Other targeted drugs in melanoma

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Abstract: Targeted therapy drugs are developed against specific molecular alterations on cancer cells. Because they are "targeted" to the tumor, these therapies are more effective and better tolerated than conventional therapies such as chemotherapy. In the last decade, great advances have been made in understanding of melanoma biology and identification of molecular mechanisms involved in malignant transformation of cells. The identification of oncogenic mutated kinases involved in this process provides an opportunity for development of new target therapies. The dependence of melanoma on BRAF-mutant kinase has provided an opportunity for development of mutation-specific inhibitors with high activity and excellent tolerance that are now being used in clinical practice. This marked a new era in the treatment of metastatic melanoma and much research is now ongoing to identify other "druggable" kinases and transduction signaling networking. It is expected that in the near future the spectrum of target drugs for melanoma treatment will increase. Herein, we review the most relevant potential novel drugs for melanoma treatment based on preclinical data and the results of early clinical trials.

Keywords: Melanoma; pathways; targeted; therapy

Introduction

Melanoma is one of the most aggressive tumors; until very recently, there were no active treatments for this disease and median survival time was less than 1 year (1).

Advances in translational research have led to the development of very active drugs for several tumor types, such as EGFR inhibitors for EGFR-mutated lung cancer (2) or imatinib for GIST tumors harboring KIT or PDGFR mutations (3). Recently the identification of the BRAF mutation in 50% of melanomas has led to development of the first targeted drugs for this disease (4). The combination of BRAF and MEK inhibitors has demonstrated an even higher activity in BRAF mutant melanomas with response rates close to 80% and a significant improvement in median survival (close to 2 years) (5-7). Nevertheless, new therapies are required due to the ability of melanomas to quickly develop resistance to current therapies. Understanding of the signaling pathways and the interactions between them could therefore provide new targets for drug development in melanoma.

Herein we review the main signaling pathways in melanoma and some of the advances in new targets and drug development for this disease (*Table 1*).

Table 1 New targeted drugs

Target	Drug	Phase of studies
pan RAF	TAK-632	Preclinical
	ARQ736	I
ERK1/2	MK-8353	I
	BVD-523	I
	AEZS-131	Preclinical
	SCH772984	Preclinical
CDK4/6	Dinaciclib (MK-7965)	II
	P276-00	II
	LEE011	Ib/II
	Palbociclib (PD-0332991)*	I, III (breast cancer)
PanPI3K	Buparlisib (BKM120)	I, III (breast cancer)
	Pictilisib (GDC-0941)	I
	PX-866	I
PI3K specific isoforms	BAY-80-6946	I
	BYL-719	I
PI3K/MTOR	BGT-226	I
	BEZ-235	I (clinical development stopped)
	GSK-2126458	I
AKT	MK-2206	I
MET	ARQ-197	I
MET, VEGFR2	E7050	II
Ac anti MET	Onartuzumab	I
IGFR1	Ganitumumab (AMG479)	I
panFGFR	SU5402	Preclinical
	BGJ398	I
HSP90	Ganetespib (STA-9090)	II
	XL888	I
VEGFR1, 2, 3, PDGFR, CKIT	Pazopanib*	II
VEGFR2, MET, AXL, RET,KIT, FLT3	Cabozantinib*	I, II
BRAFV600, BRAF, CRAF, VEGFR2, PDGFR, CRAF, CSF, RET, ckit, SRC	RAF-265	II
NOTCH1	RO-4929097	II (clinical development stopped)
	PF-03084014	I
TGF-β	Trabedersen (AP12009)	I-II
	Tasisulam (LY573636)	III (prematurely closed due to toxicity)
	EW7203	Preclinical
	Galunisertib (LY2157299)	Preclinical
STAT/JAK3	WP1066	I
YAP-TEAD	Verteporfin	II

*, FDA approval in other tumors.
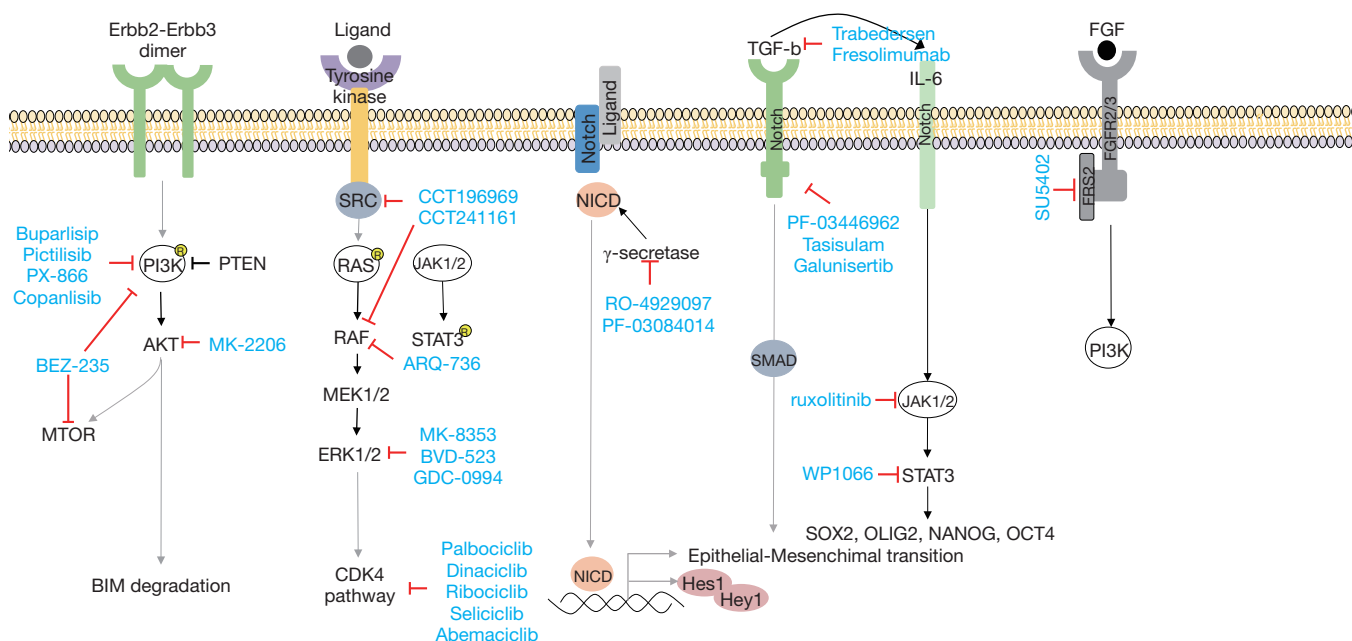
Figure 1 New targeted drugs.

PAN-RAF selective inhibitors

Activation of the RAS/RAF/MEK/ERK pathway occurs in NRAS and BRAF mutant melanomas. These tumors, especially those with BRAF mutation, are dependent on ERK signal for proliferation and survival (8). The most frequent mechanisms of resistance to BRAF inhibitors are the acquisition of a new NRAS mutation, overexpression of COT, or new splicing forms of BRAF (9-11). BRAF inhibitors such as vemurafenib or dabrafenib induce this paradoxical RAF activation via RAF dimerization in BRAF wild type cells (9). This process is known as "drug-mediated RAF dimers transactivation" (12). Therefore, BRAF inhibitors are could be potentially deleterious in NRAS mutant melanomas or melanomas with NF-1 loss.

Several pan-RAF inhibitors have been developed. These new drugs inhibit all or most of the members of the RAF family, including rare variants of BRAF, splicing variants, BRAF fusion proteins, NRAS mutants (*Figure 1*). They, therefore, block this phenomenon and could theoretically be effective in NRAS mutant melanomas or in many BRAF mutant melanomas resistant to BRAF inhibitors (13).

TAK-632 is active for NRAS and resistant BRAF mutant melanoma cell lines either due to the development of splicing forms or to an acquired NRAS mutation (13,14). The combination of this drug with the MEK inhibitor TAK-733 has a synergistic effect on cell lines. However,

there are currently no ongoing clinical trials with this drug.

ARQ-736 is another panRAF inhibitor currently in development. A phase I clinical trial for BRAF resistant melanomas or NRAS mutant melanomas has now been completed (NCT01225536) and results are awaited.

CCT196969, CCT241161: these panRAF inhibitors also inhibit RTK/SRC-family kinase (SFK) signaling. These compounds do not drive paradoxical pathway activation and inhibit MEK/ERK in BRAF and NRAS mutant melanoma. They do inhibit melanoma cells and patient-derived xenografts resistant to BRAF and BRAF/MEK inhibitors (15).

Other panRAF inhibitors in development: LY3009120, CCT3833.

ERK1/2 inhibitors

When acquired resistance to BRAF inhibitors occurs, MAPK pathway is reactivated in 80% of cases through different mechanisms. Since they all converge downstream activating ERK, ERK inhibitors may play a role in these situations (*Figure 1*).

Unlike BRAF or MEK inhibitors, most ERK inhibitors are still in preclinical phases of development. These inhibitors generally show activity as single agents in preclinical studies, especially in BRAF resistant mutant melanoma (16). The combination of these drugs

Page 4 of 17

González-Cao et al. New targeted drugs in melanoma

with BRAF inhibitors is particularly interesting since they have an opposite effect on BRAF mutant cells and it is expected that the combination is less toxic than monotherapy with BRAF inhibitors or ERK inhibitors alone. The potential advantage of the combination of BRAF inhibitors and ERK inhibitors is based on the fact that ERK inhibitors block the phosphorylation of ERK, reducing the chances of developing feedback. But the question is if this combination will be superior to the standard combination of BRAF and MEK inhibitors. Some of the intrinsic differences among ERK inhibitors are related to their biochemical activities with kinetic differences in phosphorylation and dephosphorylation of ERK that suggest a range of conformational effects. Also *in vitro* profiles of ERK inhibitors can differ with effects on phospho-ERK levels different ERK between different inhibitors: most chemical series show rapid and sustained increase in phospho-ERK, but for example, GDC-0994 has little acute effect on phospho-ERK *in vitro*, in contrast to SCH772984 that is reported to decrease phospho-ERK (17). It is well-known that inhibition of phospho-p90RSK correlates well with biochemical potency, and could be a subrogated marker of activity.

It is expected that dose limiting toxicities (DLT) due to inhibition of ERK do not allow a narrow therapeutic index. The combination with BRAF inhibitors in intermittent schemes could minimize side effects and delay the onset of resistance (18).

SCH772984 is a new selective inhibitor of ERK1/2. In addition to inhibiting ERK kinase activity directly, also it inhibits ERK phosphorylation mediated by MEK, unless it is not a direct inhibitor of MEK (19). In preclinical studies, SCH772984 has demonstrated a response rate of 50% in NRAS mutant melanomas cell lines and especially in BRAF resistant melanoma through acquisition of new mutations in RAS or MEK1, splicing variants of BRAF or BRAF amplification (17) .

VTX11e is an inhibitor of ERK. In laboratory models, sensitivity is recovered when VTX11e is added to the combination of MEK inhibitors and BRAF inhibitors in resistant melanomas with NF1 loss (20).

MK-8353 is an ERK1/2 inhibitor that has completed a phase I clinical trial in multiple solid tumors (NCT01358331).

BVD-523 is an ERK1/2 inhibitor currently in a phase I study in solid tumors including untreated BRAF mutant melanomas, BRAF mutant melanomas with resistance to BRAF inhibitors and NRAS mutant melanomas (NCT01781429).

AEZS-131 is an ERK inhibitor with demonstrated activity in colorectal and breast cancer models. However, there are no clinical trials currently ongoing.

FR-180204 is an ERK1/2 inhibitor (21). There are no clinical studies currently ongoing.

GDC-0994 is a highly selective inhibitor of ERK1/2 (22). Two phase I trials are ongoing in solid tumors, one as a single agent (NCT 01875705) and the other one in combination with cobimetinib (a MEK inhibitor) (NCT02457793).

CDK4/6 inhibitors

The p16-cyclin D-CDK4/6-retinoblastoma protein (RB) pathway (CDK4 pathway) is altered in 90% of melanomas. When CDK4 is activated, it leads to phosphorylation and, therefore, to inhibition of the RB that permits G1-S cell-cycle transition (23).

Cyclin synthesis and consequently the activation of cyclin-dependent kinase (CDK) is specific of the stages of the cell cycle, and coordinates DNA replication and cell division. CDK activity is regulated at many levels through different mechanisms including the number of regulatory cyclin subunits, its association with the catalytic unit of CDK, and the members of the two families of CDK inhibitory proteins. This family includes INK4A (p16), INK4B (p15 and CDKN2B), INK4C (p18 and CDKN2C) and INK4D (p19 or CDKN2D) and CIP, as well as the KIP family which includes p21 (CDKN1A), p27 (CDKN1B) and p57 (CDKN1C) (23).

CDKs are often overactive in cancer due to genetic and epigenetic alterations that affect either the CDKs, the CDKs activators, the endogenous inhibitors or the upstream mitogenic pathways. Although CDKs are not usually mutated in tumors, they are normally amplified or overexpressed, as are their respective cyclin activators, while their inhibitors are usually lost. In summary, the cascade of cyclin D-CDK4/6, INK4A (p16), RB and transcription factor E2F are altered in most tumors.

The relationship between the risk of melanoma and the alteration of the CDK pathway was first described when germline mutations in the CDKN2A (p16INK4A) and CDK4 were discovered (24). Currently, it is well known that loss of p16INK4A occurs in approximately 80-90% of metastatic melanomas, commonly through deletion (50%), mutation (10%) or promoter methylation (20-30%). The loss of p16 from benign nevi to metastatic melanoma is a progressive phenomenon: p16 is positive in 80% of nevi, in 30-80% of primary melanomas and in 10-20% of metastatic

melanomas. This loss at different stages of disease reflects the important role of the CDK pathway in melanoma progression (23).

From the CDK family, CDK2 plays a fundamental role in the development and progression of melanoma and its expression in melanoma is regulated by the transcription factor MITF (25). Melanoma cell lines with low expression of CDK2 have increased sensitivity to CDK inhibitors (25).

CDK inhibitors are small molecule inhibitors of cyclin kinase which have been tested in several tumors as single or combination agents. In breast cancer, the combination with hormone therapy has recently achieved FDA approval (26,27).

Combination therapies with other target drugs in melanoma are supported by solid preclinical studies, both *in vitro* and *in vivo*. Trametinib, a MEK inhibitor, in combination with the CDK4/6 inhibitor palbociclib demonstrated high activity in NRAS mutant models, with 30% of complete responses in animal models (28). The first compound that inhibited CDKs was flavopiridol (Alvocidib) from Sanofi-Aventis. Unfortunately, clinical studies showed poor activity in melanoma and other tumors, probably because flavopiridol is not a specific inhibitor of CDK4 (29). Other inhibitors of CDK4 have recently entered clinical development.

Palbociclib (PD-0332991) is an inhibitor of CDK4 and CDK6 which has recently been approved for treatment of hormone receptor positive breast cancer on the basis of the survival benefit demonstrated in a phase III trial in advanced disease (27). The DLT is hematological. In melanoma, palbociclib is being tested in several phase I trials as single agent in tumors with mutant or amplified CDK4 (NCT01037790), in solid tumors in combination with the MEK inhibitor trametinib (NCT02065063) and in BRAF mutant melanoma with CDNKN2A loss in combination with the BRAF inhibitor vemurafenib (NCT02202200).

Dinaciclib (MK-7965) (MSD) is a non-selective inhibitor of CDK4/6 which also inhibits CDK2, CDK9, CDK5 and CDK1 and has a better toxicity profile than flavopiridol. Treatment of human melanoma mouse xenografts with dinaciclib led to tumor regression associated with reduced RB phosphorylation and Bcl-2 expression. It is believed that the pro-apoptotic activity is mediated by inhibition of CDK7 and CDK9 that regulates expression of the antiapoptotic proteins Bcl-2 and Mcl-1. Further mechanistic studies revealed that dinaciclib induces p53 expression whilst simultaneously down-regulating expression of the anti-apoptotic factors Mcl-1 and XIAP (30). It has been demonstrated to have single agent activity in relapsed

myeloma in a phase II trial (31). In advanced melanoma, a phase II clinical trial has concluded and results are pending (NCT00937937).

Ribociclib (LEE011) (Novartis) has been evaluated in melanoma in several phase I trials in combination with BRAF or MEK inhibitors (NCT01777776, NCT01781572, NCT01820364). Early data from a phase Ib/II study has shown that the combination with the MEK1/2 inhibitor binimetinib (MEK162) is active in patients with NRAS-mutant melanoma, with objective response in seven patients from 21 evaluable (32). Seliciclib (roscovitine, CYC202) has shown clinical benefit as a single agent in phase I and II clinical studies in nasopharyngeal cancer and non-small cell lung cancer (NSCLC). Interim phase II data showed that 7 of 10 previously-treated NPC patients had stable disease with 2 staying on treatment for over 8 and 1 for over 24 months (33). In a phase I study shown a significant transcriptional down-regulation of genes related to cellular proliferation and survival in some patients post treatment. Topline results from a double-blinded, randomized phase IIb study in patients with at least two prior treatments for NSCLC showed no difference in progression free survival but an increase in median overall survival favoring seliciclib over placebo (http://investor.cyclacel.com/releasedetail.cfm?ReleaseID=614395).

Other specific inhibitors of CDK4/6 in development are LY2835210 and P276-00. These inhibitors are less toxic, but still show hematological toxicity.

Abemaciclib (LY2835210) (Lilly) is one of the most promising CDK inhibitors. Results from a phase I trial including 132 patients with five tumor types, were presented during the 2014 Association for Cancer Research meeting, showing anti-tumor activity for LY2835210 in HR+ metastatic breast cancer patients, with a response rate of 25% (9 out of 36 patients) (34). Data from a phase I study suggested a higher activity in KRAS mutant tumors, with a demonstrated a response rate of 55% in 29 KRAS mutant patients *vs.* 38% in those that were wild-type KRAS (35). It is being tested in several phase III clinical trials in breast and lung cancer, but there are not ongoing studies in melanoma.

P276-00 has been tested in a phase II clinical trial in melanoma with expression of cyclin D1 protein (NCT00835419).

Inactivation of RB appears to predict resistance to CDK4 and CDK6 inhibitors. Other promising biomarkers, such as loss of p16INK4a and gains of cyclin D1, failed to predict a benefit with palbociclib in the phase III trial in estrogen-receptor-positive breast cancer (27).

Page 6 of 17

González-Cao et al. New targeted drugs in melanoma

PI3K/AKT/mTOR inhibitors

PI3K activation initiates a signal transduction cascade, of which the major effectors are the kinases AKT and mTORC1. PTEN is a tumor-suppressor gene that functions as a phosphatase and is the primary negative regulator of PI3K through hydrolysis of PIP3 (36).

Deregulation of the PI3K pathway has frequently been implicated in a wide range of malignancies, including melanoma. Alteration of the pathway commonly occurs through mutation or amplification of PIK3CA that encodes the p110α catalytic subunit, loss of function of PTEN (mutation, deletion, or lower expression), alterations in the INPP4B and PHLPP phosphatases, mutations of the PI3K regulatory subunits, through activation of upstream receptor tyrosine kinases or cross-talk with the RAS pathway.

In the case of BRAF mutant melanoma, PI3K reactivation is involved in 20% of patients with resistance to BRAF and MEK inhibitors (9). Also, some melanoma cases present constitutive activation of the PI3K pathway through PTEN loss (20-30% of melanomas) (37). PTEN loss frequently coexists with a BRAF, but not NRAS, mutation. In the case of melanomas with mutated BRAF and loss of PTEN, pharmacological inhibition of BRAF or MEK is less effective than in the case of melanoma without loss of PTEN (38). The combination of BRAF inhibitors with PI3K inhibitors has demonstrated activity in melanomas with PTEN loss, showing increase of BIM expression (37,39,40). Responses to PI3K pathway inhibitors have been reported in BRAF-mutant cancer cell lines, including those without any known PI3K pathway aberration (41).

In addition to mutant BRAF melanomas, inhibition of PI3K also is effective in NRAS mutant melanomas. NRAS mutation simultaneously activates the MAPK and the PI3K pathway (42), so the most logical treatment for RAS mutant tumors would be the combination of drugs, inhibiting both pathways. In mice with KRAS activation, NVP-BEZ235, a dual pan-PI3K/mTOR inhibitor, did not reduced tumor volume, however, when these tumors were treated with the PI3K inhibitor combined with a MEK inhibitor, they underwent regression (43,44). Also, there are multiple feedback loops in the PI3K pathway that can be activated when a PI3K inhibitor is used in monotherapy, so adequate inhibition of this pathway requires the combination of several drugs.

There are many PI3K inhibitors in development, although none have yet been approved. Only mTOR inhibitors have been approved for breast and renal cancer (45-47).

For pan-class I PI3K inhibitors, the importance of the therapeutic window with regard to their pharmacodynamic effect is critical ≥90% inhibition of AKT phosphorylation is necessary to inhibit cancer cell proliferation, implying potentially important differences between the drugs (48).

Buparlisib (BKM120) (Novartis) is an oral pan-class I PI3K inhibitor (*Figure 1*). Preclinical data in murine models demonstrated significant activity in several tumors, including melanoma (49). DLT were hyperglycemia, rash, mood alteration and epigastralgia (50). Combination with the MEK inhibitor trametinib has demonstrated activity in KRAS mutant ovarian cancer (51). Currently, there is an ongoing phase III trial in metastatic breast cancer in combination with hormonotherapy (52). In BRAF mutant melanoma there are ongoing phase I trials in combination with BRAF inhibitors (NCT01512251 and NCT01820364) and MEK inhibitors (NCT013363232).

Pictilisib (GDC-0941) (Genentech) is an oral selective inhibitor of class I PI3K. Since it has been shown to achieve 100% AKT inhibition in two patients at the MTD, it seems a promising inhibitor. It has a good toxicity profile as seen in the first in human trial, and antitumor activity as shown by partial response in two patients. One of whom had V600E BRAF-mutant melanoma; the other had platinum-refractory epithelial ovarian cancer exhibiting PTEN loss and PIK3CA amplification (53). Pictilisib-related hyperglycemia was limited to grades 1-2 elevations, with grade ≥3 hyperglycemia being observed in only one patient (53). In melanoma the combination with cobimetinib (GDC-0973), a MEK inhibitor, demonstrated activity in RAS and BRAF mutant melanoma using intermittent dosing schedule (54).

Other pan-class I PI3K inhibitors that have undergone phase I clinical evaluation include SAR245408 and the irreversible inhibitor wortmannin derivative PX-866 (Oncothyreon). Both drugs were associated with minimal hyperglycemia, but differences were observed in the frequency of rash, which occurred in 26% with SAR245048 and not with PX-866. DLT of PX-866 was diarrhea and elevation of aspartate aminotransferase (55). A phase I trial in melanoma with PX-866 in combination with vemurafenib has been completed (NCT01616199).

A second group of PI3K inhibitors are those that inhibit only some isoforms in a selective way (56). These PI3K inhibitors have limited toxicity even at high doses. In tumors with PI3Kα mutation or those in which PI3K signaling is driven by activated RTKs, PI3Kα is the dominant isoform, whereas in tumors with PTEN

mutations, PI3Kβ is dominant (57-59). These data suggest that selective inhibitors of PI3Kβ, but not of PI3Kα, will effectively inhibit PI3K signaling in melanomas in which PTEN is inactivated.

Taselisib (GDC-0032) (Genentech) is a class I selective oral inhibitor of PI3K isoforms-α-δ, and -γ. Its main toxicity is hyperglycemia and asthenia (60). There are ongoing studies in breast cancer, but not in melanoma.

Copanlisib (BAY 80-6946) (Bayer) is a class I selective inhibitor of PI3K isoform-α and β (61) which has shown activity in several types of tumor cell lines, including melanoma (62). A phase I clinical trial has shown good tolerability profile with DLT including left ventricular dysfunction, hyperglycemia, rash, renal and hepatic dysfunction (63). A phase I trial in combination with a MEK inhibitor has been completed (NCT01392521).

BEZ-235 (Novartis) is a dual inhibitor of PI3K (p110 isoforms-α, -β, -γ, -δ) and mTOR with DLT of mucositis (64). Novartis has decided to discontinue its clinical development.

Alpelisib (BYL-719) is an oral selective inhibitor of PI3K isoform-α that is active against the somatic PI3Kα mutations and wild-type PI3Kα (65). Significant anti-tumor activity has been observed in mice bearing PIK3CA-dependent tumor xenograft models and good tolerance (65). DLTs are hyperglycemia and diarrhea (66). Clinical trials ongoing are mainly in breast cancer, but in the phase I study in combination with a MEK inhibitor, melanoma patients were also treated (NCT01449058).

BGT-226 (Novartis) is a dual inhibitor of pan-class I PI3K and mTOR. Its main toxicity is gastrointestinal (nausea and vomiting). It had limited activity in the phase I trial in solid tumors (67). There are no clinical trials ongoing.

GSK-2126458 (GSK) is a selective inhibitor of PI3K isoforms p110-α, -β, -γ, -δ, mTORC1 and mTORC2. The phase I trial of the combination with a MEK inhibitor was stopped due to toxicity and lack of efficacy (NCT01248858).

MK-2206: is a pan-AKT inhibitor (*Figure 1*). In preclinical studies it has demonstrated synergy with different compounds included chemotherapy (68). A phase I trial has demonstrated tolerability testing different schedules (69). Recently there have been reported two cases with response in wild type BRAF melanoma (70). There are no clinical trials ongoing in melanoma.

AZD8186 (AZ) is a selective inhibitor of PI3K isoforms p110-β and -δ (71). Combined inhibition of both isoforms in PTEN-deficient models, results in major tumor regressions. Combination with inhibition of PI3K isoforms p110-α in prostate model plus hormonotherapy demonstrated high activity (72). It has recently entered phase I clinical trials for PTEN deficient solid malignances (NCT01884285).

Notch pathway inhibitors

The Notch signaling pathway plays a key role in the processes of differentiation and maintenance of cancer stem cells. There are four Notch receptors (Notch 1, 2, 3 and 4) and five ligands (including delta ligand DLL1, DLL3, DLL4 and Jagged JAG 1 and 2). These receptors and their ligands are transmembrane proteins that activate intercellular signaling connections between neighboring cells. Upon binding of the ligand to the receptor, ADAM (also called TACE "metalloproteinase tumor necrosis factor alpha converting enzyme") is activated. This activation leads to the release of the NICD into the cytoplasm and it translocates to the nucleus where it binds to the transcription factor CSL, activating gene transcription Notch dependent and genes encoding the HES protein include p21, her2, ciclinaD1 and 3, c-myc, neuregulin and some members of NF-κB pathway. Clinical activity of LY2835219, a novel cell cycle inhibitor selective for CDK4 and CDK6, is in patients with non-small cell lung cancer.

The Notch pathway plays an important role in epithelial-mesenchymal transition (EMT) (73). Recently, cross talk between Notch and Hedgehog pathway have been described since HES1, a target of Notch, directly modulates the transcription factor Gli-155 (74,75).

In solid tumors, although mutations in Notch1 are infrequent, there are frequent alterations in the expression of wild-type Notch receptors and their ligands (76). In melanoma, high levels of HES1 correlate with a poor prognosis (77). Tumors are frequently composed of heterogeneous cell types including cancer stem cells that are resistant to conventional therapies. Therapeutic strategies that effectively target Notch signaling could have a major impact on cancer patient survival by eliminating cancer stem cells. The clinical development of drugs that inhibit the Notch pathway has encountered serious difficulties due to gastrointestinal toxicity. This has been managed by trying discontinuous dosing schedules, concomitant corticosteroid therapy and developing more selective drugs (78).

Drugs targeting the Notch pathway can be generally classified in two groups: γ-secretase inhibitors (GSI) and monoclonal antibodies against the interaction of receptors and ligands of Notch (74).

A wide variety of GSI have entered clinical development; because of the diversity of GSIs and their substrates, the

Page 8 of 17

González-Cao et al. New targeted drugs in melanoma

targeting for Notch cleavage is often not highly specific (*Figure 1*).

MK-0752 (Merck) is a GSI drug. In a phase I study it demonstrated activity in cerebral tumors (79). The daily treatment scheme is highly toxic but the intermittent scheme seems more appropriate (79). Currently there are no studies open in melanoma, although there are in other tumors types such as pancreatic cancer and breast cancer.

RO-4929097 (Roche) is a GSI that in early clinical studies demonstrated activity in several tumor subtypes, including melanoma (80). Initial clinical testing suggested a favorable toxicity profile, with low grade toxicities of fatigue, thrombocytopenia, rash, chills, and anorexia (80). Activity was seen in patients with refractory metastatic or locally advanced solid tumors; tumor responses included one complete response in a patient with melanoma (80). In a phase II study of 33 evaluable patients with metastatic colorectal cancer, there were no objective radiographic responses and six patients had stable disease (81). Development of RO4929097 has been discontinued.

PF-03084014 (Pfizer) is a non-competitive reversible GSI which has shown activity in triple-negative breast cancer and desmoid tumors in a phase I study (82). There are no ongoing specific studies in melanoma.

Other GSI in early clinical development are: BMS-708 163, BMS-906 024, LY3039478, LY900009 and MK-0752.

The monoclonal antibodies against receptor ligands of Notch are in early stages of clinical development in various tumor types (though not specifically in melanoma): OMP-59R5 (tarextumab), OMP-21M18 (demcizumab), REGN421/SAR153192, OMP-52M51 (83).

TGF-β pathway inhibitors

TGF-β has biphasic effects during tumorigenesis. In early stages of tumor development, it acts as a tumor suppressor but in the later stages it contributes to its development, inducing EMT (84).

EMT is a dynamic process in which cells undergo bidirectional differentiation from epithelial to mesenchymal state. Cells in different stages of epithelial-mesenchymal differentiation usually co-exist within the same tumor. Cells with mesenchymal differentiation are more invasive. EMT is associated with the acquisition of properties of cancer stem cells and, therefore, worse response to treatment. TGF-β is an important signaling pathway involved in the EMT phenomenon, and a number of transcription factors such as Twist, Snail, Slug and ZEB1 are involved in gene expression changes associated with the mesenchymal phenotype (85). In melanoma, transcription factors ZEB2 and Snail 2 are expressed in normal melanocytes and act as tumor suppressors to activate the program of melanocytic differentiation dependent on MITF. During tumor progression there is an increase in expression of ZEB1 and TWIST1 (86). Melanomas with EMT activation have low levels of MITF.

EMT activation also makes tumors resistant to immunotherapy. Therapeutic strategies have been proposed in laboratory models inducing melanoma differentiation, which reinstates MITF and reverses resistance to immunotherapy (87).

TGF-β ligands (TGF-β1, TGF-β2, TGF-β3) bind the transmembrane serine/threonine kinase TβRI (ALK5), which in turn activate the receptor type II. The heterodimer complex (TβRI with TβRII) phosphorylates the intracellular proteins SMAD2 and downstream effectors SMAD3, SMAD4 that activate signaling to induce several nuclear transduction proteins (88).

In addition, TGF-β is a potent immunosuppressive cytokine that inhibits T cell function and antigen presenting capacity of dendritic cells. In melanoma murine models immunoevasion mechanisms increasing TGF-β in the population of dendritic cells and up-regulation of the enzyme IDO in dendritic cells have been described (89,90).

Many tumors have overexpression of TGF-β. In advanced melanoma overexpression of TGF-β1, TGF-β2 and TGF-β3 is common. Constitutive SMAD signal occurs in response to autocrine secretion of TGF-β. GLI2, a transcription factor involved in the Hedgehog pathway and a transcriptional target of the TGF-β/SMAD, is overexpressed in invasive melanoma (91).

There are several different types of TGF-β inhibitors: small molecule inhibitors of TGF-β receptor type I and II, monoclonal antibodies against TGF-β ligands, and antisense oligonucleotides that block production of TGF-β ligands (*Figure 1*). However, none of these treatments has yet achieved approval for clinical use (92,93).

Trabedersen (AP12009) is an antisense molecule against TGF-β2 which has been studied in phase I-II clinical trials in several tumors, demonstrating a good toxicity profile. In melanoma patients it demonstrated activity with a median survival close to 1 year (94). There are no clinical trials ongoing.

Fresolimumab (GC1008) (Genzyme) is a monoclonal antibody against anti-TGF-β that neutralizes all isoforms of TGF-β. A phase I study has shown activity in patients

with melanoma, with good tolerability. The development of reversible cutaneous keratoacanthomas/squamous-cell carcinomas and hyperkeratosis was the major adverse event (95). There is currently an open phase I trial in combination with radiation therapy in breast cancer that has recently completed recruitment (NCT01401062). There are no ongoing studies in melanoma.

Other antibodies developed by the same pharmaceutical company are CAT-152 (Lerdelimimab) and CAT-192 (Metelimumab), which inhibit TGF-β1 and TGF-β2 respectively.

PF-03446962 (Pfizer) is a humanized monoclonal antibody against ALK-1 (a specific receptor of TGF-β). It has demonstrated safety and but limited clinical activity in phase I-II studies in solid tumors (96,97). There is only one clinical trial ongoing in colorectal cancer.

SB-431542 and SB-505124 (GSK) are small molecule inhibitors of ALK5. They inhibit angiogenesis, and increase maturation of dendritic cell activity and CD8 T cells (98-100). No clinical studies are underway.

YR-290 is a tyrosine kinase inhibitor of ALK5 that has demonstrated activity in breast cancer in animal models (101).

Tasisulam (LY573636) is a tyrosine kinase inhibitor of TGF-β/ALK5. In melanoma, a phase III trial *vs*. chemotherapy was closed early due to toxicity, with three deaths related to the drug (thrombocytopenia) and no activity compared with chemotherapy (102).

EW-7203, 7195-EW and EW-7197 are potent oral inhibitors of TGF-β/ALK5 (103-106), which have shown activity in animal models of melanoma (106).

Galunisertib (LY2157299) (Lilly) is a small molecule inhibitor of ALK5. As with other ALK5 inhibitors, it is not only blocks the phosphorylation of R-Smads, but also induces degradation of Smad4, mainly CD8+ T cells, and increases anti-melanoma CTL response (106). It is the first small molecule inhibitor that has passed to advanced clinical development. In the first in-human trial, it has demonstrated tolerability and preliminary activity in gliomas (107). It has been tested in combination with TKIs such as gefitinib or crizotinib in tumors with MED12 loss (MED12 loss produces activation of TGF-β and activates the EMT process), demonstrating suppression of ERK. It has been suggested that this combination could be active in tumors with high TGF-β signal (108). Currently there is an ongoing phase I study of LY2157299 in combination with the anti PD-1 antibody nivolumab in solid tumors (NCT02423343).

Other drugs from this group are in preclinical development: KI26894, LY364937, LY2109761, SD093, SD208 and LY580276.

Inhibitors of YAP-TEAD1: the Hippo pathway

The Hippo pathway maintains the transcriptional activators Yes-associated protein (YAP) and TAZ in phosphorylated forms in the cytoplasm, preventing cell proliferation. When the Hippo pathway is inactivated, YAP and TAZ are translocated to the nucleus and induce the expression of a variety of proteins concerned with entry into the cell division cycle, such as cyclin D1 and Fox M1, as well as the inhibition of apoptosis (109). Involvement of Hippo pathway in the development of melanoma has been demonstrated, activating processes of invasion, metastasis and resistance (109,110).

Verteporfin inhibits the assembly of a functional YAP-TEAD transcription factor and has been developed mainly in ophthalmology for the treatment of macular degeneration. A phase I-II clinical trial in advanced melanoma was conducted with photodynamic therapy (NCT00007969).

Heat shock protein 90 (Hsp90) inhibitors

Hsp90 is a cellular chaperone that stabilizes and activates over 200 proteins known as "HSP90 clients". Hsp90 is acetylated by HDAC6 and HDAC, resulting in protein degradation by the proteasome "clients" of Hsp90 as Erb1, Erb2, AKT, CRAF, Flt-3 or BRAF mutant. Hsp90 is necessary for the maturation and function of many cellular proteins, including some that are involved in the pathogenesis of melanoma such as BRAF, CRAF, IGF1R, cyclin D1, CDK4 and AKT (111). Also, tumor cells use Hsp90 as a biochemical buffer to protect their mutated oncoproteins from degradation via the proteasome, thereby facilitating tumor survival driven by an oncogene. Overexpression of Hsp90 in tumors has been associated with drug resistance and poor prognosis (112). Hsp90 inhibition may be especially interesting in resistant BRAF mutant melanoma, as preclinical studies indicate that resistance to BRAF inhibitors when mediated by overexpression COT could be reversed by treatment with Hsp90 inhibitors (113).

The first inhibitor developed was tanespimycin (17-AAG), an analog of geldanamycin from BMS. In a phase II study with 17 patients with metastatic melanoma, there was not observed significant activity (114).

BIIB021 is an oral inhibitor of Hsp90. The phase I

study showed acceptable tolerance, with the most frequent toxicity being gastrointestinal. Stabilizations were observed in 11% of cases, none in patients with melanoma (115,116). There are no ongoing studies with this drug in melanoma.

Luminespib (AUY922) is an inhibitor of geldanamycin second generation non-isoxazole Hsp90. In the phase I study in more than 100 patients with various solid tumors there were no objective responses, although it had a good toxicity profile and stabilizations (117). Ongoing clinical studies are focusing on lung cancer.

SNX-5422 in the phase I study treatment-related adverse events were mainly low grade, including diarrhea and nausea. Reversible grade 1-3 nyctalopia (night blindness) was reported by four patients. Objective responses in breast cancer and prostate cancer patients were observed (118).

Ganetespib (STA-9090) is active in resistant BRAF mutant melanoma cell lines with loss of PTEN, inactivation of RB1 or overexpression of COT (111,119). Ganetespib also shows greater activity than AZD6244 or vemurafenib against melanoma cell lines with BRAF mutation. The combination of ganetespib with a MEK inhibitor in BRAF resistant melanomas is active in preclinical models (120). Two phase II clinical trials have been completed in advanced melanoma, as well as in ocular and cutaneous melanoma (NCT01200238, NCT01551693).

XL888 has demonstrated activity in resistant BRAF mutant melanoma cell lines and animal models (121). Currently there is a phase I study in melanoma combining XL888 and vemurafenib (NCT01657591).

Inhibitors of growth factors and their receptors

Development of adaptive resistance with a rapid overexpression of RTKs and/or their stimulating factors as PDGFBR, IGFR1, hepatocyte growth factor (HGF)/c-MET, EGFR3/neuroregulin, FGFR1-4/FGF2 and AXL/Gas6 is common during treatment of BRAF mutant melanomas with BRAF and MEK inhibitors (28). This resistance is mediated by overexpression of RTKs and could be inhibited by combining BRAF inhibitors with RTK inhibitors. In BRAF mutant melanomas, the production of HGF confers resistance to vemurafenib (122); in NRAS mutant melanomas the role of MET/HGF is well known. Recently, six different melanocytic tumors with genomic rearrangements of MET fusing the kinase domain of MET in-frame to six different N-terminal partners have been identified (123). Some MET receptor tyrosine kinase inhibitors are in clinical development.

Tivantinib (ARQ197) in combination with sorafenib has shown clinical activity in a phase I trial with two partial responses and four stabilizations in six patients with NRAS mutant melanomas. Two phase III trials in NSCLC have recently reported an improvement in DFS (124,125).

PHA665752 reduces the proliferation and migration of NRAS mutant cell lines (126).

Onartuzumab (Roche) is a monoclonal antibody against MET that inhibits the binding of HGF to its receptor (127). Recruitment has been recently completed for a phase I clinical trial of onartuzumab in combination with cobimetinib and vemurafenib (NCT01974258).

Crizotinib (Pfizer) is an inhibitor of c-MET, ROS1 and ALK. In melanoma activity in preclinical studies in uveal melanoma has been demonstrated (128). A phase II trial is currently recruiting patients with uveal melanoma after definitive adjuvant treatment with crizotinib (NCT02223819).

E7050 (Eisai) is an inhibitor of c-MET and VEGFR2 (129). A phase II clinical trial in combination with E7080 in advanced melanoma has recently completed recruitment (NCT01433991). Other RTKs involved in melanoma progression and resistance, are PDGFR, IGFR, EGFR, FGFR and Erb3.

Ganitumab (AMG479) (Amgen) is a IGFR1 inhibitor that has demonstrated activity in melanoma cell lines (130). The combination of a MEK inhibitor with a IGF1R inhibitor demonstrated activity in preclinical models (131). In melanoma patients, a phase I study in combination with MEK162 has been completed (NCT01562899).

MEHD7945A (Genentech) is a monoclonal bispecific antibody that inhibits EGFR and HER3. Melanoma cells often express HER3 and neuroregulin which, acting through HER3 activates the PI3K/AKT pathway. Erb3 is overexpressed in BRAF mutant melanomas in response to inhibition of the MAPK pathway (132). MEHD7945A has demonstrated activity in EGFR inhibitor resistant tumors but has not been studied in melanoma (133,134). A phase I trial in solid tumors demonstrated a good toxicity profile with grade 3 toxicity only in one patient with diarrhea and nausea (135). Clinical trials were focused mainly on squamous head and neck and colorectal cancer due to demonstrated activity in these tumors (135). MEHD7945A is currently being studied in a phase I clinical trial in KRAS mutant tumors in combination with cobimetinib, a MEK inhibitor (NCT01986166).

Other EGFR inhibitors have shown activity in

experiments with cell lines and mouse models in melanoma. Lapatinib, a TKI of EGFR and HER2, in combination with BRAF inhibitor reverse resistance in BRAF mutant melanomas (136,137). A phase II trial in Erb4 mutant melanoma patients has been terminated and no clinical responses observed (NCT01264081).

Expression in melanoma cell lines of FGFR1, FGFR4 and FGF family ligands is particularly high. Pharmacological inhibition of FGFRs restores sensitivity in melanoma cells resistant to vemurafenib (138). There are many FGFR inhibitors in development. Among non-selective FGFR TKI are Dovitinib (TKI258), Lucitanib (E3810), Nintedanib (BIBF1120), Brivanib (BMS582664), Lenvatinib (E7080), Ponatinib (AP24534), Orantinib (TSU68) and ENMD-2076. Selective FGFR TKI are BJG398, AZD4547, Debio1347, LY287445, ARQ087, JNJ-42756493 and TAS120 Also there are antibodies and FGF-ligand TRAPas MGFR1877S (RG744) and FP-1039 (GSK3052230) (139). In melanoma models, SU5402, a small molecule FGFR inhibitor, has demonstrated activity (140). In melanoma patients the panFGFR inhibitor BGJ398 (Novartis) is being tested in combination with the BRAF and MEK inhibitor in a phase I trial (NCT02159066) (*Figure 1*).

Other targeted drugs

Among multikinase inhibitors Pazopanib is an inhibitor of VEGFR1, VEGFR2, VEGFR3, PDGFRβ, PDGFRα and c-kit that has FDA approval for sarcoma and renal cell carcinoma. In the phase I trial the most common toxicities were alopecia, fatigue, hypertension, nausea, diarrhea, dysgeusia, neutropenia, myalgia, hair color changes, and peripheral neuropathy, with a manageable safety profile (141). Responses have been reported in melanoma when combining with chemotherapy (141,142). Pazopanib is being tested in a phase II trial in melanoma in combination with chemotherapy (NCT01107665).

Cabozantinib (Exelixis) is a multikinase inhibitor of MET, VEGFR2, RET, KIT, AXL and FLT3, FDA-approved for progressive medullary thyroid cancer. It is active in melanomas with MET fusion proteins (TRIM4-MET and ZKSCAN1-MET) (123). Cabozantinib demonstrated 5% of objective responses in a phase II trial in cutaneous and ocular melanoma (143). A trial comparing with chemotherapy in ocular melanoma is ongoing (NCT 01835145) and a phase I trial in combination with the MEK inhibitor MEK162 has been terminated (NCT01835184).

RAF265 (Novartis) is an inhibitor of wild type and mutant BRAF, VEGFR2, CRAF, PDGFR, CSF, RET, ckit and SRC. It demonstrated activity in the first in-human study both in BRAF mutant and wild type tumors (144). A phase II trial in melanoma has been completed testing different schedules (NCT 00304525).

JAK/STAT inhibitors are under development in multiple tumors including melanoma. WP1066 is a STAT3 inhibitor with activity in resistant BRAF melanoma, there is an ongoing clinical trial in melanoma patients with brain metastases (NCT 01904123) (*Figure 1*). Melanoma cells are sensitive to other STAT3 inhibitors such as curcumin and their analogues, and to other natural inhibitors such as BBMD3, a derivative of the natural bis-benzylisoquinoline alkaloid berberine (145).

Other therapeutic targets in melanoma are have been recently defined, such as IL-2 inducible T-cell kinase (ITK), a known immune cell-specific protein I is inhibited by the small-molecule inhibitor BI 10N that is active in mouse melanoma models (146).

Conclusions

In recent years the clinical development and approval of BRAF and MEK inhibitors for treatment of BRAF mutant melanoma has led to increased interest in drug discovery for molecular targets implicated in melanoma development and progression.

Although the main focus of interest in this area has moved towards immunotherapy, several new drugs that inhibit the main activated pathways in melanoma are now in clinical development. PanRAF inhibitors and ERK inhibitors could be of special interest for treatment of BRAF mutant melanoma as they can circumvent the main resistance mechanisms to BRAF inhibitors. Evidence precludes the development of several combinations as MAPK inhibitors with PI3K inhibitors or RTK inhibitors, as TGFR inhibitors with immunotherapy. Ever-growing understanding of melanoma molecular biology will help us design the most active and specific drugs for continued improvements in treatment in the coming years.

Acknowledgements

Footnote

Conflicts of Interest: The authors have no conflicts of interest

Page 12 of 17

González-Cao et al. New targeted drugs in melanoma

to declare.

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Page 14 of 17

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Page 16 of 17

González-Cao et al. New targeted drugs in melanoma

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