RNA interference resulted in more apoptosis [72]. However, siRNA therapy remains challenging because the nuclear acid molecules are unstable in the circulation during delivery.

In conclusion, therapies targeting RAS protein in melanoma is relatively ineffective. One explanation is that different isoforms of RAS protein, like NRAS and KRAS, can be geranylgeranylated if the farnesyl transferase is inhibited, and these prenylated RAS proteins are still able to localize to cell membrane and still be functional [73]. The clinical benefits of RAS inhibition require more investigation, whereas RAS inhibitors still hold promise as therapeutic approach for melanoma.

3 Targeting RAF

3.1 Overview

As the downstream target of RAS in the MAPK-ERK cascade, RAF family proteins play an important role in this signal transduction. RAF proteins include three members, A-RAF, B-RAF, and C-RAF (or RAF-1) [74]. All RAF isoforms contain three highly conserved regions, CR1, CR2, and CR3, in which CR3 contains the kinase domain and key phosphorylation sites that regulate enzymatic activity [75]. Normal nonmutated RAF proteins can be activated by RAS proteins through translocation to plasma membrane, dimerization, and phosphorylation [76, 77].

BRAF is the most commonly mutated gene in MAPK pathway and has been shown to be critical in the pathogenesis of melanoma [78, 79]. Oncogenic *BRAF* mutation in melanoma was originally identified by a systematic genome-wide screen using genomic DNA extracted from 15 cancer cell lines (with only one melanoma cell line) [78]. Subsequent screening of additional 530 cancer cell lines (including 34 melanoma cell lines) demonstrated approximately 66 % melanomas harbored *BRAF* mutation. Majority of *BRAF* mutations (~80 %) arise as a result of substitution of glutamic acid for valine at codon 600 (BRAF V600E). $BRAF^{V600E}$ is 10.7-fold more active than wild-type BRAF and does not require upstream RAS-induced membrane translocation to exert enzymatic activity, thus constitutively activates subsequent downstream signal [78]. Over 50 distinct *BRAF* gene mutations have been identified so far [74]. V600K and V600D are shown in common and represent 16 and 3 % of all *BRAF* mutations, respectively in melanoma [80] (Fig. 2). Interestingly, *BRAF* mutations occurs in more than 80 % melanocytic nevi [81, 82], which are even more prevalent than in melanomas, suggesting that these genetic alterations are early events in melanoma development.

Since *BRAF* mutations play such an essential role in melanoma, the question is whether the mutation itself is sufficient to cause melanoma. Surprisingly, two groups separately found that individuals with germline *BRAF* mutations develop cardiofaciocutaneous syndrome but not cancer [83, 84]. Multiple lines of evidence showed that *BRAF* mutations are insufficient but has to collaborate with other gene/protein alterations to transform melanocytes. Garraway et al. found that ectopic microphthalmia-associated transcription factor (MITF) expression together with *BRAF V600E* mutation transformed primary human melanocytes [85]. Jane-Valbuena et al. demonstrated that ETS translocation variant 1 (which is one of ETS transcription factors) overexpression in cooperation with *NRAS* or *BRAF* mutation was able to transform immortalized human melanocytes in the presence of MITF protein [86]. It appears that *NRAS G12D* mutation was more efficient than *BRAF V600E* mutation in terms of genesis of melanoma. By screening *BRAF* mutations in a series of melanoma samples, Tsao et al. raised the possibility for cooperation between BRAF activation and PTEN loss in melanoma tumorigenesis [87]. Direct effects of activating BRAF in nevus and melanoma have been investigated in various animal models. In transgenic zebrafish model, Patton et al. showed that expression of BRAF V600E resulted in dramatic patches of ectopic melanocytes, which was termed fish nevi; while BRAF V600E induced formation of melanocyte lesions rapidly developed into invasive melanomas in p53-deficient fish [88]. In BRAF V600E knock-in marine model, two distinct results emerged [89, 90]. In consistent with the hypothesis by Tsao et al., Dankort et al. showed that BRAF V600E mice developed benign melanocytic

hyperplasia and failed to form tumor, whereas expression of BRAF V600E combined with PTEN gene silencing led to metastatic melanoma [90]. In contrast, Dhomen et al. demonstrated that ~70 % of mice generated melanoma with BRAF V600E expression alone [89].

To characterize the functional impact of genetic alterations associated with melanoma, Chudnovsky et al. regenerated human skin *in vivo* on nude mice by inducing melanocytes selectively engineered to express specific mutations frequently observed in human melanoma [91]. They found that BRAF V600E failed to induce melanoma, but merely mild junctional melanocytic nesting. In contrast, both activated RAS and PI3K along with retinoblastoma protein-p53 inhibition as well as human telomerase reverse transcriptase expression are able to yield invasive melanoma [91]. Unlike *BRAF* mutations, *NRAS* mutations and PI3K induction occurs more frequently in melanoma than in benign nevi [81, 92, 93] suggesting that BRAF, NRAS, and PI3K activation represents event that can be triggered in different timing of melanoma development. This fact may have implications on relatively low oncogenic activity of BRAF compared to RAS and PI3K in the study described above.

Although discrepancy still exists on exact function and effects of *BRAF* mutations, it is unquestionable that *BRAF* mutations are critical early events in the initiation of melanocytic neoplasia. Therefore, intense preclinical and clinical studies targeting BRAF have been carried out. Multiple studies showed that knocking down BRAF by RNA interference resulted in reduced melanoma cell growth and induction of apoptosis [94–96]. Moreover, inhibiting oncogenic BRAF rendered tumor regression in inducible short hairpin RNA xenograft models [97]. Numerous small molecule RAF inhibitors showed potency in suppressing melanoma cell growth, whereas only the *BRAF* mutant melanoma cell lines exhibit sensitivity to those inhibitors [98].

3.2 BRAF inhibitors

3.2.1 Sorafenib

The first BRAF inhibitor studied in clinical trial was sorafenib (BAY43-9006, Nexavar), which is an oral multikinase inhibitor [95]. Besides RAF, sorafenib also inhibits activity of VEGFR 1, 2, and 3, c-KIT (CD117 or stem cell factor receptor), p38, FGFR-1, platelet-derived growth factor receptor (PDGFR), and tyrosine kinase FLT3 [99], thus inhibits both tumor growth and angiogenesis [100–102]. In 2006, a phase II randomized trial failed to show any antitumor activity of sorafenib in patients with metastatic melanoma as a single agent [103]. Combination of sorafenib and other chemotherapeutic drugs have been tested for melanoma treatment. Phase III placebo-controlled trials of sorafenib in combination with carboplatin and paclitaxel (CP) failed to show any benefit for advanced melanoma patients [104]. Another recently published randomized phase III trial of carboplatin and paclitaxel with or without sorafenib in 823 patients also showed that sorafenib does not improve melanoma patient survival in combination with CP [105]. Combination of sorafenib and dacarbazine in patients with advanced melanoma has been investigated in a double-blind randomized phase II trial. Dacarbazine is the first chemotherapeutic agent approved by the FDA for the treatment of metastatic melanoma, with a response rate of 10–20 % and median survival of 5.6–7.8 months after initiation of the treatment [5, 106–108]. The results indicated an encouraging improvement in progression-free survival (21.1 weeks in the sorafenib plus dacarbazine versus 11.7 weeks in placebo plus dacarbazine); however, no significant difference (median, 51.3 versus 45.6 weeks) in overall patient survival was observed [109].

3.2.2 Dabrafenib

Dabrafenib (GSK2118436, GlaxoSmithKline) is a reversible, ATP-competitive inhibitor that selectively inhibits $BRAF^{V600E}$ kinase activity both *in vitro* and *in vivo*. It has been shown that Dabrafenib possesses significant antitumor activity in human tumor cell lines and xenografts [99, 110]. Early-phase clinical trials indicated that with administration of 150 mg twice daily, objective responses were seen in 53–69 % of melanoma patients with *BRAF V600E* or *V600K* mutations (NCT00880321) [111]. Further, phase III trial compared dabrafenib with dacarbazine in 733 patients with *BRAF V600E* mutant metastatic melanoma (NCT01227889) [112]. The results showed that dabrafenib improved response rate (RR, 53 vs 19 %) and progression-free survival (PFS, 5.3 vs 2.7 months) compared with dacarbazine. More recently published article showed the results of another phase II trial with dabrafenib in patients with *BRAF V600* mutations and brain metastases (NCT01266967) [113]. Intracranial response rate were 39.2 % (29/74) in *BRAF* mutant melanoma patients and 30.8 % (20/65) in patients with progressive brain metastases. Similar clinical efficacy of dabrafenib was exhibited compared with vemurafenib; however, dabrafenib has relatively mild and manageable toxicity profile. The most common side-effects are cutaneous squamous cell carcinomas and pyrexia [111]. Several other clinical studies on debrafenib mono/combination therapies are ongoing to test the potential of dabrafenib in treating melanoma patients with BRAF mutations and brain metastases.

3.2.3 Vemurafenib

Vemurafenib (PLX4032, Hoffmann-La Roche) is an oral potent inhibitor of mutated BRAF, which was the first drug

approved for the treatment of *BRAF*-mutated metastatic melanoma in USA in 2011 [114, 115]. *In vitro* studies showed that vemurafenib induced G1 phase cell cycle arrest and apoptosis, which associated with increased BIM expression [116]. Early *in vitro* studies showed that vemurafenib inhibited ERK phosphorylation and proliferation of cancer cell lines that harboring *BRAF* mutations, but not those cells with wild-type *BRAF*. Vemurafenib also inhibited melanoma xenografts containing mutated *BRAF* and prolong the delay of tumor growth after ending of drugs usage [117].

In a phase I trial, 32 genotype-selected metastatic patients were treated with vemurafenib at the maximum dose of 960 mg twice daily, and 80 % of them responded to it including two complete responses [118] (Table 2). A phase II trial with melanoma patients with *BRAF V600E* mutation showed a response rate of 53 % and a median duration of response of 6.7 months (NCT00949702) [119]. Following phase III randomize clinical trial, vemurafenib was compared with dacarbazine in 675 patients with previously untreated, metastatic melanoma with *BRAF* mutation (NCT01006980). The results showed that vemurafenib improved overall survival to 84 % compared with 64 % in dacarbazine group; the RR was 48 % for vemurafenib and 5 % for dacarbazine. The analysis for PFS showed that vemurafenib was associated with a relative reduction of 63 % in the risk of death ($P<0.001$) and of 74 % in the risk of either death or disease progression ($P<0.001$) as compared with dacarbazine [120]. The compelling results validated the clinical efficacy of vemurafenib and led to the approval of this drug by FDA thereafter.

Although the clinical studies of vemurafenib showed a high objective tumor response rate and marked improvement in overall survival, the severe adverse effects and drug resistance observed remain major problem of this drug. Besides the common side effects, secondary nonmelanoma skin cancers, including cutaneous squamous cell carcinoma and keratoacanthomas have been reported in 15–30 % patients treated with vemurafenib, usually in 2–3 months of therapy [118]. A recent study has also showed that vemurafenib activates ERK and enhances cell proliferation as well as migration in melanoma cells containing wild-type BRAF [121]. It remains controversial that whether its clinical efficacy is because of its selective inhibition of BRAF V600E or is due to inhibition of targets other than BRAF V600E. Vemurafenib might be inducing nonmelanoma skin cancer through activation of ERK in normal cells.

The mechanisms underlying vemurafenib-induced drug resistance is complicated and various models have been established to forestall drug resistance. One latest study by Thakur et al. succeeded to derive drug-resistant xenograft by continuous vemurafenib administration. Surprisingly, they found that vemurafenib-resistant melanomas became drug dependent, evidenced by the regression of established drug-

Table 2 Clinical trials with Vemurafenib on BRAF-mutated melanoma

Phase	NCT ID	Drugs	Conditions	Patient no.	Findings
II	NCT00949702	Single agent	Previously treated metastatic melanoma	132	Median overall RR was 53 %, PFS was 6.8 months, improved overall survival (15.9 months)
II	NCT01474551	Single agent	Unresectable or metastatic melanoma with BRAF V600E mutation	22	Developed keratoacanthomas and squamous cell carcinoma
II	NCT01586195	Single agent	Advanced melanoma with activating Exon 15 BRAF mutations other than V600E	50	Ongoing
II	NCT01378975	Single agent	Metastatic melanoma with brain metastases	132	Ongoing
III	NCT01006980	Vemurafenib vs dacarbazine	Previously untreated metastatic melanoma	675	Higher response rate (48 %), improved overall survival (84 %) in vemurafenib group, compared with dacarbazine
III	NCT01597908	Vemurafnib vs dabrafenib+ trametinib	Unresectable or metastatic cutaneous melanoma with BRAF V600E mutation	694	Ongoing
III	NCT01689519	Vemurafenib vs vemurafenib+GDC-0973	Untreated advanced or metastatic melanoma with BRAF V600E mutation	500	Ongoing
III	NCT01667419	Vemurafenib vs placebo	Resected cutaneous BRAF mutant melanoma	725	Ongoing

resistant tumors upon cessation of vemurafenib administration [122]. More importantly, the study indicated that intermittent dosing strategy significantly delays the onset of vemurafenib-induced drug resistance, and therefore suggested that altered dosing regimen may sustain the durability of the vemurafenib response for the subset of melanoma patients with BRAF mutations [122].

3.2.4 RAF265

Raf265 (CHIR-265, Novartis) is an orally available, broad-spectrum kinase inhibitor with IC_{50} ranging from less than 20 to more than 100 nM. RAF265 inhibits activity of multiple intracellular kinases, including all three RAF isoforms (as well as *BRAF V600E* mutation), VEGF receptor2, PDGFR, c-KIT, and SRC (proto-oncogene tyrosine–protein kinase) [123, 124]. Cell-based assay indicates that RAF265 is more effective on inhibiting $BRAF^{V600E}$ and VEGFR2, than PDGFR and C-KIT [123]. Su et al. studied the response of orthotopically implanted melanoma from 34 patients to RAF265 in nude mice and correlated the reaction with gene mutation profile [124]. RAF265 caused more than 50 % reduction of tumor growth, whereas $BRAF^{WT}$ tumors appeared more sensitive than $BRAF^{V600E}$ tumors. Furthermore, this study showed that response to RAF265 correlated with reduced phosphor-MEK1, rather than phosphor-ERK1/2. As a derivative of sorafenib, RAF265 may also have anti-angiogenesis activity by VEGFR2 suppression. The clinical activity of RAF265 is under evaluation in phase I trial in patients with locally advanced or metastatic melanoma (NCT00304525) and combined with MEK inhibitor MEK162 in advanced solid tumors harboring *RAS* or *BRAF V600E* mutations (NCT01352273).

3.2.5 XL281

XL281 (Exelixis) is another potent and selective RAF inhibitor targeting both wild type and mutant RAF kinases [125]. Unlike vemurafenib, XL281 does not require patient genotype selection. A phase I clinical trial has examined the efficacy of XL281 in patients with advanced solid tumors including seven colorectal, five thyroid cancer and five melanoma patients (NCT00451880). Some initial results showed that only one ocular melanoma had a partial response and six patients had minor tumor regression [126]. In addition, this drug induced squamous cell carcinomas and caused systemic toxicity.

To summarize, BRAF inhibitors achieved a remarkable success in treating metastatic melanoma, although limited duration of clinical response and drug resistance are still challenging aspects which need to be overcome. Additional compounds have been developed include GDC-0879 [127], SB590885 [128], LGX818 [129], PLX4720 [130], ARQ-736, and CEP-32496 [131] (Table 1). The ongoing studies and clinical trials will shed more light on the future strategy against those limitations.

4 MEK inhibitor

4.1 Overview

MEK inhibition is another important strategy to target RAS/RAF/MEK/ERK pathway in melanoma treatment. MEK-1 and MEK-2 are members of the dual-specificity tyrosine/threonine protein kinase family, which act as MAP kinase kinases [48]. They share approximately 80 % structural similarity and phosphorylate downstream ERKs, which is the only known group of substrates [132]. Unlike *NRAS* and *BRAF*, *MEK* mutations have only recently been reported in melanoma. In 2011, a whole-exome sequencing of seven metastatic melanoma lines identified two samples with somatic *MEK1* and *MEK2* mutations. Then, this Swiss group performed Sanger sequencing of the *MEK1* and *MEK2* in 127 additional melanoma samples from 121 affected individuals, 8 % of samples (10) were found to harbor mutations either in *MEK1* or *MEK2*, which cause constitutive ERK phosphorylation and higher resistance to MEK inhibitors [133]. *MEK* mutation was also found by a massively parallel sequencing of 138 cancer genes in a melanoma sample obtained from a patient who developed resistance to BRAF inhibitor PLX4032 after an initial marked response. *MEK1 C121S* mutation was identified and *in vitro* studies showed this *MEK* mutation enhanced kinase activity and may contribute to the resistance to both RAF and MEK inhibitors [134].

The impact of *MEK* mutation in melanoma development is uncertain, but the key role of MEK in MAP kinase signal transduction has long made them attractive therapeutic targets. The first generation of MEK inhibitors were developed for treatment of cancers with *RAS* mutations. However, melanoma cells harbor *BRAF* mutations were found more sensitive to MEK inhibition than cells with *NRAS* or *KRAS* mutation [135]. This may be due to the fact that *BRAF* mutant cells are more dependent on MEK activity, while *RAS* mutations are able to activate additional signalling pathways bypassing MEK. Therefore, most studies on MEK inhibitors focus on *BRAF* mutant cancers and the *BRAF* mutation status is critical when treat melanoma patients with MEK inhibitors. Several small-molecule MEK inhibitors have been developed and under investigation (Table 1). Although none of MEK inhibitors has been approved by FDA, preclinical *in vitro* and *in vivo* studies have demonstrated significant activity in melanoma cell lines and mouse xenograft models, and clinical trials in patients with metastatic melanoma are ongoing [135, 136].

4.2 MEK inhibitors

4.2.1 PD98059 and U0126

PD98059 is the first small-molecule inhibitor of MEK1/2. The IC50 of PD98059 has been determined for the purified MEK proteins and is 4 μM for MEK1 and 50 μM for MEK2. Although early *in vitro* studies showed that PD98059 inhibited cell migration on UM-SCC-1 cell line, the poor pharmacokinetics and solubility limited the *in vivo* and clinical application of this molecule [137, 138]. Compared to PD98059, U0126 is a stronger MEK inhibitor, which has approximately 100-fold higher affinity for MEK. *In vitro* studies showed that U0126 inhibited melanoma cell invasion, suppressed the protein expression of c-Jun, uPA, and MMP-9 [139]. Due to poor bioavailability and therapeutic efficacy in early clinical trial, this compound has been abandoned for further clinical application, but the molecular structure could serve as a basis for future molecules which could have therapeutic effects. Although PD98059 and U0126 were not moved to clinical trials, they are widely used as powerful tools for *in vitro* and cellular study of MAPK signal pathway in multiple diseases.

4.2.2 CI-1040

CI-1040 (PD184352) is a highly potent, selective oral inhibitor of MEK1/2 with an IC50 of 17 nM on purified MEK1 [136]. CI-1040 is the first small molecule MEK inhibitor showing antitumor activity in preclinical cancer models and entered clinical trial stage [140]. A phase I clinical trials of CI-1040 in 77 patients with advanced cancers showed a median 73 % inhibition of phopho-ERK1/2 expression in tumor biopsies with acceptable adverse effects [140]. Based on this encouraging study, 67 patients with advanced cancers were recruited for a multicenter phase II trials to assess the antitumor activity and safety of CI-1040 [141]. The results showed that CI-1040 was well tolerated but demonstrated poor bioavailability and insufficient antitumor activity. As a result, the clinical development of CI-1040 has been closed. However, these early studies indicated the clinical activity of MEK inhibition in humans, and the potential use of MEK inhibitor in cancer.

4.2.3 PD0325901

The second generation MEK inhibitor PD0325901 shows 100-fold more potent against MEK and has significantly improved bioavailability and metabolic stability, resulting in longer inhibition of MEK at reduced doses compared to CI-1040 [125]. But the pilot study (NCT00147550) in 13 patients with metastatic measurable disease (seven melanomas, three breast cancers, and thre colon cancers) indicated that PD0325901 causes more severe toxicity than CI-1040, including gait disturbance, memory impairment, confusion, mental status changes, visual disturbances, and muscular weakness at doses of ≥15 mg twice daily [142]. Further development of this drug in melanoma patients was thus terminated for the toxicity concerns. One study on PD0325901 and isotopomerase II inhibitor irinotecan in combination with PI3K/mTOR inhibitor PF-05212384 was currently under phase I clinical trial on advanced cancers (NCT01347866).

4.2.4 AZD6244

AZD6244 (selumetinib, ARRY-142886, Array BioPharma) is an oral, selective, non-ATP-competitive MEK inhibitor, and the IC_{50} against pure MEK is about 14 nM [143]. Preclinical studies indicate that increasing the concentration of selumetinib to 1 μM and phosphorylation of the MEK1/2 substrate ERK1/2 can be completely abrogated in 12-*O*-tetradecanoylphorbol-13-acetate-stimulated peripheral blood mononuclear cells. Up to 10 μM of concentration, AZD6244 shows very high specificity for MEK1/2 over 40 other kinases including epidermal growth factor receptor, ERBB2 (HER2), MAPK14, MAPK1 (ERK2), and MAP2K6 [143]. *In vitro* and *in vivo* studies revealed that AZD6244 inhibits MEK activity and ERK phosphorylation in melanoma cell lines and mice xenograft model [144, 145]. Interestingly, AZD6244 shows a cytostatic effect through G1 phase cell cycle arrest as a monotherapy in melanoma, but shows a cytotoxic effect when combined with chemotherapy drug docetaxel, leading to tumor regression [144]. This finding also suggests the potential clinical benefit of MEK inhibitor and chemotherapy drug combinations.

Phase I clinical studies determined the tolerated dose of AZD6244 was 100 mg twice a day in free-base formulation and 75 mg twice a day in Hyd-sulfate formulation [146, 147]. By investigating the bioavailability of two formulas, Hyd-sulfate capsule was found significantly higher than free-base suspension. Therefore, the subsequent clinical trials used more effective Hyd-sulfate formulation of AZD6244. Another phase I study of the choice of combination of AZD6244 and other molecularly targeted drugs, like dacarbazine, docetaxel, erlotinib, or temsirolimus, is ongoing [148]. The activity of AZD6244 was compared with that of temozolomide in 200 chemo-naive unresectable stages III or IV melanoma patients in a randomized phase II study [149]. Of the patients, 104 received AZD6244, while 96 patients received temozolomide. The results showed no significant difference between the two treatment arms in progression free survival and overall survival. The response rate of patients was not statistically significant between AZD6244 (5.8 %) and temozolomide (9.4 %). However,

notably five of six patients with a partial response to AZD6244 had tumors with *BRAF* mutation. This observation is consistent with a phase I study which demonstrated that in 18 analyzed patients, all 5 who had partial response also had *BRAF* mutation [145]. It seems that patients with mutated-*BRAF* melanoma are more sensitive to AZD6244, but due to small patient number, more conclusive studies are required. Several other phase II clinical trials on AZD6244 are underway, and the results are eagerly expected.

4.2.5 Trametinib

Trametinib (GSK1120212, JTP-74057) is an orally available, potent, selective, allosteric MEK1/2 inhibitor, with an IC_{50} of 0.7–14.9 nM for MEK1/2 [150]. Unlike other MEK inhibitory compounds, GSK1120212 has been shown to be well tolerated and have relatively long circulating half-life, making it a promising drug to develop [150]. Preclinical studies have shown that trametinib inhibits proliferation of multiple tumor cell lines with mutant *BRAF* or *RAS* causes G1 cell cycle arrest and reduces tumor growth in xenografted models [150]. Notably, the maximum effects were observed among *BRAF* or *RAS* mutated tumors. A phase I dose-escalation trial (NCT00687622) showed that 3 mg once daily is the most tolerated dose and identified 2 mg daily as the recommended dose for phase II trials [151]. This study also showed that GSK1120212 was more effective in the patients with *BRAF*-mutated melanoma but were BRAF inhibition naive (RR, 33 %), than in the cohort harbor *BRAF*-mutated melanoma and received previous BRAF inhibitor treatment (RR, 17 %), or with wild-type *BRAF* (RR, 10 %) [152]. These findings suggest that the *BRAF* genotype and whether previously received BRAF inhibitor treatment should be considered in further studies of trametinib. The subsequent phase II clinical trial (NCT01037127), using the dosage of 2 mg GSK1120212 daily, grouped 97 metastatic melanoma patients into two cohorts: cohort A (n=40) previously treated with a BRAF inhibitor; cohort B treated with chemotherapy and/or immunotherapy, BRAF inhibitor naive. Significant better clinical activity was observed in cohort B (RR, 25 %; PFS, 4.0 months) than cohort A (RR, 0 %; PFS, 1.8 months), suggesting that resistance to MEK inhibitor monotherapy may attribute to the mechanisms implying BRAF inhibitor resistance [153]. Consistent with this clinical study, Flaherty et al. reported significant improved survival with trametinib (4.8 months) compared with chemotherapy drugs (1.5 months) in a phase III clinical study on 322 metastatic melanoma patients with V600E or V600K *BRAF* mutation [154]. Clinical data supported that trametinib could be a useful drug for melanoma patients with *BRAF* V600E or V600K mutation in single-agent or combined therapy.

4.2.6 MEK162

MEK162 (ARRY-162) is a novel, orally available, potent, and selective inhibitor of MEK1/2 with an IC_{50} of 12 nM. Preclinical studies showed MEK 162 has significant antitumor activities in cell lines and animal models [155]. Promising data on MEK162 in an ongoing phase II clinical trial of patients with *BRAF*- and *NRAS*-mutated melanoma (NCT01320085) was shown at the 2012 American Society of Clinical Oncology Annual Meeting. Efficacy of MEK162 (at a starting dose of 45 mg twice daily) was evaluated in 35 *BRAF*-mutated and 13 *NRAS*-mutated melanoma patients. In the *BRAF* arm, one confirmed and six unconfirmed partial responses and nine patients with stable disease were recorded; two confirmed and one unconfirmed partial responses and four patients with stable disease were recorded in *NRAS* arm [156]. The median PFS was 3.55 months (95 % CI, 2.00–3.81 months) for patients with *BRAF* mutation and 3.65 months (95 % CI, 2.53–5.39 months) for patients with *NRAS* mutations. MEK162 showed clinical activity and good tolerability, and notably, this is the first therapy showed marked clinical response in patients with *NRAS* mutation melanoma [157]. Further clinical studies are currently ongoing. For example, a two-arm, randomized, phase III study to compare the efficacy and safety of MEK162 (45 mg BID) versus dacarbazine (1,000 mg/m^2 every 3 weeks) in *NRAS* Q61 mutation-positive melanoma patients were initiated (NCT01763164).

In conclusion, MEK inhibitors are very promising in clinical practice, both as single agent and in combination treatment, and will serve as important options to overcome BRAF inhibitor induced resistance.

5 Combined therapy

To date, although novel single-agent-targeted therapies, such as vemurafenib and trametinib, showed considerable improvement of response rate and overall survival for patients with metastatic melanoma, the duration of response is relatively short, clinical adverse effects were observed, and treatment resistance were frequently reported. Disease progression was observed in approximate 50 % patients who were treated with BRAF or MEK inhibitors within 6–7 months after initiation of treatment [112, 158]. Several possible mechanisms mediating BRAF/MEK inhibitor resistance have been described. (1) Tumor cells have elevated CRAF (RAF1) expression, resulting in a switch from BRAF to CRAF dependency, so that bypass BRAF to activate MEK/ ERK [159]. (2) Overexpression of mitogen-activated protein kinase kinase kinase 8 (also known as MAP3K8 or COT), maintains downstream MEK and ERK phosphorylation in a RAF-independent manner, conferring

resistance to RAF inhibitors [160]. (3) High level of *NRAS* (Q61K) or *MEK1* (C121S, P124L or Q56P) mutation leads to reactivation of MAPK pathway [134, 161]. (4) Dimerization of aberrantly spliced *BRAF* V600E independent with *RAS* causes insensitivity to BRAF inhibitor [162]. (5) Secretion of hepatocyte growth factor (HGF) results in activation of the HGF receptor MET, reactivation of MAPK and PI3K-AKT signalling pathways, thus confers to BRAF inhibitor resistance [163]. (6) Increased activation of the receptor tyrosine kinase PDGFR β is able to confer resistance independent of MAPK pathway [161]. (7) Increased IGF-1R and pAKT levels are also involved in acquired resistance to BRAF inhibitor [164]. (8) PTEN (phosphatise and tensin homolog) loss activates PI3K pathway and contributes to partial resistance to BRAF inhibition in *BRAF* V600E mutant melanoma cells [165]. These previous studies indicate that multiple mechanisms of resistance can be developed in one melanoma patient. To overcome drug resistance, intense investigations and clinical studies of combination therapies are ongoing. We discuss the promising combination strategies as below.

5.1 Combination of BRAF and MEK inhibitors

Restoration of MAPK signalling by diverse mechanisms has been associated with BRAF inhibitor resistance. Complete shutdown of MAPK pathway by addition of MEK inhibitors could be an effective strategy overcoming the resistance of BRAF single-agent therapy appears on average 6–7 months after initiation of therapy. Several clinical trials already showed the promise of this combination regimen. In phases 1 and 2 trial (NCT01072175) involving 247 patients with metastatic melanoma and *BRAF* V600 mutation, the combination of dabrafenib (selective BRAF inhibitor) and trametinib (selective MEK inhibitor) demonstrated improved median PFS to 9.4 from 5.8 months compared with the monotherapy (HR, 0.39; 95 % CI, 0.25–0.62; $P<0.01$) [166]. The rate of complete or partial response was also increased in combination group (54–76 %, $P=0.03$). Cutaneous squamous cell carcinoma was observed in 7 % of patients receiving combination therapy, as compared with 19 % receiving monotherapy ($P=0.09$), while pyrexia was more common in combination group [166]. This study showed that dabrafenib and trametinib could be safely combined at their single-agent dose, resulting in better clinical outcome and less toxicity. Trametinib may reduce the chance of patient to develop proliferative skin lesions by suppressing dabrafenib-induced activation of MAPK signalling. Due to limited clinical data, the activity of this combination needs more investigation. Two phase III clinical studies comparing this combination therapy with single agent therapy in patient with metastatic melanoma are underway (NCT01584648 and NCT01597908). The other combination of BRAF inhibitor and MEK inhibitor include vemurafenib and trametinib (NCT01597908), vemurafenib and GDC0973 (NCT01271803) are ongoing.

5.2 Combination with PI3K inhibitors

The MAPK and PI3K pathways both are critical for cell survival and proliferation, and highly activated in most melanomas. Therefore, combining the inhibitors of both pathways may result in enhanced suppression effects. Previous studies revealed that increased phosphor-AKT expression and PTEN loss induced PI3K signalling confer to resistance to BRAF inhibitor, which provides the rationale to combine inhibition of both MAPK and PI3K-AKT pathway in melanoma therapy. Two important PI3K pathway inhibitors include Temsirolimus and Everolimus, which are directly targeting mTOR. mTOR is a serine/theronine kinase downstream of AKT that regulates protein synthesis, cell cycle progression, and angiogenesis [167]. However, due to the broad inhibition spectrum, the specificity of mTOR inhibitors is relatively high, limiting the application of these drugs in melanoma therapy. Recent studies of combined treatment with mTOR inhibitor rapamycin and PI3K inhibitor PX-886 induced cell death in *BRAF* mutant, *PTEN* wild-type melanoma cell lines, supporting the notion that targeting PI3K pathway could be an option to overcome BRAF inhibitor resistance [168]. Early clinical trials have been designed to test the effects of combination therapy with both MAPK and PI3K pathway.

5.3 Combination with immunotherapies

Melanoma is one of the most immunogenic tumors; therefore, immunotherapies to overcome melanoma immune escape have been developed to treat melanoma patients. Based on the finding that IFN-α generated 15–20 % response rates with systematic administration, FDA-approved IFN-α for adjuvant treatment of stages IIB–III resectable melanoma in 1995 [169]. In 2011, ipilimumab (Yervoy) demonstrated significant prolongations in patient survival, thus was approved by FDA for the treatment of metastatic melanoma patients [170]. Ipilimumab is a fully humanized immunoglobulin G1 κ monoclonal antibody that blocks cytotoxic T-lymphocyte-associated antigen 4, which increases T cell activity and antitumor activity [171]. Phase III clinical studies compared ipilimumab with dacarbazine and glycoprotein 100 peptide vaccine (gp100). Ipilimumab demonstrated an improved overall survival versus gp100 and dacarbazine single agent treatment, 10.1 vs 6.4 and 11.2 vs 9.2 months, respectively [170]. However, aside from the relative modest

response rate and improvement in OS, toxicities were observed, such as immune-related enterocolitis, hepatitis, and dermatitis. It has also been recently reported that *BRAF V600E* mutation-induced MAPK pathway activation contributes to immune escape of melanomas by suppression of MDA [32]. Immune cells recognize melanoma cells through MDAs; BRAF or MEK inhibitors are able to increase expression of MDAs without weakening T cell function, suggesting the promise of combination of MAPK-targeted therapy with immunotherapy drugs, such as ipilimumab [32]. The phase I and II clinical trials with combination of vemurafenib and ipilimumab in advanced or metastatic *BRAF*-positive melanoma patients are designed to test the safety and efficacy (NCT01400451).

5.4 Combination with chemotherapeutic drugs

Although vemurafenib and ipilimumab have been registered for the treatment of metastatic melanoma, both drugs showed limitations. Vemurafenib has short response duration, while ipilimumab shows low response rate. Therefore, DTIC or other chemotherapeutic drugs are still used in most patients with advanced melanoma. The combined treatment of sorafenib with carbopaltin and paclitaxel was tested in 823 patients with advanced melanoma [105]. These two chemotherapy agents have been known to generate synergistic results in nonmelanoma models and sorafenib is able to enhance the activity of each agent. However, combined sorafenib with carbopaltin and paclitaxel does not improve overall survival in chemotherapy-naive patients (NCT00110019).

6 Conclusion

The MAPK pathway is a key signal cascade in melanoma development, thus specific inhibitors targeting important molecules in this pathway have been developed and extensively tested. Recent studies and clinical trials showed exciting progress on the targeted therapy in malignant melanoma. The success of BRAF inhibitor vemurafenib has brightened the future for the oncogene-targeted melanoma therapy, and brings hope and promise to patients and clinicians. Unintended outcomes on the inhibition of MAPK pathway show the complexity of this pathway. Further investigations aim to understand and overcome drug resistance pathways and improve the efficacy of MAPK-targeted compounds are eagerly needed.

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