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Utilizing Systems Level Approaches to Identify Key Mechanisms of Drug Resistance in BRAF Mutated Melanoma

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Utilizing Systems Level Approaches to Identify Key Mechanisms of Drug Resistance in BRAF Mutated Melanoma

by

Kim H. T. Paraiso

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Cell Biology, Microbiology, and Molecular Biology with a concentration in Cancer Biology College of Arts and Sciences University of South Florida

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Dedication

I dedicate this work to Isabella Mai Paraiso. Your imagination, enthusiasm and laughter are infectious. Your sincerity, compassion and generosity are inspiring. With you, every day is an adventure full of endless possibilities. You had me at alum.

"Ten tiny little fingers that always want to play, that never stop exploring the wonder of today. Ten tiny little fingers that from the very start, will reach out for tomorrow yet always hold your heart." -Unknown
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Abstract

In the last four years, seven new drugs have been FDA approved for the treatment of late stage melanoma, for the field of melanoma, this marks an incredibly exciting. Three of these new therapies, vemurafenib, dabrafenib and trametinib are small molecule kinase inhibitors that target the MAPK pathway and as such have been approved for the treatment of BRAFV600 mutant melanomas. Yet despite recent advances, mechanisms of intrinsic and acquired BRAF inhibitor resistance continue to undermine uniform and long-lasting therapeutic responses. Several studies have shown that the reactivation of MAPK signaling is a critical event leading to BRAF inhibitor resistance. These studies lead to the evaluation and subsequent FDA approval of frontline BRAF (dabrafenib) plus MEK (trametinib) inhibitors to delay drug resistance. Though this approach has meaningful clinical benefit, there are still a number of patients who do not respond to therapy or who, through unknown mechanisms, succumb to refractory disease. In an effort to identify drivers of MAPK inhibitor resistance, several studies have relied on traditional genomics methods. While gene-based approaches have guided precision medicine, they do not address the dynamics of the global signaling changes that occur following acquired resistance.

The dissertation herein will describe our efforts to fill these gaps of knowledge and will expand upon the evolution and development of our understanding of intrinsic and acquired MAPK pathway inhibitor resistance. This work will elaborate on our early understanding of single agent BRAF inhibitor resistance, the use of genomic and proteomic approaches to further elucidate these mechanisms, and evidence based
approaches to delay and overcome single agent BRAF inhibitor resistance. This work will describe global phosphoproteomic and bioinformatics methodologies to elucidate the underlying processes of both single (BRAF) and dual agent (BRAF plus MEK) inhibitor resistance as well as strategies to constrain dual agent BRAF plus MEK inhibitor resistance.
Chapter 1

Introduction

Note to Reader

Portions of this chapter have been previously published in Skin Cancer and Melanoma, Biotargets of Cancer in Current Clinical Practice, M. Bologna Editor, Springer/Humana Press, New York, April 2012 \(^1\) and have been reproduced with permission from Humana Press Publishing. Author contributions: Paraiso KH (writing and figures), John J (writing), Smalley KS (writing, concept and design). Portions of this chapter have also been previously published in Fibroblast-mediated drug resistance in cancer. Biochem Pharmacol. 2013 Apr 15;85(8):1033-41 \(^{1,2}\) and have been reproduced with permission from Elsevier Publishing. Author contributions: Paraiso KH (writing, figures, concept and design), Smalley KS (writing, concept and design).

Melanoma: Incidence, Epidemiology, and Risk Factors

Skin cancer is the most common type of cancer whose major risk factor is ultraviolet (UV) radiation \(^3\). Non-melanocytic and melanocytic skin cancers follow distinct biological behaviors and clinical courses. For example, while basal cell carcinomas (BCC) are the most common form of skin cancer, accounting for approximately 56% of skin cancers, they rarely metastasize and are infrequently fatal. In contrast, melanomas account for less than 2% of all skin cancers yet are frequently aggressive, therapy resistant, and responsible for the majority of skin cancer related deaths \(^3,4\).
In 2014, it was estimated that 76,100 new cases of melanoma would be diagnosed in the US, with 63,770 of these cases being melanoma in situ. In addition, it was predicted that approximately 9,710 deaths would occur due to melanoma \(^4,5\). While overall rates of cancer deaths continue to decrease, the risk of death from melanoma, particularly in male patients, continues to rise with a 7% increase in male deaths from 1990–2006 \(^6\). The incidence of melanoma is both gender and age related with frequencies being higher in women than men who are less than 40 years of age and then significantly higher in men than women who are 40 years of age or older \(^7,8\). The incidence of melanoma has been steadily increasing over the last 30 years with frequencies in younger adults (age 20-39) rising at alarming rates \(^9,10\). Among teens aged 15-19, melanoma accounts for 6% of all cancer cases compared to 4.6% for patients of all age groups \(^11\).

The two main risk factors for developing melanoma are UV exposure (e.g., sunlight and the use of tanning beds) and a family history of melanoma, with skin type, number of nevi, age and ethnicity also playing a role in melanoma development \(^7,10\). Ultraviolet radiation is known to damage genomic DNA leading to mutations, and as such, significant correlations have been found between average annual UV exposure and melanoma risk \(^12\). Similar melanoma risk correlations are also related to latitude (with UV radiation being most intense at the equator) and altitude (where UV exposures are greater at higher elevations) \(^12\). The causative role of UV exposure in melanoma development is supported further by the findings that individuals with poor tanning responses (such as those with fair skin and a tendency to freckle), and a presumably higher rate of UV-induced DNA damage, have the highest risk of melanoma.
development. Support for these epidemiological studies also comes from whole genome-sequencing studies where multiple UV-signature mutations were detected in human melanoma cell lines. Additionally, the reduction of UV exposure through the regular use of sunscreens has been shown to significantly reduce melanoma development by ~50%.

The second largest risk factor for developing melanoma is family history, with approximately 10% of all melanomas occurring in familial clusters. High-penetrance genes, encoding for the cyclin-dependent kinase inhibitor 2A (CDKN2A: p16) and cyclin-dependent kinase 4 (CDK4), respectively, have been associated with the development of hereditary melanoma. Of these, the incidence of CDKN2A mutation is more prevalent and thought to account for 20-40% of all familial melanomas. In comparison, CDK4 mutations have only been identified in 15 families worldwide, making them relatively rare.

Another predictive factor for melanoma development is the presence of large numbers of nevi. Commonly acquired nevi are melanocytic proliferations that generally develop on areas of sun-exposed skin. Although most nevi are benign, they can, in rare cases, develop into melanoma. There is a good correlation between nevus number and melanoma development, with a 20-fold increased risk of developing melanoma in individuals who have >120 nevi.

**Melanoma Diagnosis and Prognosis**

Currently accepted prognostic markers for melanoma are vertical tumor thickness (Breslow thickness), mitotic rate (measured as the number of mitoses per mm²), presence of ulceration, degree of lymph node involvement, invasion level (Clark
level), the absence or presence of vertical growth phase and regression. Breslow thickness is measured in millimeters from the granular layer of the epidermis down to the deepest point of invasion (Fig. 1.1) while Clark level describes the level of anatomical invasion of the melanoma in the skin (epidermis, papillary dermis, reticular dermis, subcutis). The current version of the AJCC melanoma staging and classification defines tumor thickness, mitotic rate and ulceration as the most powerful predictors of survival in patients with localized melanomas (stages I and II). For patients with nodal metastases (stage III), the number of regional lymph nodes harboring metastatic disease, regional node tumor burden, and ulceration of the primary tumor are the most powerful independent predictors of survival. Among patients with stage IV disease, the anatomic site of distant metastases was the most significant predictor of survival. In those with distant skin, subcutaneous tissues, and/or lymph node basin metastasis 62% had a one-year survival rate. Pulmonary metastasis was associated with a one-year survival rate of 53% while non-pulmonary visceral metastases and/or an elevated serum lactate dehydrogenase (LDH) have the worst one-year survival of 33%. 

The risk of metastasis to lymph nodes is directly related to Breslow thickness, mitotic rate and ulceration of the primary melanoma. Metastases are rare for thin melanomas (<1 mm) while the risk for tumors 1-4 mm thick is about 5%. Melanomas with an intermediate thickness (1-4 mm) have a risk that starts at about 8% for 1.0-mm tumors, this risk increases steadily to 30% with increasing depth. In addition to a high risk of systemic spread, melanomas >4.0 mm have a risk of approximately 40% nodal involvement. Though the survival benefit of sentinel lymph node dissection is highly
debated, the status of the sentinel lymph nodes (which receive direct lymphatic drainage from the primary tumor site) does provide accurate prognostic information for overall and disease-free survival for melanomas stage T1b or greater \(^{20,28-30}\).

![Diagram showing the layers of the skin and its constituent cells.](image)

**Fig. 1.1.** Schematic showing the progression of melanoma by Clark level Breslow thickness and mitotic rate. Diagram shows the layers of the skin and its constituent cells.

Although most melanomas can be adequately diagnosed through histological criteria, there are subsets of melanoma that are difficult to distinguish from benign melanocytic nevi. Specific examples include certain types of nevi, such as dysplastic nevi and Spitz nevi, as these share overlapping histopathological features with melanomas. Diagnosis of these cases is especially difficult as none of the histochemical or immunohistochemical markers used in routine diagnosis can sufficiently differentiate between nevi and melanoma \(^{31-33}\). The one exception to this is the marker HMB45, which, in benign and dysplastic nevi, shows a gradient of strong staining in the superficial cells and weak-to-negative staining in the deeper tumor cells. In melanoma,
HMB45 shows strong staining in the deep tumor cells. Since misdiagnosis can have potentially serious consequences, there have been a number of attempts to develop diagnostic markers that allow for the differentiation of benign and malignant melanocytic lesions. A study by Kashani-Sabet et al. defined a panel of five molecular markers that included developmental WNT pathway member-2 (WNT-2), fibronectin (FN1), actin-related protein 2/3 complex subunit 2 (ARPC2), secreted phosphoprotein-1 (SPP1), and regulator of G-protein signaling 1 (RGS1) 34. Using a sample set of 693 melanocytic lesions (composed of Spitz nevi, melanomas, nevi, and misdiagnosed melanomas), the authors successfully used their marker panel to differentiate benign melanocytic lesions from melanoma with a specificity of 95% and a sensitivity of 91% 34.

The diagnosis of amelanotic melanomas can also be difficult. In these cases, the approach of choice is the immunohistochemical staining of lesions for components of the pigmentation machinery including the S100 protein, gp100 (HMB-45 antigen), and melanoma antigen recognized by T-cell one (MART-1; Melan-A protein). MART-1/Melan-A, S100, and gp100/HMB-45 show high sensitivity for melanoma (75-92%, 97-100%, 69-93%). However, since all of these markers are also found in melanocytic nevi, their specificity to distinguishing melanoma from nevi is low 31-33. MART-1/Melan-A and S100 strongly stain both benign and malignant melanocytic neoplasms.

Melanomas frequently metastasize to distant sites (Table 1.1 35-37), therefore it is critical to identify patients who are at risk for relapse and dissemination. Although biomarker strategies have been used successfully for prognostic and diagnostic purposes in other tumor types, no reliable biomarkers have yet been identified that are both highly sensitive and melanoma specific 38,39. A recent meta-analysis of the
literature identified over 515 publications \(^{40}\) that described novel melanoma biomarkers. As yet, none of these have found their way into routine clinical practice for either diagnostic or prognostic purposes. The reasons for this lack of translation were manifold and included the lack of statistical power in the sample size and inadequate validation techniques. Of the initial 515 studies under consideration, only 37 of these were judged to be worth analyzing. The molecules identified in this subset of publications included melanoma cell adhesion molecule (Mel-CAM), matrix metalloproteinase-2 (MMP-2), the proliferation markers Ki67 and proliferating cell nuclear antigen (PCNA), and the tumor suppressor locus p16 INK4A \(^{40}\).

Table 1.1. Sites and frequencies of melanoma metastases. Several factors such as integrins, chemokines/chemokine receptors and growth factor/growth factor receptors have been associated with homing of disseminated melanoma cells to specific tissues.

<table>
<thead>
<tr>
<th>Site</th>
<th>Associated factors</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenals</td>
<td></td>
<td>36-54</td>
</tr>
<tr>
<td>Bone</td>
<td></td>
<td>23-49</td>
</tr>
<tr>
<td>Brain</td>
<td>NGF-R</td>
<td>36-54</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td>40-45</td>
</tr>
<tr>
<td>Intestine</td>
<td>(\alpha_4\beta_1,\ \text{CCR9:CCL25})</td>
<td>26-58</td>
</tr>
<tr>
<td>Kidneys</td>
<td></td>
<td>35-48</td>
</tr>
<tr>
<td>Liver</td>
<td>CXCR4:CCL25, C-MET:HGF, IGF1R:IGF1</td>
<td>54-77</td>
</tr>
<tr>
<td>Lung</td>
<td>(\alpha,\ \beta_1)</td>
<td>70-87</td>
</tr>
<tr>
<td>Pancreas</td>
<td></td>
<td>38-53</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>(\alpha_4\beta_1,\ \text{VEGFR3:VEGF-C, CCR7:CCL21})</td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td></td>
<td>50-75</td>
</tr>
<tr>
<td>Subcutaneous tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid</td>
<td></td>
<td>25-39</td>
</tr>
</tbody>
</table>

The Ki-67 protein is expressed at all stages of the cell cycle except for the G0 quiescent phase and is considered to be a sensitive marker of cell proliferation. Expression of dermal Ki-67 is related to the development of melanoma metastases, with high dermal expression of Ki-67 of \(>20\%\) being demonstrated to be an independent prognostic factor \(^{41}\). A 10-year retrospective study of 396 patients with thin melanomas stained for Ki-67 identified two groups with high risks of metastasis: the first consisted of
men and women with a dermal mitotic rate of >0 and a dermal Ki-67 positivity of >20% and the second group consisted of men with a mitotic rate of >0 and a dermal Ki-67 of <20%, with 10-year metastasis rate of 39% and 20%, respectively.

The increased invasive potential of melanoma cells compared to melanocytes is due in part to altered expression of cell–cell and cell–matrix proteins, and a number of these molecules have been investigated as potential prognostic biomarkers for melanoma. Melanoma cells are known to express increased levels of receptors of the immunoglobulin gene superfamily of cell adhesion molecules (CAMs), such as melanoma cell adhesion molecule (MCAM, Mel-CAM, MUC18, CD146), L1 cell adhesion molecule (L1-CAM, CD171), activated leukocyte cell adhesion molecule (ALCAM, CD166), vascular cell adhesion molecule 1 (VCAM-1, CD106), intercellular cell adhesion molecule 1 (ICAM-1, CD54), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1, CD66a). Of these, Mel-CAM is required for homologous melanoma/melanoma and heterologous melanoma/endothelial cell/cell interactions. In melanocytic cells, expression of Mel-CAM is initially found in nevi, when the cells have separated from the epidermal keratinocytes and have migrated into the dermis. As the tumor progresses, Mel-CAM expression gradually increases and is at its highest in melanoma metastases. Two recent studies (n = 76 and n = 170, respectively) have shown Mel-CAM expression to independently predict for development of lymph node metastases and worse overall survival (after adjustment for age, Breslow index, and Clark level). It was noted that Mel-CAM-negative patients had a 5-year survival of 92% compared to 40% for patients who were Mel-CAM positive. One further study, on a larger cohort of patients (n = 340) showed Mel-CAM
expression to predict for disease-free and overall survival in a univariate analysis but not when a multivariate analysis was performed. The discrepancy between this and the two previous studies was suggested to be a consequence of differences in the antibodies used and the methods of sample preservation.\(^{51}\)

L1-CAM is a neuronal cell adhesion molecule that is expressed in melanoma cells.\(^{51,52}\) It mediates adhesion through hemophilic (L1-CAM/L1-CAM) and heterophilic (L1-CAM/-/\(\alpha_\nu\beta_3\) integrin) mechanisms and allows for melanoma/melanoma and melanoma/endothelial cell interactions through its binding to \(\alpha_\nu\beta_3\) integrin.\(^{53,54}\) The interaction of L1-CAM and \(\alpha_\nu\beta_3\) integrin plays an important role in transendothelial migration of melanoma cells, while overexpression of L1-CAM in the absence of upregulated \(\alpha_\nu\beta_3\) integrin expression, promotes the conversion of melanomas from radial to vertical growth phase.\(^{55,56}\) L1-CAM immunoreactivity is also increased in melanoma compared to nevi.\(^{57}\) A study that systematically identified novel melanoma-specific genes confirmed that L1-CAM was not expressed in normal skin and melanocytic nevi but was highly and differentially expressed in primary melanoma tissues and melanoma lymph node metastases.\(^{58}\) A recent study, evaluating 12 nevi, 67 primary melanomas, 40 sentinel lymph nodes and 35 distant metastases, showed L1-CAM to be a highly sensitive (90–93%) and specific (100%) diagnostic marker for melanoma.\(^{33}\) A 10-year retrospective biomarker study, evaluating 100 melanoma specimens, showed the expression of L1-CAM in human primary cutaneous melanoma to be associated with metastatic spread and an independent predictor for metastasis.\(^{52}\)

Intercellular adhesion molecule (ICAM)-1 binds to integrin \(\alpha_\nu\beta_2\) (lymphocyte function associated antigen 1, LFA-1) and Mac1 on lymphocytes.\(^{59}\) Its expression is
known to correlate with melanoma progression and the increased risk of metastasis \(^{60}\). Increases in ICAM-1 expression parallel the transition from nevi to melanoma metastasis and correlate with Breslow index in primary melanomas \(^{61-64}\). The role of ICAM-1 in melanoma progression and metastasis was also supported by the fact that stage I patients with ICAM-1-positive melanomas had a significantly shorter disease free interval and overall survival than those with ICAM-1 negative tumors \(^{62}\). Further, the suppression of ICAM-1 in an animal model study reduced the metastatic capacity \(^{65}\). However, the exact role of ICAM-1 in melanoma progression remains obscure with contradictory reports showing that ICAM-1 promotes both the aggregation of melanoma cells with leukocytes, thereby enhancing survival of tumor cells in the vascular system, and that ICAM-1 is shed from melanoma cells, possibly in a form that inhibits lymphocyte–tumor cell interactions \(^{66-68}\).

CEACAM1, CD66a, is a member of the immunoglobulin family of cellular adhesion molecules involved in intercellular adhesion. In epithelial cells, CEACAM1 acts as a growth suppressor with its expression being either lost or significantly down- or deregulated in carcinomas of liver, prostate, endometrium, breast, and colon \(^{42}\). CEACAM1 interacts with the \(\beta3\) integrin subunit via the CEACAM1 cytoplasmic domain colocalizing at the tumor–stroma interface, suggesting a role for the CEACAM1-integrin \(\beta3\) interaction in melanoma cell migration and invasion and the development of metastases \(^{69,70}\). Forced overexpression of CEACAM1 in CEACAM1-negative melanocytic cells and melanoma cell lines leads to increased migratory and invasive growth potentials in vitro, supporting the role of CEACAM1 in melanoma progression and metastasis \(^{71}\). An evaluation of 12 nevi, 67 primary melanomas, 40 sentinel lymph
nodes, and 35 distant metastases showed CEACAM1 to be a highly sensitive (93-97%) and specific (63%) diagnostic marker for melanoma \(^{33}\).

E-cadherin is expressed on the cell surface of both keratinocytes and melanocytes and is the major adhesion molecule mediating the interaction between these two cell types in the epidermis \(^{72}\) \(^{73}\). In culture, melanoma cells undergo a cadherin switch. This loss of E-cadherin and gain of N-cadherin expression allows melanoma cells to associate with fibroblasts and vascular endothelial cells \(^{72}\). Although experimental studies confirm that E-cadherin loss is critical to melanoma progression, the clinical data are conflicting and show that E-cadherin expression is not decreased in many cases of advanced melanoma \(^{74}-^{76}\). A study evaluating 144 primary melanomas, 53 metastases, and 8 nevi reported E-cadherin expression to be significantly correlated with primary tumor depth, but not predictive of patient outcome \(^{77}\). However, when the E-cadherin expression data was combined with that of the calcium-binding protein S100A4 data, a significant positive correlation between S100A4 negative, E-cadherin high biopsies and disease-free survival was revealed \(^{77}\). Another study evaluating 115 melanoma samples (55% of which were acral lentiginous melanomas), with a range of 4 to 85 months follow-up (median 69 months), reported that 91% of the tumors showed reduced E-cadherin expression; however, in this study there was no significant correlation between the level of E-cadherin expression and patient survival \(^{78}\).

**Molecular Subtypes of Melanoma**

**BRAF Mutant Melanoma**

A significant advance in our understanding of melanoma initiation and progression was the discovery of activating \textit{BRAF} mutations that occur in over 50% of
Rapidly growing fibrosarcoma (derived from Raf) proteins constitute a three member family of serine/threonine kinases (ARAF, BRAF, and CRAF) with closely overlapping functions. So far, over 50 distinct mutations in BRAF have been identified. Of these, the BRAFV600E mutation, resulting from a valine to glutamic acid substitution, is the most common and accounts for over 80% of all reported BRAF mutations. Most of the tumor initiating activity of the BRAFV600E mutation is thought to result from the stimulation of the MAPK pathway (Fig. 1.2). Constitutive activity in the RAS/RAF/MEK/ERK MAPK pathway contributes to the oncogenic phenotype of melanoma by increasing cell proliferation, invasion, and survival (Fig. 1.3).

Fig. 1.2. Cell signaling scheme of pathways activated in melanoma cells through either receptor tyrosine kinases (RTKs), Ras, or BRAF. Irrespective of the activating oncogenic mutation, nearly all melanoma cells are known to have constitutive signaling in the BRAF/MEK/ERK and PI3K/AKT pathways. Together, these pathways drive the uncontrolled growth of melanoma cells and prevent the induction of apoptosis.
Regulation of cell growth, particularly at the G1 cell-cycle checkpoint is the most well characterized role for downstream MAPK signaling in melanoma. Progression through the G1 restriction point into S-phase is driven by cyclin-dependent kinases (CDK) 4 and 6 which interact with cyclin D1, as well as by CDK2 which interacts with cyclins A/E (Fig. 1.2)\textsuperscript{85}. Inhibition of either BRAF or MEK in melanoma cell lines using siRNA strategies and pharmacological inhibitors leads to a profound G1 phase cell-cycle arrest. In melanoma, cell cycle progression is also associated with upregulated cyclin D1 and downregulated p27 expression\textsuperscript{86}.

Fig. 1.3. Constitutive MAPK signaling plays a key role in the oncogenic behavior of melanoma cells. Increased activity in the MAPK pathway is known to decrease control by local skin keratinocytes by downregulating E-cadherin expression. Pathway activity also stimulates melanoma growth by increasing cyclin D1 expression and invasion through the modulation of integrin expression and increased matrix metalloproteinase (MMP) release.
In experimental systems, the role of mutated \textit{BRAF} in melanoma is convincing. In vitro studies have shown that V600E mutant \textit{BRAF} is an oncogene in immortalized mouse melanocytes and that selective downregulation of the V600E-mutated \textit{BRAF} using RNAi leads to reversal of the melanoma phenotype\textsuperscript{87,88}. Increased BRAF activity also suppresses the activity of the melanocyte-specific transcription factor microphthalmia (MITF), diverting the melanoma cells from a differentiated state into one of rapid proliferation\textsuperscript{89}. There is also evidence implicating BRAF in the of anoikis, with siRNA studies showing a link between mutated BRAF down-regulation of the pro-apoptotic BH3-family (Bcl-2 family) proteins BIM, BAD, and Mcl-1\textsuperscript{90-92}.

Acquisition of the \textit{BRAF}V600E mutation appears to be an early event in melanoma development with a high percentage of nevi found to be \textit{BRAF}V600E mutation positive\textsuperscript{93}. In line with observations that nevi only rarely develop into melanoma, the presence of a \textit{BRAF}V600E mutation alone is not sufficient to oncogenically transform primary human melanocytes into melanoma and instead leads to an irreversible growth arrest which is characteristic of senescence\textsuperscript{94}. Clinical studies have confirmed these findings and have shown that most nevi are growth-arrested and stain positively for the senescence marker \(\beta\)-galactosidase\textsuperscript{94}. This phenomenon, which is termed “oncogene-induced senescence” is an important mechanism by which cells protect themselves from oncogenic transformation by activating pathways leading to irreversible cell cycle exit, such as the ARF/p53/p21 axis and the cyclin-dependent kinase inhibitor p16 INK4A\textsuperscript{94}. As both silencing and mutation of the \textit{p16} gene is a common event in some inherited forms of melanoma, \textit{BRAF} mutations were initially thought to occur in tandem with p16 inactivation\textsuperscript{95}. Interestingly, this seems not to be
the case with the introduction of mutated \textit{BRAF} leading to an irregular pattern of p16 induction \textsuperscript{94}. Further \textit{in vitro} studies confirmed the clinical findings and showed that siRNA knockdown of \textit{p16} in melanocytes did not lead to malignant transformation when combined with the \textit{BRAFV600E} mutation \textsuperscript{94}. In addition introduction of \textit{BRAFV600E} alone not found to activate the ARF/p53/p21 axis \textsuperscript{94}.

A preponderance of evidence now supports the idea that multiple signaling pathways must be activated to drive melanoma development. The other major signal transduction cascade known to contribute to melanoma initiation and development is the PI3K/ AKT pathway. Activation of PI3K/AKT signaling occurs via multiple mechanisms, and in \textit{BRAFV600E}-mutated melanocytes, melanoma arises through loss or inactivation of \textit{PTEN}, and mutations in \textit{AKT3} (Fig. 1.2) \textsuperscript{95-97}. The strongest supporting evidence for the dual requirement of \textit{BRAF} and PI3K/AKT signaling in melanoma initiation comes from mouse modeling studies showing that the mutant \textit{BRAF} alone can only lead to the development of melanocytic hyperplasia while mutant \textit{BRAF} paired with \textit{PTEN} inactivation leads to melanoma development \textsuperscript{98}.

Less frequently, \textit{BRAF} mutations also occur in positions other than 600. In isolated kinase assays, many of non-V600 \textit{BRAF} mutants tend to show impaired BRAF kinase activation and are dubbed “low-activity \textit{BRAF} mutants”. Similar to \textit{NRAS} mutants, low-activity \textit{BRAF} mutants require the presence of CRAF to transactivate their MAPK signaling \textsuperscript{83}. Analysis of a large panel of melanoma cell lines and tissues revealed that \~1\% of melanoma cell lines had either D594G or G469E mutation in \textit{BRAF}, respectively, and that 1\% of melanoma specimens harbored a G469A mutation in \textit{BRAF} \textsuperscript{99}. These low-activity \textit{BRAF}-mutated cell lines differed in their signaling from
BRAFV600E mutants and showed high levels of phospho-ERK, low levels of phospho-MEK, and resistance to MEK inhibition\textsuperscript{99}.

**NRAS Mutant Melanoma**

The first activating oncogenic mutation to be reported in melanoma was in *NRAS*\textsuperscript{100,101}. *NRAS* mutant melanomas constitute the most significant subgroup of *BRAF* wild-type melanomas identified so far. RAS proteins are a large family of low molecular weight GTP-binding proteins (or GTPases). Three of the RAS family members, *NRAS*, *HRAS*, and *KRAS*, are often mutated in human cancers, and >20% of all lesions harbor an activating RAS mutation\textsuperscript{102}. Since their discovery in 1984, *NRAS* mutations have been identified in 15-20% of all melanomas and are typically the result of a point mutation leading to the substitution of leucine to glutamine at position 61\textsuperscript{79,103}. *NRAS* mutations have also been reported at positions 12 and 13\textsuperscript{104}. Mechanistically, the acquisition of point mutations in *NRAS* leads to impaired GTPase activity, leading to more abundant levels of GTP-bound NRAS than GDP-bound NRAS. This facilitates the recruitment of adapter proteins leading to an increase in intracellular signaling. In addition to *NRAS*, 1-2% of melanomas have *KRAS* mutations while 2% having *HRAS* mutations\textsuperscript{79,103}. The predominance of mutations in *NRAS* (compared to the other isoforms of RAS) may be due in part to the overexpression of *NRAS* in melanocytes relative to other RAS isoforms. It is also possible that NRAS possesses distinct signaling properties from the other RAS isoforms that favor melanocyte transformation\textsuperscript{105}. In agreement with this, *NRAS* has a greater transforming activity than *KRAS* in mouse melanoma models even though both mutated GTPases stimulate RAF signaling in mouse melanocytes\textsuperscript{105}. In its GTP-bound state, RAS binds to and activates a number
of effector signaling pathways involved in proliferation. The most well characterized of these is the serine/threonine kinase RAF\textsuperscript{106}. Most of RAF’s oncogenic activity is mediated through activation of the mitogen activated protein kinase (MAPK) cascade, which regulates the cell-cycle entry through control of cyclin D1 expression (Fig. 1.2)\textsuperscript{107}. RAS is also known to activate the PI3K/AKT pathway which contributes to tumor progression via the modulation of growth and survival of transformed cells (Fig. 1.2)\textsuperscript{107}.

Although \textit{BRAF} and \textit{NRAS} mutant melanomas tend to show constitutive activation of RAF/MEK/ERK and PI3K/AKT signaling, there are important differences in how these pathways are regulated. Melanomas harboring activating \textit{NRAS} mutations are different from melanomas with \textit{BRAF} mutations in that they rely upon CRAF to induce their MAPK pathway activity\textsuperscript{107}. In normal melanocytes, receptor tyrosine kinase (RTK)-induced activation of RAS leads to the stimulation of both \textit{BRAF} and \textit{CRAF}\textsuperscript{107}. Under these conditions, constitutive protein kinase A (PKA) activity leads to the phosphorylation and inactivation of CRAF and therefore, activation of the MAPK pathway proceeds through \textit{BRAF} signaling. In melanomas with \textit{NRAS} mutations, the cyclic AMP/PKA system is deregulated so that PKA no longer suppresses CRAF, thereby allowing CRAF-mediated MAPK activation to occur\textsuperscript{107}.

\textit{c-KIT Mutant Melanoma}

The \textit{KIT} gene was originally identified as the viral oncogene \textit{v-KIT}, derived from the feline sarcoma virus HZ 4-FeSV, and then subsequently as the proto-oncogene form \textit{c-KIT}. \textit{c-KIT} is an RTK member of the platelet-derived growth factor (PDGF) family. Structurally, it is composed of five immunoglobulin-like motifs in the extracellular portion and a hydrophilic kinase insert domain that forms the intracellular portion (Fig.
Its ligand is the glycoprotein stem cell factor (SCF), which is also known under a variety of other names including mast cell growth factor and steel factor (SF). SCF activates c-KIT through binding and the induction of a process that leads to receptor dimerization and autophosphorylation. Sequencing of c-KIT exons 11, 13, 17, and 18 revealed the most prevalent mutations in melanoma to be K642E, L576P, D816H, and V559A substitutions.

![Diagram of c-KIT receptor and signaling pathways](image)

**Fig. 1.4.** Structure of the c-KIT receptor and signaling pathways activated downstream of KIT. Upon phosphorylation, the KIT receptor stimulates the MAPK pathway (Ras/Raf/MEK/ERK) through recruitment of an adaptor complex consisting of Grb2/Sos/Shc. Other phosphorylation sites on the KIT receptor are also known to recruit Src family kinases and the p85 subunit of PI3K. Activation of all of these pathways leads to increased growth and survival of melanoma cells.

It was also shown that the presence of a c-KIT mutation is typically accompanied by an increase in KIT gene copy number and genomic amplification as identified by array comparative genomic hybridization (a-CGH). There were cases where c-KIT was amplified in the absence of a mutation, and therefore it was reported that a total number of c-KIT aberrations (either amplification and/or mutation), were 39% for
mucosal, 36% for acral, and 28% for melanomas arising from sun-damaged skin. Subsequent studies have shown that c-KIT is expressed in 88% of oral mucosal melanomas and that at least 22% of these harbor activating c-KIT mutations. Another study also reported the presence of the activating L576P mutation in c-KIT in 15% of anal melanomas, a mutation that was shown to be imatinib sensitive in vitro.

It should however be noted that acral lentiginous and mucosal melanomas are relatively rare and when combined account for only ~4% of all melanomas, therefore the total number of melanoma patients presenting with activating mutations in c-KIT are likely to be quite low.

Another subset of BRAF WT melanomas that express high levels of c-KIT and CDK4 have also been reported. These melanoma cell lines lacked activating c-KIT mutations and showed no evidence of an SCF/c-KIT autocrine loop. They were however, found to have constitutive c-KIT receptor signaling as shown by the presence of high-levels of phospho-c-KIT expression suggesting that the signaling activity may have arisen as a consequence of very high receptor expression levels leading to spontaneous receptor dimerization. Similar findings have been reported in non-small cell lung cancer where very high epidermal growth factor (EGF) receptor expression levels lead to constitutive signaling activity.

**BRAF/NRAS Wild-type**

Melanomas that are genetically classified as BRAF/NRAS wild-type (WT) comprise a considerable proportion (13-26%) of all melanomas. Tumor-initiating events for this subgroup are largely unknown and as such efforts are currently underway to study this significant subpopulation of melanomas. However, a
recent examination of the whole exomes of 34 patient-derived fresh-frozen primary cutaneous melanomas revealed that the majority of \textit{BRAF/NRAS} WT melanomas lacked single driver mutations and instead carried an overall higher mutational burden when compared to \textit{BRAF} or \textit{NRAS} mutated melanomas \textsuperscript{115}. These mutations were consistent with UV-induced damage with a higher proportion occurring as C>T transitions and CC>TT dinucleotide transitions \textsuperscript{115}. These mutations were reported to occur within “hotspots” of the \textit{NF1} and \textit{RAC1} genes \textsuperscript{114,115}.

Recent studies utilizing patient-derived melanoma cell lines also support the hypothesis that \textit{NF1} mutations could contribute to tumorigenesis in cutaneous \textit{BRAF/NRAS} WT melanoma \textsuperscript{116}. Loss of \textit{NF1} (a tumor suppressor gene that encodes a protein required for hydrolyzing GTP to GDP) was associated with RAS upregulation and downstream ERK activity. \textit{BRAF/NRAS} WT, \textit{NF1} mutants were found to be resistance to RAF inhibition but maintained MEK dependency as seen by sensitivity to the MEK inhibitor, trametinib, wherein treatment with the MEK1/2 inhibitor lead to durable ERK downregulation and growth arrest \textsuperscript{116,117}. However, cell death was not induced in studies of single agent MEK inhibitors. Therefore it remains to be seen whether further genetic subgrouping of this heterogeneous population of melanomas will be able to guide prospective therapeutic interventions.

\textbf{Pathological and Prognostic Features of Melanomas with Different Activating Mutations}

Both \textit{NRAS} and \textit{BRAF} mutant melanomas are found on sun-exposed skin, whereas \textit{c-KIT} mutant melanomas arise on sun-protected sites \textsuperscript{118,119}. Therefore, \textit{BRAF} mutant melanomas rarely occur on the palms of the hands, the soles of the feet (or as
acral melanomas occurring on subungual sites), or on mucous membranes (mucosal melanomas)\textsuperscript{108}. Evidence is emerging that the duration and frequency of sun exposure may determine the nature of initiating oncogenic event, with \textit{BRAF} mutant melanomas tending to occur in younger patients who have had lower lifetime UV exposure and \textit{NRAS} mutant melanomas more likely to develop in older patients who have had a longer history of sun exposure \textsuperscript{120}. Additionally, \textit{BRAF} mutations are more frequently found on skin that has had intermittent sun exposure and is infrequently found on skin that exhibits signs of chronic sun damage (as shown by increased solar elastosis) \textsuperscript{121,122}.

Careful pathological examinations of large numbers of \textit{BRAF}, \textit{NRAS}, and \textit{c-KIT} mutant melanoma specimens have revealed significantly different mutation-specific biological behaviors \textsuperscript{123}. It was found that \textit{BRAF} mutated melanomas had an increased tendency to upward migration and nest formation and gave rise to larger, rounded, and more pigmented tumor cells \textsuperscript{123}. In contrast, \textit{NRAS} mutated melanomas were not found to exhibit these morphological and phenotypic characteristics \textsuperscript{123}. With regard to the possible prognostic value of mutation status, there is some suggestion that \textit{NRAS} mutant primary melanomas may pose a higher risk of metastasis as they tend to be more deeply invasive at the time of initial diagnosis than \textit{BRAF} mutated melanomas and tend to have a higher mitotic rate. The effects of the different initiating mutations upon melanoma prognosis and biological behavior remain an area of intense study. As things stand, the available evidence suggests that both \textit{BRAF} and \textit{NRAS} mutant melanomas ultimately follow a similar clinical course, with little differences in overall survival noted \textsuperscript{124}.
**Personalized Therapy**

Currently, there are few effective treatments for disseminated melanoma and as such, patients with metastatic disease have a median survival ranging from only 6 to 10 months. While primary melanoma is easily curable through surgery, treatment of advanced disease remains a challenge with therapeutic strategies employed over the past 30 years not significantly improving cure rates. Until very recently, all major chemotherapy drugs, immunotherapies, and radiotherapies failed to prolong survival when tested in large-scale phase III clinical trials\(^{125}\).

However, there are now encouraging signs that the impasse in the therapeutic management of disseminated melanoma may soon be broken. The past decade has seen breakthroughs in personalized cancer medicine, with new targeted therapies being developed that inhibit cellular proliferation and survival in tumors with specific oncogenic mutations. Targeted agents, such as crizotinib (Xalkori™) for the treatment of echinoderm microtubule associated protein like 4/anaplastic lymphoma kinase (EML4-ALK) rearrangement positive non-small lung cancer (NSLC) and dabrafenib (Tafinlar™) in combination with trametinib (Mekinist™) for the treatment of \(BRAFV600\) mutated melanomas mark a revolutionized era in precision medicine\(^{126,127}\).

It is evident that melanomas constitute a heterogeneous group of tumors, with different patterns of oncogenic mutation, overexpression, and genomic amplification\(^{108,114,118}\). However, in the next section I will focus my discussion on \(BRAFV600\) mutated melanomas in the context of drug development and resistance.
Targeting *BRAF* Mutant Melanoma

**BRAF inhibitors**

The dependency of melanoma on MAPK signaling for survival and growth has made it an attractive candidate for targeted kinase therapy. Indeed the hallmark discovery, made 13 years ago, that the valine to glutamic acid substitution at codon 600 in *BRAF* (*BRAF*V600E) is a melanoma driver mutation has led to the rapid development of numerous RAF inhibitors. The most thoroughly studied of these is the pan-kinase inhibitor, sorafenib (Nexxavar). Originally developed as a CRAF inhibitor, sorafenib was also found to inhibit BRAF with moderate potency and was evaluated as a potential first proof-of-concept for BRAF inhibition in melanoma. Though sorafenib was found to have some activity in cell culture experiments and caused growth arrest in human melanoma xenograft models, it was eventually shown to have little activity in melanoma patients. Additional preclinical investigations showed sorafenib to be a relatively weak inhibitor of BRAF, with many off-target effects including inhibition of VEGFR, PDGFR, and p38 MAP kinase.

In the wake of the clinical disappointment of sorafenib, a new wave of more promising BRAF inhibitors were developed which boast higher specificities for oncogenic *BRAF* and fewer off-target side effects. These potent and selective next generation BRAF inhibitors include vemurafenib, dabrafenib, XL281, ARQ-736, RO521254, RAF265, LGX818, SB590885 and AZ628. Among these, dabrafenib and vemurafenib are currently FDA approved as single-agents. LGX818 is currently being evaluated in various stages as a single agent (LGX818), while all three
inhibitors are in current clinical testing in various inhibitor combinations (Table 1.2)\textsuperscript{45,132-140}.

Table 1.2. BRAF inhibitor combinations in current clinical trial.

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<td>SAR260301</td>
<td>PI3K</td>
<td></td>
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<tr>
<td>NCT01835184</td>
<td>Vemurafenib</td>
<td>XL184</td>
<td>c-MET</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>NCT01657591</td>
<td>Vemurafenib</td>
<td>XL888</td>
<td>HSP90</td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Vemurafenib and dabrafenib are both adenosine triphosphate (ATP)-competitive RAF inhibitors (vemurafenib: mutant BRAF IC\textsubscript{50} = 31 nM, CRAF IC\textsubscript{50} = 48 nM, WT BRAF IC\textsubscript{50} = 100 nM; dabrafenib: mutant BRAF IC\textsubscript{50} = 0.8 nM, CRAF IC\textsubscript{50} = 4.8 nM, WT BRAF IC\textsubscript{50} = 3.2 nM) that selectively target BRAF\textsubscript{V600E}\textsuperscript{142,143}. In preclinical models, treatment of patient-derived \textit{BRAF\textsubscript{V600E}} cell line xenografts with either vemurafenib or dabrafenib lead to highly specific and potent inhibitions of BRAF, downregulation of ERK activity and partial to complete responses in all tumors\textsuperscript{143,144}. Vemurafenib-induced tumor regression was also associated with increased BIM expression and the induction of apoptosis\textsuperscript{143}. In phase III clinical trials, both vemurafenib (BRIM-3) and dabrafenib (BREAK-3) showed significant improvements in objective response (OR) rate, progression free survival (PFS) and overall survival (OS) compared to the alkylating chemotherapeutic, dacarbazine (DTIC) (Table1.3); furthermore, objective clinical responses to vemurafenib were associated with >80% MAPK signaling blockade.
In the phase III study, common side effects for vemurafenib included cutaneous events, photosensitivity skin reactions (grade 2 or 3), and arthralgia. Of 336 patients treated with vemurafenib, 61 (18%) developed either secondary cutaneous squamous-cell carcinoma or keratoacanthoma. For dabrafenib, common adverse events included pyrexia, fatigue, nausea, headache, chills, diarrhea, arthralgia, rash, and hypertension. Overall, a higher percentage of patients responded to dabrafenib (54%) versus vemurafenib (48%), perhaps due to the higher potency of dabrafenib. In addition, secondary skin lesions occurred less frequently in dabrafenib vs. vemurafenib treated patients (9% vs. 18%, respectively).

Table 1.3. Summary of clinical trial results for dabrafenib, vemurafenib and trametinib.

<table>
<thead>
<tr>
<th>Dabrafenib + Trametinib</th>
<th>Dabrafenib</th>
<th>Trametinib</th>
<th>Vemurafenib</th>
<th>DTIC</th>
<th>Dabrafenib + Trametinib</th>
<th>PFS (months) OR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9.3</td>
<td>8.8</td>
<td>8.0</td>
<td>5.0</td>
<td>67.0</td>
<td>51.0</td>
</tr>
<tr>
<td></td>
<td>51.0</td>
<td>6.0</td>
<td>48.0</td>
<td>5.0</td>
<td>22.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.9</td>
<td>2.7</td>
<td>5.3</td>
<td>1.6</td>
<td>4.8</td>
<td></td>
</tr>
</tbody>
</table>

Intriguingly, the clinical response rates correlated with cell line based studies showing that not all \(\text{BRAF}\) mutated melanomas are sensitive to \(\text{BRAF}\) inhibition, with a significant proportion of \(\text{BRAF}\) mutated melanomas having variable degrees of intrinsic resistance. A recent genetic study, which looked for patterns of mutations and genomic amplifications between vemurafenib-sensitive and vemurafenib-resistant cell lines was unable to identify any unifying differences between the two groups. However, phenotypic studies from our own group (discussed in Chapter 2) implicated the loss of the tumor suppressor PTEN as being a potential mechanism of intrinsic resistance. In these studies, loss of PTEN (which is observed in ~20% of melanomas) was associated with increased PI3K/AKT signaling following \(\text{BRAF}\) inhibition. Increased AKT activity lead to suppressed of BIM and BAD expression, impaired apoptosis and decreased sensitivity to \(\text{BRAF}\) inhibition. In addition to
PTEN, it is also known that BRAFV600E mutated melanomas show alterations in cyclin D1, CDK2, CDK4, MITF, and AKT3. Clinical trials are currently underway that will address some of the aforementioned mechanisms of intrinsic BRAF inhibitor resistance (Table 1.2). In addition, BRAF inhibitors are currently being combined with immunotherapeutics (Table 1.4). A previous trial combining vemurafenib with ipilimumab was terminated due to unpredicted high levels of hepatotoxicity that was associated with the concurrent dosing regimen. Many of the current clinical evaluations are still in early phases, therefore it will be of great interest to see how combinatorial strategies such as metronomic dosing will be tolerated.

Table 1.4. BRAF inhibitor/immunotherapy combinations in current clinical trial.

<table>
<thead>
<tr>
<th>Trial ID</th>
<th>BRAF inhibitor</th>
<th>2nd drug</th>
<th>Immunotherapy</th>
<th>2nd immunotherapy</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT01767454</td>
<td>Dabrafenib</td>
<td>Trametinib</td>
<td>Ipilimumab (anti-CTLA4)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>NCT02027961</td>
<td>Dabrafenib</td>
<td>Trametinib</td>
<td>MEDI4736 (anti-PDL1)</td>
<td></td>
<td>1, 2</td>
</tr>
<tr>
<td>NCT02130466</td>
<td>Dabrafenib</td>
<td>Trametinib</td>
<td>Pembrolizumab (anti-PD1)</td>
<td></td>
<td>1, 2</td>
</tr>
<tr>
<td>NCT01585415</td>
<td>Vemurafenib</td>
<td></td>
<td>Adoptive cell therapy</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>NCT01656642</td>
<td>Vemurafenib</td>
<td></td>
<td>MPDL3280A (anti-PDL1)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>NCT01943422</td>
<td>Vemurafenib</td>
<td></td>
<td>High-dose interferon alfa-2b</td>
<td></td>
<td>1, 2</td>
</tr>
<tr>
<td>NCT01959633</td>
<td>Vemurafenib</td>
<td></td>
<td>PEG-interferon</td>
<td></td>
<td>1, 2</td>
</tr>
<tr>
<td>NCT01603212</td>
<td>Vemurafenib</td>
<td></td>
<td>Interleukin 2</td>
<td>Interferon alfa-2b</td>
<td>1, 2</td>
</tr>
<tr>
<td>NCT01659151</td>
<td>Vemurafenib</td>
<td></td>
<td>Adoptive cell transfer</td>
<td>High-dose interleukin 2</td>
<td>2</td>
</tr>
<tr>
<td>NCT01754376</td>
<td>Vemurafenib</td>
<td></td>
<td>Interleukin 2</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

An unanticipated clinical observation was that BRAF inhibitors actually induced the activation of the MAPK signaling pathway in BRAF WT melanoma (Fig1.5). In cell line models, the paradoxical activation of MAPK signaling arose in BRAF WT melanoma cell lines that harbored either an oncogenic Ras mutation or had upstream constitutive RTK activity (events that can occur due to selection pressures brought on by BRAF inhibition). In these studies, BRAF inhibition lead to the formation of BRAF/CRAF dimers that cooperated with oncogenic RAS to activate MAPK signaling (Fig1.5). Activation of RAF following BRAF inhibition resulted in enhanced proliferation.
(increased MAPK signal), survival (increased anti-apoptotic Mcl-1 expression), and invasion (increased FAK activity) of RAS mutated cells \[^{159}\].

**Fig. 1.5.** BRAF inhibition causes paradoxical MAPK activation in BRAF WT cells. In the absence of BRAF inhibition, WT RAF signal output is normal (left). WT BRAF inhibition drives BRAF/CRAF dimerization and cooperates with RAS or RTK activation leading to increased MAPK pathway signaling (middle). Paradox breakers inhibit MAPK signal output by inhibiting mutant and WT BRAF, CRAF and SFK kinase activity (right).

There is increasing clinical evidence that squamous cell carcinomas (SCC), keratoacanthoma (KA), new nevi and secondary (BRAF wild-type) melanomas may arise as a result of the paradoxical activation of MAPK signaling. Indeed almost all of the clinically evaluated BRAF inhibitors, including sorafenib, vemurafenib, dabrafenib and XL281, have been associated with the development of these proliferative skin lesions \[^{127,138,148}\]. Sequencing of spontaneous KA’s from RAF inhibitor treated patients revealed that these patients had a greater likelihood of developing KA’s with RAS mutations than non-RAF inhibitor treated patients (21.1% RAFi vs. 3.2% non-RAFi, p<0.01) \[^{160}\]. Interestingly, compared to vemurafenib treated patients, fewer patients developed secondary SCC or KA’s when treated with dabrafenib, perhaps owing to the higher potency of dabrafenib for non-mutated BRAF (vemurafenib: CRAF IC\(_{50}\) = 48 nM,
WT BRAF IC\textsubscript{50} = 100 nM; dabrafenib: CRAF IC\textsubscript{50} = 4.8 nM, WT BRAF IC\textsubscript{50} = 3.2 nM)\textsuperscript{142,143}.

**Paradox Breakers**

To address the issue of paradoxical activation, a new class of non-agonistic BRAF inhibitors, known as paradox breakers or pan-RAF inhibitors (CCT196969, CCT241161, PLX7904 and PLX8394) have been developed\textsuperscript{161-163}. Recent preclinical studies have shown that treatment with PLX7904, PLX8394, CCT196969 or CCT241161 could effectively inhibit MAPK signaling in \textit{BRAFV600E} melanomas without agonistic effects on WT RAF signaling (Figure 1.5)\textsuperscript{161-163}. Studies conducted with PLX7904 demonstrated that paradox breaker mediated inhibition leads to G1/S cell cycle arrest and growth inhibition in melanoma cell lines with acquired vemurafenib resistance (secondary \textit{NRAS} mutation)\textsuperscript{161,162}. Additionally, treatment with PLX8394 lead to growth arrest and cell death in melanoma cell lines harboring vemurafenib resistant, \textit{BRAF} splice variants\textsuperscript{161,162}.

The pan-RAF inhibitors, CCT196969 and CCT241161 have also shown impressive preclinical efficacy in both \textit{in vitro} and \textit{in vivo} models\textsuperscript{163}. To evaluate BRAF inhibitor resistance, these studies utilized xenografts derived from \textit{BRAFV600} tumors of stage IV melanoma patients with either acquired (partial or complete responses) or intrinsic resistance (no therapeutic response) to vemurafenib. In addition, patient-derived xenografts (PDX) were also established from a patient who had a partial response to combined dabrafenib plus trametinib followed by relapse. In these mouse models, CCT196969 and CCT241161 were well tolerated at their effective dose of 20mg/kg with no evidence of weight loss, clinical signs of adverse effect during
treatment or post-mortem microscopic tissue changes. Importantly, in all PDX models, treatment with CCT196969 and CCT241161 lead to significant tumor growth inhibition (compared to vemurafenib or dabrafenib plus trametinib) which was associated with inhibition of MAPK and src family kinase (SFK) activity. The studies conducted with the CCT196969 and CCT241161 inhibitors have garnered much excitement and it is hoped that their improved inhibitory profile may translate into first line therapies for treatment of naïve melanomas or second line therapies for treatment refractory lesions.

**MEK inhibitors**

The first MEK inhibitor studies preceded the discovery of BRAF activating mutations in melanoma and emphasized the role of MAPK in suppressing differentiation. Many of these studies, which utilized inhibitors such as PD98059, PD0325901, CI-1040, UO126 and AZD6244 (selumetinib), found that inhibition of MEK in melanoma cells resulted in G1 phase cell cycle arrest correlating to MAPK inhibition, cyclin D1 downregulation, p27 upregulation and hypophosphorylation retinoblastoma (Rb) protein. Although PD0325901 and selumetinib are highly potent MEK1 inhibitors (MEK1 IC50=0.33nM and 14nM, respectively), clinical testing of early MEK inhibitors were met with poor clinical efficacy and further clinical evaluations were abandoned. In fact, prior to the development of trametinib, MEK inhibitors were generally found to induce only modest levels of apoptosis in the most sensitive of BRAF mutated melanoma cell lines. However, the understanding that near complete MAPK blockade is required for tumor regression, that reactivation of MAPK signaling is critical to BRAF inhibitor resistance, and a dire need for therapies to manage NRAS mutated melanomas has prompted renewed interest in the further development of MEK inhibitors.
To date, the FDA approved highly selective allosteric MEK1/2 inhibitor (IC$_{50}$: MEK1 = 0.92 nM, MEK2 = 1.8 nM), trametinib, is the most potent and well-studied inhibitor in its class. In the phase III trial of 322 $BRAFV600E/K$ melanoma patients, trametinib was shown to have the best single agent activity of any MEK inhibitor evaluated so far (trametinib vs. DTIC: PFS = 4.8 months vs. to 1.5 months; OS = 81% vs. 67%; OR = 22% vs. 8%) (Table 1.3). The most common toxic effects observed in trametinib treated patients were rash, diarrhea, and peripheral edema. Importantly, secondary skin neoplasms were not observed. Trametinib in combination with dabrafenib as a first-line therapy for treatment naïve $BRAFV600$ mutant melanomas has also been recently FDA approved. Clinical comparisons of dabrafenib plus trametinib vs. dabrafenib monotherapy indicated that the addition of a second MAPK pathway inhibitor could improve PFS by 3.6 months and improve response rates by 22% (Table 1.3). Notably, although there was still some evidence of paradoxical activation of MAPK signaling, as seen by the development of secondary cutaneous SCC’s in patients receiving combinatorial drugs, the addition of a MEK inhibitor was able to significantly lessen development of secondary tumors (patients with SCC or KA: dabrafenib plus trametinib = 2 %; dabrafenib = 9%; trametinib = no secondary skin lesions reported). Similar clinical outcomes have also been reported for the combination of vemurafenib plus the MEK inhibitor, cobimetinib (Genetech: GDC-0973, Exelixis: XL518).

**ERK inhibitors**

Another class of drugs targeting the MAPK pathway are the ERK inhibitors which include SCH772984 (IC$_{50}$: ERK1 = 4 nM, ERK2 = 1nM) and VX-11e (IC$_{50}$: ERK2 =
Studies evaluating ERK inhibitors as a monotherapy have primarily focused on the ATP-competitive compound, SCH772984. Recent *in vitro* studies have indicated that sensitive and resistant (*BRAF* amplification, *MEK1* mutation, *RAS* mutation) V600E melanomas may be amenable to ERK inhibition \(^{186,187}\). In these studies, treatment of melanoma cell lines with SCH772984 lead to cell cycle arrest and apoptosis \(^{186,187}\).

Similar to previous findings utilizing SCH772984, recent data from our own group (\(^{188}\) and data not shown) have demonstrated that VX-11e may be combined with a MEK inhibitor such as trametinib or selumetinib in *NRAS* mutant melanomas to induce growth arrest and apoptosis. Similar effects were also seen when VX-11e was combined with a BRAF and MEK inhibitor (vemurafenib and selumetinib) in treatment sensitive V600E, BRAF inhibitor resistant V600E (data not shown) and *NRAS* mutant melanomas. In all studies of the SCH772984 and VX-11e ERK inhibitors, cytostatic and cytotoxic effects were associated with decreased MAPK activity and downregulated cyclin D1 expression (\(^{188,186,187}\) and data not shown). The evaluation of ERK inhibitors is still in its relative infancy therefore it will be of interest to see if these compounds can be utilized clinically as frontline monotherapies for *BRAF* and *NRAS* melanomas or in combinations with MEK and/or *BRAF* inhibitors to increase MAPK blockade.

**HSP90 inhibitors**

In cancer, HSP90 stabilizes a multitude of client proteins known to be essential for cellular signaling pathways such as growth, survival, differentiation and DNA damage response making HSP90 an attractive therapeutic target. In addition, several proteins known to be important for melanoma signaling, including *BRAF*V600E, have
been identified as HSP90 clients. In oncogenic cells, HSP90 client-chaperone complexes are enriched, are functionally distinct, and have greater binding affinities for HSP90 inhibitors (Figure 1.6).

**Fig. 1.6.** In stress environments, HSP90 exists in an activated state allowing for increased binding and stabilization of oncogenic proteins.

Under homeostatic conditions, HSP90 proteins exists in a latent state, interacting with client proteins in a low-affinity, ATP-dependent manner. However, under stress conditions (typified by the hypoxic and nutrient deprived cancer milieus), HSP90 interactions become deregulated. In this environment, HSP90 proteins have a higher affinity for client and co-chaperone proteins (HSP70, HSP40, cdc37) leading to aberrantly elevated and stable formations of HSP90/onco-client/co-chaperone complexes. Additionally, HSP90 inhibitors have been shown to preferentially bind to oncogenic clients (e.g. BRAFV600E and Bcr-Abl) with greater affinity and are therefore selectively retained in tumorigenic tissues. Currently, this observation is being explored to improve drug delivery methods.

Historically, attempts to utilize HSP90 inhibitors to treat melanoma were marred with difficulties in achieving effective tolerable doses; however, 2nd generation HSP90 inhibitors have now been developed which exhibit greater bioavailability, higher potency.
and lower toxicity profiles resulting in greater efficacy (Table 1.5)\textsuperscript{195,196}. Work by our group and others have shown that HSP90 inhibitors can induce cell death in melanoma through simultaneous degradation or inhibition of several key client proteins that are necessary for cancer survival, proliferation and metastasis\textsuperscript{197-199}. Recent clinical evaluations of the HSP90 inhibitors have also demonstrated that HSP90 inhibition could overcome multiple mechanisms of resistance across multiple cancer types e.g., trastuzumab resistance in HER-2 positive breast cancer and bortezomib resistance in multiple myeloma\textsuperscript{196,200}.

<table>
<thead>
<tr>
<th>HSP90 inhibitor</th>
<th>Class</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retaspimycin hydrochloride (IPI-504)</td>
<td>Hydroquinone derivative</td>
<td>Infinity</td>
</tr>
<tr>
<td>SNX-5422</td>
<td>Indazol-4-one</td>
<td>Seranex</td>
</tr>
<tr>
<td>XL888</td>
<td>Other chemotype</td>
<td>Exelixis</td>
</tr>
<tr>
<td>DS2248</td>
<td>Other chemotype</td>
<td>Daiichi Sankyo</td>
</tr>
<tr>
<td>Debio 0932</td>
<td>Purine-Scaffold</td>
<td>Debiopharm</td>
</tr>
<tr>
<td>MPC-0767</td>
<td>Purine-Scaffold</td>
<td>Myrexis</td>
</tr>
<tr>
<td>MPC-3100</td>
<td>Purine-Scaffold</td>
<td>Myrexis</td>
</tr>
<tr>
<td>PU-H71</td>
<td>Purine-Scaffold</td>
<td>Samus</td>
</tr>
<tr>
<td>AUY922</td>
<td>Resorcinol-Isoxazole</td>
<td>Novartis</td>
</tr>
<tr>
<td>Ganetespib (STA9090)</td>
<td>Resorcinol-Triazole</td>
<td>Synta</td>
</tr>
<tr>
<td>AT13387</td>
<td>Resorsinol containing synthetic agent</td>
<td>Astex</td>
</tr>
<tr>
<td>HSP990</td>
<td>Resorsinol containing synthetic agent</td>
<td>Novartis</td>
</tr>
<tr>
<td>KW2478</td>
<td>Resorsinol containing synthetic agent</td>
<td>Kyowa Hakko Kirin</td>
</tr>
</tbody>
</table>

Our own data, (discussed in Chapters 4 and 5) demonstrated that treatment of single and dual agent MAPK inhibitor resistant melanoma cells with HSP90 inhibitors leads to the rapid degradation or inhibition of key HSP90 client proteins and results in marked inductions of apoptosis\textsuperscript{197,198}. These findings support our hypothesis that resistance proteins are critically dependent upon HSP90 for stabilization and that the HSP90 machinery is indispensable for the mechanisms that promote oncogenesis. Moreover, our studies demonstrate that combined BRAF and HSP90 inhibition may dramatically delay the onset of BRAF inhibitor resistance\textsuperscript{193,197,198}. 

33
**Mechanisms of BRAF inhibitor Resistance**

**Tumor Intrinsic Mechanisms**

The clinically approved BRAF inhibitors, vemurafenib and dabrafenib, are the most well studied with phase III reports indicating that these highly potent and selective compounds can significantly delay the time to relapse and improve therapeutic responses (Table 1.3). Although the results have been encouraging and provide strong evidence that melanoma is amenable to targeted therapy, response rates are still well below 100% (dabrafenib 51%, vemurafenib 48%). Median progression-free survival was also reported as 6.9 months for dacarbazine and 5.3 months for vemurafenib, with refractory disease being the eventual outcome for the majority of patients. Therefore, the two major hurdles to effective therapeutic intervention are intrinsic and acquired vemurafenib resistance. These observations mirror the pattern of response seen to targeted therapy in NSCLC, chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (GIST), where an initial period of tumor regression is later followed by relapse. Imatinib resistance in CML and GIST and erlotinib resistance in NSCLC generally emerges following the acquisition of secondary mutations “gatekeeper” sites. Mutations of these target kinase ATP binding residues prevent the binding of drug to the hydrophobic pocket. Though BRAF gatekeeper mutations have been predicted *in silico* and generated drug resistance *in vitro*, similar drug-insensitive BRAF mutations have not yet been detected in melanoma patients.

Melanoma is a notoriously heterogeneous disease with diverse and complex adaptive mechanisms. As such, resistance is often regulated by multiple factors.
occurring both upstream and downstream of mutated $BRAF^{\text{134,206,209-215}}$. In cancer cell lines, adaptive growth factor signaling can be activated through signal transduction inhibition (such as inhibition of mTOR, MEK or BRAF). In some cases, pathway inhibition leads to the de-repression of negative feedback signaling and reciprocal RTK signaling (Fig. 1.7) $^{216-218}$.

Fig. 1.7. Derepression of negative feedback signaling. Following BRAF inhibition, one mechanism for tumor adaptation is through the relief of negative feedback signaling (inhibition of ERK) which in turn allows for reciprocal RTK activation.

Studies from several independent groups have shown that BRAF inhibitor resistance is mediated through increased RTK signaling (e.g. HER3, PDGFRβ, IGF1R, c-MET, EGFR and EphA2) $^{212,219-223}$. In the case of IGFR1, downstream MAPK signaling was reactivated following the rerouting of signaling from mutated BRAF to ARAF and CRAF $^{212}$. However, cell line studies from our own group (data not shown) indicate that upregulated expression of PDGFRβ, IGF1R, EGFR or EphA2 does not confer resistance to BRAF inhibitors in the canonical sense. Though inhibition of RTK's lead to decreased kinase activity (as measured by receptor tyrosine dephosphorylation), siRNA
knock-down of RTK’s (PDGFRβ or EphA2), or treatment with small molecule inhibitors (IGF1R: NVP-ADW-742 \(^{150}\); EGFR: lapatinib or erlotinib) in respective RTK overexpressing resistant cell lines did not lead to significant levels of cell cycle arrest or apoptosis. Further data from our group (Chapter 5) have also shown that receptor tyrosine kinases are not singly overexpressed following acquired resistance, with several cell lines observed to have increased activity in more than one RTK \(^{197,198}\). Together, these observations indicate that targeting individual RTK’s may not be a viable treatment option for overcoming dynamic BRAF inhibitor resistance mechanisms.

Fig. 1.8. Overview of tumor intrinsic mechanisms of BRAF inhibitor resistance. Reported tumor-mediated mechanisms of resistance are shown in yellow.
Rather than having canonical effects upon cellular growth and survival, recent data from the Marais lab and our own group have shown that RTK upregulation (EGFR and EphA2) leads to an adaptive switch whereby resistant cells adopt a more aggressive invasive and migratory phenotype\textsuperscript{221,224}. Unlike HER3, IGF1R and EGFR upregulation (which is likely due to the relief of feedback suppression) the processes underlying increased EphA2 overexpression are still unclear with evidence from our group (discussed in greater detail in Chapter 6), and others, which indicate that EphA2 expression may be epigenetically controlled and is reliant upon histone deacetylase (HDAC) and DNA methyltransferase activity\textsuperscript{224,225}.

Melanoma cells can also circumvent BRAF inhibition through downstream MAPK pathway signaling (Figure 1.8). A recent study demonstrated that increased COT (MAP3K8) expression drives BRAF inhibitor resistance through the RAF-independent activation of ERK\textsuperscript{211}. The clinical relevance of increased COT expression in the resistance phenotype was confirmed in a limited number of melanoma samples from patients failing BRAF and MEK inhibitor treatment\textsuperscript{211}. A number of other vemurafenib resistance mechanisms have also been reported for melanoma including upregulated receptor tyrosine kinase signaling secondary mutations (\textit{NRAS}-Q61K and \textit{MEK1}-C121S), amplifications (\textit{BRAF} and \textit{cyclin D1}), loss of tumor suppressors (\textit{PTEN}: discussed in Chapter 3 and \textit{NF1}) and truncated BRAF (p61) expression\textsuperscript{134,176,210,223,226}.

\textit{Tumor Extrinsic Mechanisms}

Recent studies have also implicated the role the tumor microenvironment in mediating BRAF inhibitor resistance. While resident fibroblasts actively suppress tumor initiation through the maintenance of tissue homeostasis, their function can switch
dramatically upon oncogenic transformation\textsuperscript{227,228}. The central role of cancer associated fibroblasts (CAFs) in tumor development and progression is suggested by their contribution to each of Hanahan and Weinberg's original hallmarks of cancer; sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, activating invasion and metastasis, inducing angiogenesis and resisting cell death\textsuperscript{229} (Fig. 1.8).

\textbf{Fig. 1.9.} Carcinoma-associated fibroblasts in the tumor microenvironment. Activated tissue associated fibroblasts and myofibroblasts are recognized sources of carcinoma associated fibroblasts (CAFs). However, CAFs may also arise from diverse lineages including endothelial cells, mesenchymal stem cells (MSC) and epithelial cells. In addition to mitogenic factors and proteases, CAFs promote cancer progression through the secretion of various structural extracellular matrix (ECM) components such as fibronectin, collagen, laminin, proteoglycans, tenascin and osteopontin. In this manner, signal transduced, direct cell–cell and ECM mediated communication can occur between tumor cells and their associated fibroblasts leading to enhanced tumor survival, proliferation and metastasis.
Melanomas exhibit varying degrees of stromal infiltration with tumors having either the absence or presence of fibroblasts. Stromal patterns seen in melanoma specimens can also vary and are characterized as desmoplastic (fibroblasts with strands of ECM) or mixoid (stellate fibroblasts with the deposition of proteoglycans). Generally, the earliest stages of melanoma (RGP phase) show much less fibroplasia than intermediate (VGP phase) lesions (which often exhibit a pattern of concentric fibroplasia around nests of melanoma cells).

Numerous studies have now reported that the host microenvironment provides a protective niche allowing minor populations of cancer cells to evade therapy. The most thoroughly studied aspect of environmentally mediated drug resistance has been therapeutic escape via the adhesion of cancer cells to the extracellular matrix (ECM). This phenomenon, also known as cell adhesion mediated drug resistance (CAM-DR), was first seen in multiple myeloma, where it was observed that adhesion of myeloma cells to the ECM protein fibronectin decreased sensitivity to the chemotherapy drug melphalan. Mechanistically, the protection conferred by fibronectin resulted from the β-integrin-mediated amplification of IL-6 signaling leading in turn to increased cell survival mediated through increased anti-apoptotic Bcl-XL and decreased pro-apoptotic BIM expression. CAM-DR has also been observed in many other tumor types, with similar protective effects noted when NSCLC, uveal melanoma, ovarian and cutaneous melanoma cell lines were adhered onto ECM proteins such as lamin, collagen IV, fibronectin and Tenascin-C.

Recent studies from our own lab have also demonstrated that the adhesion of melanoma cells to fibroblast monolayers significantly reduces the cytotoxic effects of the
chemotherapy drug cisplatin<sup>237</sup>. Although the mechanism underlying this protection is not yet known, there is evidence that melanoma cells and fibroblasts directly adhere to each other through N-cadherin, and that homotypic cadherin binding increases melanoma survival through increased AKT and BAD signaling<sup>238</sup>. In addition to fibroblasts, other host cells are also known to be involved in microenvironment-mediated drug resistance. Astrocytes can also reduce the efficacy of targeted therapy agents through direct cell adhesion and secretion of paracrine survival factors. A recent series of intriguing experiments showed that conditioned media derived from primary human astrocytes was more effective at stimulating pro-survival signals in melanoma cells than conditioned media from normal skin fibroblasts<sup>239</sup>. It was further shown that astrocyte-conditioned media could increase melanoma invasion in comparison to fibroblast-conditioned media<sup>239</sup>.

The release of pro-survival growth factors, can arise from tumor or stromal cells<sup>240</sup>. In addition, growth factors can be also be released from “depots” in the ECM through the activity of enzymes such as heparanase<sup>241,242</sup>. Host fibroblasts constitute an important source of growth factors that is permissive for therapeutic escape (Fig. 1.10)<sup>223</sup>. Recent cell proliferation based studies, using a high throughput screen of multiple cancer cell lines paired with multiple stromal cell types identified a number of pairings in which the host cells conferred drug resistance<sup>223</sup>. It was generally observed that the stromal cells conferred the most resistance to targeted therapy agents (such as kinase inhibitors) as opposed to chemotherapies. For melanoma cells treated with vemurafenib, fibroblasts were the major host cell type implicated in transmitting BRAF inhibitor resistance, with 6 different fibroblast cell lines conferring resistance to 7
different \textit{BRAF} V600E mutant melanoma cell lines \textsuperscript{223}. Of the many growth factors evaluated, HGF was identified as the key factor mediating resistance to BRAF inhibitor therapy. In total, 20 different \textit{BRAF} melanoma cell lines were rescued from BRAF inhibition following co-treatment with exogenous HGF \textsuperscript{223}. Further analysis of melanoma patients receiving BRAF inhibitor therapy showed that individuals with increased stromal HGF secretion responded most poorly to the drug \textsuperscript{223,240}. These data fit with earlier preclinical studies showing that exogenous growth factors and cytokines could reactivate MAPK signaling and rescue melanoma cells from BRAF siRNA silencing-induced cell death \textsuperscript{243,244}. However, a recent study of 41 melanoma patient specimens (which did find evidence of elevated HGF expression at disease progression), was not able to correlate HGF levels with clinical outcome \textsuperscript{245}. These results could be due in part to the small cohort of patient samples evaluated, therefore larger scale biomarker studies are needed.

In another large-scale study, RNAi screen was utilized to understand the role of stromal-derived growth factors in driving therapeutic escape. In these studies, the TGF-\(\beta\) signaling regulator MED12 was identified a potential mediator of resistance to multiple kinase inhibitors, including vemurafenib \textsuperscript{246}. Functionally, MED12 was found to suppress T\(\beta\)RII expression, where loss of MED12 was associated with increased TGF-\(\beta\) signaling, and conferred resistance to BRAF inhibitors through increased MAPK activation and the induction of an endothelial to mesenchymal transition (EMT) \textsuperscript{246}. Exogenous TGF-\(\beta\) alone was growth inhibitory on the A375 melanoma cell line, however, in the presence of targeted kinase inhibition, TGF-\(\beta\) activation conferred resistance to BRAF (vemurafenib) or MEK (AZD6244) inhibitors in the same cell line \textsuperscript{246}.  

41
The authors concluded that although TGF-β signaling was growth suppressive for a subset of cancers, its effects were reversed in the context of targeted therapy.

**Fig. 1.10.** Reciprocal relationship of CAFs and their associated cancer cells. The multipotency of CAFs to enable tumorigenesis is inherent to their production of numerous anti-apoptotic, inflammatory, mitogenic, angiogenic and ECM modulating tumor-promoting factors. The secretion of CAF proteins transforms malignant cells into highly aggressive cancers that in turn produce factors that enable the transformation of host stroma into activated tumor associated fibroblasts. This highly dynamic CAF–stroma interaction is a result of their co-evolution and results in highly aggressive, treatment refractory cancers.

Taken together, it is expected that combinatorial strategies that incorporate the tumor autonomous and microenvironment-mediated drug resistance into their design will prove successful at significantly delaying or preventing the onset of resistance. In the next chapters I will discuss our early understanding of single agent BRAF inhibitor resistance, the use of proteomic approaches to elucidate mechanisms of single-agent
resistance, and evidence based approaches to delay and overcome BRAF inhibitor resistance. I will then go on to describe global phosphoproteomic and bioinformatics methodologies, use of these methodologies to elucidate the underlying processes of refractory disease (for both single agent BRAF and dual agent BRAF plus MEK inhibitor resistance) as well as strategies to constrain dual agent BRAF plus MEK inhibitor resistance.
Chapter 2

Recovery of phospho-ERK activity allows melanoma cells to escape from BRAF inhibitor therapy

Note to Reader

Portions of this chapter have been previously published in Br J Cancer. 2010 Jun 8;102(12):1724-30 \(^{133}\) and have been reproduced with permission from Nature Publishing Group. Author contributions: Paraiso KH (designed/performed experiments, interpreted data, writing, figures); Fedorenko IV, Cantini LP, Munko AC, and Hall M (performed experiments); Sondak VK, Messina JL (data interpretation, wording); Smalley KS (study concept, designed/performed experiments, interpreted data, writing, figures).

Introduction

Mutated \textit{BRAF} exerts most of its oncogenic effects through the activation of the RAF/MEK/ERK MAPK pathway \(^{247,248}\). Constitutive activation of BRAF drives the uncontrolled growth of melanoma cells via upregulated cyclin D1 expression and suppression of the cyclin-dependent kinase inhibitor \(p27^{KIP1}\) \(^{86,249}\). \textit{In vitro} and \textit{in vivo} studies from our group and others have shown vemurafenib, a BRAF inhibitor, to have excellent anti-tumor activity \(^{90,132}\). In clinical vemurafenib studies, mutated BRAF was validated as a therapeutic target in melanoma, where initial phase I responses were observed in an unprecedented 70\% of patients \(^{146}\). However, the current post clinical trial data indicate that only 48\% of patients with V600E-\textit{BRAF} mutant melanomas will
likely respond to vemurafenib monotherapy\textsuperscript{146}. For patients who do respond, tumor regression is typically quite rapid. But unfortunately initial levels of dramatic tumor regression do not typically persist with most patients eventually succumbing to progressive disease after 10 months\textsuperscript{146,250}. In this study, we have identified the rebound activation of phospho-ERK (pERK) as being a mechanism of early therapy escape and show that combined BRAF/MEK inhibition can increase rates of response, enhance levels of apoptosis and delay the onset of resistance.

\textbf{Results}

\textit{PLX4720 has selective effects on BRAF-V600E-mutated melanoma cell lines}

Treatment of melanoma cells with increasing concentrations of the BRAF inhibitor PLX4720 led to a dose-dependent reduction in the growth of BRAF-V600E-mutated melanoma cell lines (WM35, WM164 and 1205Lu) (Fig. 2.1A). In contrast, cell lines that harbored an NRAS mutation (WM1346, WM1361A and WM1366) were more resistant (Fig. 2.1A). Lower doses of PLX4720 (0.3 and 3 \(\mu\)M) led to a profound G1-phase cell-cycle arrest and a reduction of 1205Lu cells entering into S-phase (Fig. 2.1B). Increasing concentrations of PLX4720 (1 h) inhibited pERK signaling in three BRAF-mutated melanoma cell lines (WM35, WM164 and 1205Lu), but not an NRAS-mutated cell line (WM1346) (Fig. 2.1C). It was noted that PLX4720 also reduced pRB protein phosphorylation, increased p27 expression, suppressed cyclin D1 expression and induced cleavage of PARP only in melanoma cell lines harboring the BRAF-V600E mutation (Fig 2.1D). Treatment of melanoma cells with increasing concentrations of the BRAF inhibitor PLX4720 led to a dose-dependent reduction in the growth of BRAF-
V600E-mutated melanoma cell lines (WM35, WM164 and 1205Lu) (Fig 2.1A).

Fig. 2.1. PLX4720 inhibits the growth of melanoma cells harboring the BRAF-V600E mutation. (A). Increasing concentrations of PLX4720 reduced the growth of melanoma cell lines harboring the BRAF-V600E mutation (WM35, 1205Lu and WM164), whereas melanoma cell lines that were BRF wild type were relatively resistant (WM1346, WM1361A and WM1366). Cells were treated with drug (3 nM–30 µM) for 72 h, and cell numbers were quantified using the MTT assay. Bars show s.e. mean. (B) Low doses of PLX4720 are cytostatic in melanoma cells harboring the BRAF-V600E mutation. 1205Lu cells were treated were either 0.3 or 3 µM PLX4720 for 24 h before being fixed, stained with propidium iodide and analysed by flow cytometry. (C) PLX4720 inhibits MAPK signaling in BRAF-V600E-mutated melanoma cells. Cells were treated with increasing concentrations of PLX4720 (0.03–30 µM, 1 h); proteins were extracted and probed for expression of phospho-ERK (pERK). Blots were stripped once and reprobed for total-ERK to show even protein loading. (D) PLX4720 induces a concentration-dependent reduction in the phosphorylation of the retinoblastoma protein (phospho-RB), induces the cleavage of PARP, stabilizes p27 and suppresses the expression of cyclin D1 in WM164 BRAF-V600E-mutated melanoma cells. Cells were treated with increasing concentrations of PLX4720 (3 nM–30 µM) for 24 h, after which time, protein was extracted and resolved by western blotting (C=vehicle control). Blots were stripped once and probed for actin to show equal protein loading.

In contrast, cell lines that harbored an NRAS mutation (WM1346, WM1361A and WM1366) were more resistant (Fig 2.1A). Lower doses of PLX4720 (0.3 and 3 µM) led to a profound G1-phase cell-cycle arrest and a reduction of 1205Lu cells entering into S-phase (Fig. 2.1B). Increasing concentrations of PLX4720 (1 h) inhibited pERK signaling in three BRAF-mutated melanoma cell lines (WM35, WM164 and 1205Lu), but
not an NRAS-mutated cell line (WM1346) (Fig 2.1C). It was noted that PLX4720 also reduced pRB protein phosphorylation, increased p27 expression, suppressed cyclin D1 expression and induced cleavage of PARP only in melanoma cell lines harboring the BRAF-V600E mutation (Fig 2.1D).

**PLX4720-mediated apoptosis induction is BRAF-V600E-mutation specific**

Concentrations of PLX4720 >3 µM were required for apoptosis induction across a panel of three BRAF-mutated melanoma cell lines (WM35, WM164 and 1205Lu) (Fig 2.2A). The pro-apoptotic effects of PLX4720 were found to be BRAF specific, with high levels (>30%) of apoptosis only induced in the BRAF-V600E-mutated melanoma cell line panel (WM35, WM164 and 1205Lu), and not the NRAS-mutated melanoma cell lines (WM1346, WM1361A and WM1366) (Fig. 2.3). The induction of apoptosis was found to be time dependent with apoptosis observed only >24 h.

![Fig. 2.2. PLX4720 induces apoptosis in BRAF-V600E-mutated melanoma cell lines. (A) PLX4720 induces apoptosis in three BRAF-mutated melanoma cell lines. Cultures were treated with increasing concentrations of PLX4720 (0.03–30 µM, 48 h), before staining for FITC-annexin-V and flow cytometry. Data show mean of three experiments. (B) PLX4720 reduces viability and invasion of 1205Lu cells grown as 3D collagen-implanted spheroids. Preformed 1205Lu spheroids were implanted into collagen and overlayed with media. Cells were treated with PLX4720 (0.3–30 µM for 72 h) before being treated with calcein-AM and ethidium bromide. Green, viable cells; red, dead cells. Lack of green staining also indicates a loss of cell viability. Magnification × 10. * P<0.05, Significant difference from control.]
Often, the pharmacological profile of drugs in 2D culture is not predictive of response in 3D culture. Here, it was found that the concentrations of PLX4720 required (>3 \( \mu \)M) to induce apoptosis in 2D cell culture (Fig. 2.2B) were equivalent to those necessary for loss of spheroid viability (as shown by the reduction of green staining and increased red staining) (Fig. 2.2B). Interestingly, some viable melanoma cells persisted even at the highest concentrations of drug.

**Fig. 2.3.** PLX4720 selectively induces apoptosis in melanoma cell lines harboring a \textit{BRAF} V600E mutation but not those harboring an \textit{NRAS} mutation. Melanoma cell lines were treated with PLX4720 (30 \( \mu \)M) for increasing periods of time (24-72 hrs), before being stained with FITC-annexin-V.

**Some cells escape from PLX4720 treatment and become resistant**

We next asked whether \textit{BRAF}-V600E-mutated melanoma cells escaped from PLX4720 therapy and become drug resistant. Here, melanoma cell lines (WM164 and 1205Lu) were treated with PLX4720 (either 2 or 3 \( \mu \)M) over a 2-month period with fresh drug added twice per week. It was noted that after an initial round of cell death, a limited number of viable cells remained and as treatment progressed, these clones began to regrow (>28 days) and eventually repopulated the whole culture (Fig. 2.4A).
Fig. 2.4. Melanoma cells escape PLX4720 and become resistant. (A) Photomicrograph of WM164 and 1205Lu melanoma cells treated with PLX4720 (3 \(\mu\)M). (B) Western blot of pERK in naive (N) and resistant (R) (8 weeks, 3 \(\mu\)M) WM164 and 1205Lu cell lines. Resistant cells were maintained continuously in the presence of PLX4720 (3 \(\mu\)M). (C) Resistant 1205Lu and WM164 cell lines continue to incorporate BrdU in the presence of PLX4720 (3 \(\mu\)M). Treatment-naive or resistant WM164 and 1205Lu cell lines (control) treated with PLX4720 (3 \(\mu\)M) or the MEK inhibitor U0126 (3 and 10 \(\mu\)M). Cells were stained for BrdU (20 \(\mu\)M, 1 h) uptake and the cell viability marker 7-AAD and analysed by flow cytometry. (D) Sequencing trace from Exon 3 of MEK1 of 1205Lu cells chronically treated with PLX4720 for 8 weeks, arrow indicates site of P124L mutation identified previously in Emery et al (2009).
The drug-resistant phenotype of the surviving cells was demonstrated by the ability of both cell lines to maintain their pERK signaling and incorporate BrdU in the continuous presence of PLX4720 (3 μM) (Fig 2.4B and C). In contrast, PLX4720 treatment (3 μM) potently inhibited BrdU incorporation in the PLX4720-naive WM164 and 1205Lu cell lines (Fig. 2.4C). It was further shown that the proliferation of the PLX4720-resistant WM164 and 1205Lu cell lines was dependent on MAPK signaling, with MEK inhibitor treatment (U0126; 3 and 10 μM) preventing the incorporation of BrdU (Fig 2.4C).

**Prolonged PLX4720 treatment leads to a recovery of pERK signaling**

Having shown the reliance of the PLX4720-resistant melanoma cell lines on MAPK signaling, we next investigated the time course of pERK signaling recovery. Treatment of drug-naive WM164 cells with PLX4720 (3 μM) showed the pathway to be rapidly inhibited, with some recovery of signaling >24 h (Fig. 2.5A and B). The recovery of pERK signaling observed was found to be insensitive to repeated PLX4720 treatments (drug added every 24 h) (Fig. 2.5A). To explain the apparent anomaly between the recovery of pERK signaling >24 h and the profound growth arrest/apoptosis observed at 48 and 72 h (Fig 2.1 A and 2.2A), we next investigated the cell cycle and signaling profile of cells treated with PLX4720 over a 72-h period. These studies showed that even though pERK signaling recovered, the majority of the cells remained growth arrested (Fig 2.5C), and that this was associated with increased p27 expression and hypophosphorylation of the pRB protein (Fig. 2.5D). Interestingly, a minor population of cells were identified that continued to proceed through S-phase (Fig 2.5C). The existence of a minor proliferating subpopulation was also confirmed by BrdU
incorporation assays (1–4 weeks), with studies showing that 2–4% of WM164 and WM793 cells continued to incorporate BrdU in the continuous presence of PLX4720 (3 µM) (Figure 2.6). Cell counting experiments were performed to better understand how PLX4720-induced apoptosis, cell-cycle arrest and therapy escape impacted on the population as a whole (Fig 2.5 E). It was observed that after an initial drop in cell numbers, the population remained relatively stable, suggesting that the recovery of pERK signaling attenuated the anti-melanoma activity of PLX4720.

![Image of experiments and graphs]

**Fig. 2.5.** pERK signaling recovers after PLX4720 treatment. (A) Naive WM164 melanoma cells were treated with PLX4720 (3 µM, every 24 h) for increasing periods of time (0–48 h) and probed for pERK and total-ERK (tERK). (B) Recovery of pERK is observed in three naive BRAF-V600E-mutated melanoma cell lines. Cells were treated with PLX4720 for 0, 8, 24, 48 h (3 µM) and analysed as in (A). (C) Most cells remain growth arrested even when pERK recovers. WM793 cells were treated with PLX4720 as for (C); protein lysates were probed for expression of pERK, total-RB (t-RB), phospho-RB (p-RB), total retinoblastoma protein (t-RB) and p27. Equal protein loading was confirmed by stripping the blot once and probing for GAPDH expression. (E) PLX4720 treatment leads to a drop in cell numbers followed by stabilization of the population. WM793, 1205Lu and WM164 melanoma cells were treated with PLX4720 (3 µM) for 0–120 h. At each time point, the cells were removed from the plate and counted. Data show the mean±s.e.mean of three independent experiments.
Rebound pERK treatment allows for escape from PLX4720-mediated apoptosis

Having demonstrated that pERK signaling recovered after PLX4720 treatment, we next determined whether dual BRAF/MEK inhibition led to enhanced cytotoxicity. It was noted that although the recovery of pERK signaling was insensitive to repeated PLX4720 treatments (Fig. 2.5A), rebound pERK signaling was sensitive to the MEK inhibitor U0126 (3 µM) (Fig. 2.7 A and B). Combined treatment of drug-naive WM164 cells with both PLX4720 and U0126 was found to decrease the expression of cyclin D1 (Fig. 2.7C) and enhance the level of PLX4720-induced PARP and caspase-3 cleavage (Fig. 2.7C). In contrast, expression of p27, a protein relatively sensitive to BRAF/MEK inhibition, was little enhanced when PLX4720 and U0126 were combined. The western blotting results were also mirrored in apoptosis assays, with the addition of U0126 (3 µM) significantly enhancing the pro-apoptotic activity of low-dose PLX4720 (3 µM) in drug-naive WM164 cells at both 24 and 48 h (Fig. 2.7D).
**Fig. 2.7.** The function of rebound pERK signaling in the escape from PLX4720 treatment. (A) U0126 blocks the rebound increase in pERK after PLX4720 treatment. Melanoma cells were either treated with vehicle (0), PLX4720 (3 μM) or PLX4720 + U0126 (both 3 μM) for 48 h, protein was then probed for expression of pERK and tERK. (B) Melanoma cells were treated with increasing concentrations of U0126 for 1 h before being probed for pERK and tERK expression. (C) Cells were treated with increasing concentrations of PLX4720 (30 nM–30 μM) for 24 h in the absence or presence of U0126 (3 μM), after which time, protein was extracted and resolved by western blotting and probed for either cleaved PARP (cl-PARP), phospho-ERK (pERK), cyclin D1 (Cyclin D1), p27 or cleaved caspase-3 (cl-casp-3). Blots were stripped once and probed for actin to show equal protein loading. (D) Combined BRAF and MEK inhibition leads to enhanced apoptosis. WM164 cells were treated with either vehicle, U0126 (3 μM, 3U0), PLX4720 (3PLX, 3 μM) or the two inhibitors in combination for 48 h. Levels of apoptosis were measured by annexin-V staining and flow cytometry. Data show the mean of three experiments. *P<0.05.

**Combined BRAF/MEK inhibitor treatment prevents the acquisition of resistance**

In a final series of experiments, we explored whether dual BRAF/MEK inhibition blocked the MAPK-dependent escape from PLX4720 therapy and asked whether this prevented the onset of resistance. Here, WM164, WM793 and 1205Lu cells were treated with PLX4720 (3 μM), U0126 (3 μM) or the two inhibitors in combination for 4 weeks. It was noted that although PLX4720 was more effective at reducing colony formation than U0126 (Fig 2.8A), a number of clones did remain. In contrast, treatment
with U0126 and PLX4720 in combination completely inhibited the formation of all colonies. A thorough microscopic examination of the plates revealed that no cells remained (Fig 2.8A, see inset). Examination of the vehicle control plates showed the cells to be highly confluent. It was further found that the combination of PLX4720 and U0126 (both 3 \( \mu \)M) also reduced the growth and survival of melanoma cell lines grown as 3D collagen-implanted spheroid cultures (Fig 2.8B).

**Fig. 2.8.** Dual BRAF/MEK inhibition prevents escape from PLX4720 therapy. (A) WM164, WM793 and 1205Lu melanoma cells were treated with vehicle (1 week), PLX4720 (3 \( \mu \)M), U0126 (3 \( \mu \)M) or the two inhibitors in combination (both 3 \( \mu \)M) for 4 weeks. After this time, colonies were fixed and stained with crystal violet. Photographs are representative of three independent experiments. Photomicrographs show the detail of one colony each on the WM793 plate ( \( \times 4 \) ). (B) Combined PLX4720 and U0126 treatment reduce growth of melanoma cells and enhance cell death in a 3D spheroid model. WM164 spheroids were implanted in collagen and treated with PLX4720 (3 \( \mu \)M), U0126 (3 \( \mu \)M) or the two drugs in combination for 72 h. After this time, plates were washed and cells were stained with a cell viability kit. Red=dead cells, green=live cells.
**Discussion**

At the initiation of these studies in 2009 (and submission for publication in 2010), the structure of vemurafenib (PLX4032) had not yet been disclosed. Therefore most early evaluations of the Plexxikon BRAF inhibitor where conducted utilizing the less potent vemurafenib analog, PLX4720 (BRAF IC$_{50}$ = 160 nM) $^{132}$. In this study, we focused on the earliest stages of BRAF inhibitor therapy. Through an initial series of experiments, we confirmed that PLX4720 had good selectivity for BRAF-mutated melanoma cell lines over those harboring NRAS mutations (BRAF WT) and also demonstrated that PLX4720 was able to induce significant levels of apoptosis. The induction of apoptosis induced had a slow onset (>24 h), but was very BRAF specific, with very little apoptosis observed in melanoma cell lines that were BRAF wild type.

Relatively little was known about the mechanisms of therapy escape after BRAF inhibition at the time of this study. In non-melanoma systems, chronic treatment with the MEK inhibitor CI-1040 lead to resistance associated with increased KRAS and MEK expression $^{251}$. In melanoma patients, resistance to the MEK inhibitor AZD6244 is associated with C121S mutations in MEK1 $^{209}$. Growth factors and cytokines had also been shown to rescue melanoma cells from apoptosis after siRNA-induced knockdown of BRAF $^{243,252}$. Other studies showed that acquired BRAF inhibitor resistance after long-term drug treatment is associated with pathway switching, where MAPK signaling is routed from BRAF to CRAF $^{135}$.

This study made the unexpected observation that combined BRAF and MEK inhibitor treatment enhances the levels of apoptosis before resistance to BRAF inhibition is even acquired, suggesting that the recovery of melanoma signaling occurs
much earlier than previously suspected. The observation that dual MEK/BRAF inhibition blocks colony formation also argued that the rebound MAPK signaling observed has a key function in the escape from therapy. Although vertical targeting of the same pathway at two points seemed redundant, it is likely that this dual inhibition strategy counteracts feedback inhibition loops that are relieved after pathway blockade at a single point\textsuperscript{253}. Intriguingly, the possibility also exists that MEK and BRAF inhibitors may hit subtly different cellular targets.

The finding that dual BRAF/MEK inhibition prevents the onset of resistance in our \textit{in vitro} melanoma models suggested that MEK inhibitors might be of use in managing resistance to BRAF inhibitors, possibly delaying or even preventing the onset of resistance. These early findings provided a strong rationale for the clinical testing of combined BRAF and MEK inhibitors [dabrafenib + trametinib\textsuperscript{127} and vemurafenib + cobimetinib (Genetech: GDC-0973, Exelixis: XL518)\textsuperscript{183,184}], evaluations that have now proven to significantly improve response rates, progression free and overall survival for patients whose melanomas harbor V600E/K \textit{BRAF} mutations\textsuperscript{148,254,255}.

\textbf{Materials and Methods}

\textit{Cell culture and growth inhibition}

Melanoma cell lines were a gift from Dr Meenhard Herlyn (The Wistar Institute) and were genotyped as described in\textsuperscript{172}. Cells were plated into a 96-well plate at a density of $2.5 \times 10^4$ cells per ml and left to grow overnight before being treated with increasing concentrations of PLX4720 in triplicate; after 72 h, the levels of growth inhibition were examined using the MTT assay\textsuperscript{168}. Data show the mean of at least three independent experiments ± the s.e. mean. In all cases, * indicates statistical significance where
P<0.05. PLX4720 was dissolved in 100% DMSO and stored at -20°C as a 10 mM solution. U0126 was from EMD Biosciences (Carlsbad, CA, USA) and was prepared in a similar manner to PLX4720.

**Western blotting**

Proteins were extracted and blotted for as described in 256. After analysis, western blots were stripped once and reprobed for β-actin or GAPDH to demonstrate even protein loading. The antibodies to pERK, cleaved caspase-3, phospho-RB protein, total-RB protein, PARP, CRAF, cyclin D1 and total-ERK were from Cell Signaling Technology (Beverly, MA, USA) and the antibody to p27 was from BD Biosciences (Franklin Lakes, NJ, USA).

**Flow cytometry**

Cells were plated into 10-cm dishes at 60% confluency and left to grow overnight before being treated with PLX4720 (0.3 and 3 µM) for 24 h. In other studies, cells were treated with PLX4720 (3 µM) in the absence or presence of U0126 (3 µM) and harvested after 24 or 48 hours. Annexin-V labeling and propidium iodide staining were performed as described in 257.

**BrdU incorporation**

Cells were seeded in 10 cm plates at a density of 100 000 cells ml⁻¹ and grown overnight before being treated with PLX4720 (3 µM) for 72 h or 1, 2, 3 and 4 weeks. For the 1-, 2-, 3- and 4-week treatments, PLX4720 (3 µM) was added twice per week. One hour before the end of the drug treatment, BrdU (Sigma-Aldrich, St Louis, MO, USA) was added to the cells to a final concentration of 20 µM for 1 h. Cells were fixed and permeabilised with eBioscience's fixation and permeabilisation buffers. The BrdU
epitopes were exposed by incubating with DNase (Sigma-Aldrich) before staining with anti-BrdU conjugated to FITC (eBioscience, San Diego, CA, USA). In all, 7-AAD (BD Bioscience) was added to stain for DNA before acquisition on a BD Facscalibur flow cytometer.

**MEK1 sequencing**

Sequencing of MEK1 Exons 3 and 6 was performed as described in

**3D spheroid assays**

Melanoma spheroids were prepared using the liquid overlay method. Spheroids were treated with 0.03–30 μM of PLX4720 or U0126, PLX4720 (both 3 μM) and both drugs in combination for 72 h before being washed (3 × in media) and treated with calcein-AM, ethidium bromide (Molecular Probes, Eugene, OR, USA) for 1 h at 37°C, according to the manufacturer's instructions. After this time, pictures of the invading spheroids were taken using a Nikon-300 inverted fluorescence microscope.

**Colony formation**

Cells (1 × 10⁴ per ml) were seeded out into six-well plates and grown overnight before being treated with vehicle, PLX4720 (3 μM), U0126 (3 μM) or the two drugs in combination. Cells were left to grow for 4 weeks with new drug added twice per week. Media was aspirated, and plates were stained with crystal violet solution (50% methanol + 50% H₂O + 0.5% crystal violet). Control plates were grown for 1 week in the absence of any drug, until 100% confluency was reached.
Chapter 3

**PTEN loss confers BRAF inhibitor resistance to melanoma cells through the suppression of BIM expression.**

**Note to Reader**

Portions of this chapter have been previously published in Cancer Res. 2011 Apr 1;71(7):2750-60 and have been reproduced with permission from the American Association for Cancer Research Publishing. Contributions: Paraiso KH (designed/performed experiments, interpreted data, writing, figures); Xiang Y, Wood E, Abel EV, Koomen JM (designed/performed proteomic experiments); Chen YA (statistical analysis); Rebecca VW, Munko AC, Fedorenko IV (performed experiments); Anderson AR (data interpretation); Ribas A, Dalla Palma M (cell lines); Sondak VK, Nathanson KL (data interpretation, wording); Messina JL (pathology experiments, data interpretation, wording); Smalley KS (study concept, designed experiments, interpreted data, writing, figures).

**Introduction**

Clinical trial reports indicate that ~48% of patients with *BRAF* V600E mutated melanoma show some response to vemurafenib; however, this translates into >50% of patients being treated not meeting the Response Evaluation Criteria in Solid Tumors (RECIST) criteria threshold for a response. Mechanisms of intrinsic BRAF inhibitor resistance generally lead to increased cyclin D1 expression (in ~17% of *BRAF* mutated melanomas) thereby allowing for cell cycle entry when MAPK signaling is abrogated.
However, constitutive activity in other pathways, such as phospho-inositol 3-kinase (PI3K)/AKT/mTOR, also contribute to intrinsic resistance by limiting apoptotic responses \(^{153,258}\). One of the most critical negative regulators of AKT activity is the phosphatase and tensin homologue (PTEN), which hydrolyses PI-3,4,5-P\(_3\) to PI-4,5-P\(_2\), ultimately preventing the phosphorylation of AKT \(^{260}\). In this study we identify loss of PTEN expression, observed in >10% of melanoma specimens, as being responsible for increased PI3K/AKT signaling when BRAF is inhibited. Utilizing a mass spectrometry based highly sensitive, precise and selective liquid chromatography multiple reaction monitoring (LC-MRM), we further show that PTEN loss contributes to the intrinsic resistance of \(BRAF\) V600E-mutated melanoma cell lines to PLX4720 by suppressing the expression of the pro-apoptotic protein BIM.

**Results**

**The role of PTEN loss in the response to PLX4720**

Initial studies identified 6 \(BRAF\) mutated melanoma cell lines that retained PTEN expression (PTEN+; WM35, WM51, WM164, WM983A, 451Lu, SK-Mel-28) and 6 that lacked PTEN expression (PTEN−; WM239A, WM266-4, WM793, 1205Lu, WM9, M233) (Fig. 3.1A, Table 3.1). Genomic analysis showed the WM9 and M233 cell lines to be homozygously deleted for PTEN and the WM793 and 1205lu cell lines be hemizygously deleted for PTEN in conjunction with a PTEN mutation (Table 3.1). The PTEN+ cell lines had lower constitutive levels of pAKT (Ser473) compared to the PTEN− (Fig. 3.1A). Similar levels of pAKT (Thr308) were observed in the PTEN− and PTEN+ cell lines. Analysis of the growth inhibitory effects of PLX4720 by the MTT and Alamar Blue
assays (Fig. 3.1B, Fig. 3.2) did not reveal any statistically significant differences in the Gl₅₀ values between the PTEN+ and PTEN− cell lines (P=0.48, Fig. 3.3).

Fig. 3.1. PTEN predicts for PLX4720-induced apoptosis. A, basal PTEN and phospho-AKT (pAKT; S473, T308) expression in PTEN+ (WM164, 451Lu, SK-mel-28, WM983A, WM35, WM51) and PTEN− (WM239A, WM266-4, WM793, M233, WM9, 1205Lu) melanoma cell lines. B, MTT assay of PTEN+ (gray) expressing versus PTEN− (black) cell lines. C, PTEN+ cells are more sensitive than PTEN− cells to PLX4720-mediated apoptosis. Cells treated for 48 hours with 3 or 10 µmol/L PLX4720 before being stained for TMRM and Annexin-V. Apoptosis was measured by flow cytometry. Data shows mean ± SE mean of 3 independent experiments. *, PTEN+ cohort significantly different from PTEN− cohort (P < 0.05).
As increased PI3K/AKT signaling is known to limit apoptosis, we next measured PLX4720-induced apoptosis in our PTEN−/PTEN+ melanoma cell line panel (Fig. 3.1C). Here we observed that following PLX4720 treatment (3 and 10µM, 48 hrs), the PTEN− melanoma cell lines showed significantly less apoptosis than the PTEN+ (*P<0.05: Fig. 3.1C). PLX4720 mediated apoptosis was blocked by high doses (>75µM) of the capase inhibitor z-vad-fmak (Fig. 3.4).

Table 3.1: BRAF and PTEN mutation status of the cell line panel. Data shows PTEN status by MLPA, the location of PTEN mutations and PTEN protein expression status.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BRAF</th>
<th>PTEN Mutation</th>
<th>PTEN protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM164</td>
<td>V600E</td>
<td>WT</td>
<td>+</td>
</tr>
<tr>
<td>451Lu</td>
<td>V600E</td>
<td>WT</td>
<td>+</td>
</tr>
<tr>
<td>SK-MEL-28</td>
<td>V600E</td>
<td>WT</td>
<td>+</td>
</tr>
<tr>
<td>WM983A</td>
<td>V600E</td>
<td>WT</td>
<td>+</td>
</tr>
<tr>
<td>WM35</td>
<td>V600E</td>
<td>Hemizygous deletion</td>
<td>+</td>
</tr>
<tr>
<td>WM51</td>
<td>V600E</td>
<td>WT</td>
<td>+</td>
</tr>
<tr>
<td>WM239A</td>
<td>V600D</td>
<td>Homozygous deletion (Ex6)</td>
<td>–</td>
</tr>
<tr>
<td>WM266-4</td>
<td>V600D</td>
<td>Homozygous deletion (Ex6)</td>
<td>–</td>
</tr>
<tr>
<td>WM793</td>
<td>V600E</td>
<td>Hemizygous deletion (Mut W274X, Ex 8)</td>
<td>–</td>
</tr>
<tr>
<td>1205Lu</td>
<td>V600E</td>
<td>Hemizygous deletion (Mut W274X, Ex 8)</td>
<td>–</td>
</tr>
<tr>
<td>WM9</td>
<td>V600E</td>
<td>Homozygous deletion (Ex 3-9)</td>
<td>–</td>
</tr>
<tr>
<td>M233</td>
<td>V600E</td>
<td>Homozygous deletion</td>
<td>–</td>
</tr>
</tbody>
</table>

Fig 3.2: Direct comparison of the MTT and Alamar Blue reagents shows similar results between the two assays. PTEN− (blue) and PTEN+ (red) cell lines were treated with 0.003-30µM PLX4720 for 72hrs prior to the addition of MTT (dashed lines) or Alamar Blue (solid lines) reagents.
Fig. 3.3. Analysis of GI50 values in the PTEN+ and PTEN- cell line panel shows no statistical significance difference between the two groups (P=0.48).

Fig. 3.4. A reduction in apoptosis is only observed after administration of a high dose of the caspase inhibitor, z-vad-fmk. Flow cytometric analysis following PI staining of WM164 (PTEN+) cells treated for 48hrs with DMSO (Control), 10μM PLX4720 alone (PLX), 100μM z-vad-fmk alone or pretreated for 1 hr with 100μM z-vad fmk followed by treatment with 10uM PLX4720 (PLX+z-vad). Apoptosis is shown by the accumulation of the cells in sub-G1.

**Loss of PTEN expression is independent of melanoma stage**

We confirmed the incidence of PTEN loss in a tissue microarray containing a large sample of melanocytic neoplasms (n=192) drawn from all stages of tumor
progression (Fig. 3.5A-C). Results of immunohistochemical staining were graded from 0-3+ based on strength of the staining. It was observed that while non-atypical nevi rarely demonstrated loss of PTEN, >10% of atypical nevi and every stage of melanoma demonstrated loss of PTEN expression (either 0 or +1). Significantly, primary melanoma, lymph node metastases and distant metastases melanoma demonstrated loss of PTEN in 12.5%, 27% and 14% of cases each (Fig. 3.5A and C). Staining of the same TMA for pAKT demonstrated an increase in AKT activation as the tumors progressed from primary melanoma to distant metastasis (Fig. 3.5B). The level of pAKT positivity only partially correlated with PTEN expression status (Fig. 3.5C).

![Fig. 3.5. PTEN expression is lost at all stages of melanoma progression. A, Images showing representative immunohistochemical staining of PTEN. B, pAKT expression in a tissue array of nevi, atypical nevi, primary, and metastatic melanoma patient tumor samples. 0 and 1 indicate no to low PTEN expression and +3 indicates the highest expression whereas +2 to 3 relate to high expression of pAKT. Magnification 200×. C, left panel shows percentage loss of PTEN expression in each subset of patient samples as indicated in first of three columns per grouping whereas the right panel shows AKT activity in matched samples.](image-url)
PLX4720 and BRAF siRNA leads to AKT signaling in BRAF V600E-mutated/PTEN− melanoma cell lines

Treatment of the PTEN+/− cell line panels with PLX4720 increased pPDK1 and pAKT signaling only in the melanoma cell lines lacking PTEN expression (Fig. 3.6A). In contrast, PLX4720 inhibited BRAF activity in both PTEN− and PTEN+ cell lines with a similar potency (Fig. 3.7) and prevented BrdU uptake in both PTEN+ and PTEN− cell lines (Fig. 3.8).

Fig. 3.6. Loss of PTEN is associated with PI3K/AKT signalling following BRAF inhibition. A, PTEN+ (WM35, WM164, WM983A) and PTEN− (M233, WM9, WM793, 1205Lu) cells were treated with PLX4720 (24 hours: 0.03–3 µmol/L) and probed for phospho-PDK1 (pPDK1), total PDK1, phospho-AKT (pAKT), total AKT (tAKT), phospho-S6 (pS6), and total S6. Numbers indicate relative intensity of pPDK1 normalized to PDK1 and pAKT normalized to tAKT. B, PLX4720 increases pAKT following PTEN knockdown. WM35 cells were incubated with nontargeting siRNA (NT) or 2 different PTEN-specific siRNA’s (PTEN) before treatment with either vehicle or PLX4720 (3 µmol/L). C, siRNA knockdown of BRAF increases pAKT in melanoma cell lines that are PTEN−. WM164 (PTEN+) and WM793 (PTEN−) cells were incubated with lipofectamine alone (L), nontargeting siRNA (NT), or BRAF-specific siRNA (BRAF). Protein was extracted, resolved, and probed for BRAF, pAKT, total AKT, and GAPDH.
Addition of PLX4720 also led to the inhibition of mTOR activity in the PTEN+ cell lines only (Fig. 3.6A) and was associated with stimulation of LKB1 and AMPK signaling (Fig. 3.9). The requirement for PTEN in the increased AKT signaling was confirmed by studies showing that PLX4720 stimulated pAKT in WM35 cells (PTEN+) when PTEN was knocked down by siRNA (Fig. 3.6B). The effects of PLX4720 upon pAKT signaling were BRAF specific, with BRAF siRNA knockdown found to increase pAKT in PTEN− cells only (Fig. 3.6C). Mechanistically, PLX4720 increased IGF-I signaling in the PTEN− cells, with the IGFR1 inhibitor NVP-ADW-742 being found to abrogate the PLX4720-mediated increase in pAKT signaling (Fig. 3.10) 261.

![Fig. 3.7. The BRAF inhibitor, PLX4720 inhibits ERK activity in both BRAF V600E PTEN- and BRAF V600E PTEN+ cell lines. PTEN- (WM793, 1205Lu) and PTEN+ (WM35 and WM164) cells were treated for 1hr with PLX4720 (0.03-3µM) prior to probing for phospho-ERK1/2. Blots were stripped and reprobed for total ERK as a loading control.](image)

![Fig. 3.8. PLX4720 causes S-phase arrest in both PTEN- (WM793) and PTEN+ (WM164) cell lines as seen by lack of BrdU incorporation. Cells were treated for 72hrs with PLX4720 (0.3 or 3µM) prior to pulsing with BrdU for 1hr. Cells were then stained with an anti-BrdU antibody and (y-axis) and 7-AAD (x-axis) followed by flow cytometric analysis. Percentages in the upper right corner of each box correspond to the percentage of cells in S-phase.](image)
Fig. 3.9. Following inhibition of BRAF, activation of LKB1 and AMPK corresponding to inactivation of RAPTOR is observed in PTEN+ (WM164) but not PTEN- (WM793). Cells were treated for 24 hrs with PLX4720 (0.03-3µM) prior to protein extraction and Western blotting.

**PLX4720 treatment differentially regulates BIM in PTEN+ and PTEN− cells**

We next used LC-MRM to quantify the PLX4720-induced changes in the expression of 17 members of the Bcl-2 protein family (Fig. 3.11A, results for 9 proteins). BIM was the only proapoptotic protein to demonstrate significant differences between the PTEN− and PTEN+ cell lines (14- and 4-fold increases, respectively) (Fig. 3.11A).
Fig. 3.11. LC-MRM identifies differential regulation of BIM in PTEN+ and PTEN− cell lines following PLX4720 treatment. A, LC-MRM data showing the fold changes in the expression of Bak, Bax, Bcl-2, Bcl-w, Bcl-xL, BID, BIM, Bok, and Mcl-1 over internal standard in the WM164 (PTEN+) and 1205Lu (PTEN−) cell lines following treatment with PLX4720 (10 µmol/L, 0–48 hours). Statistical analyses of BIM fold change in PTEN− versus PTEN+. *, P < 0.05. B, Western blot of BIM expression following PLX4720 treatment (10 µmol/L, 0–48 hours) in PTEN− (WM793, 1205Lu) and WM164 cell lines (PTEN+). C, immunofluorescence staining of BIM expression, DAPI staining of PTEN− (M233, WM9, WM793, 1205Lu) and PTEN+ (WM35, WM164, WM983A) cells following PLX4720 treatment (3 µmol/L, 48 hours). D, Western blot of BAD phosphorylation following treatment with PLX4720 (0–48 hours) in PTEN− (WM793, 1205Lu) and PTEN+ WM164. Annexin V binding following treatment with 3 or 10 µmol/L PLX4720 (48 hours) showing increased apoptosis in WM793 stably overexpressing WT BAD. *, P < 0.05.
Western blots and immunofluorescence staining confirmed the LC-MRM data and showed a greater degree of PLX4720-induced (3 and 10 µmol/L) BIM expression in the PTEN+ cell lines compared to PTEN− cell lines (Fig. 3.11B, C and Fig. 3.12). In parallel, we observed that PLX4720 also increased the inactivation of BAD (as shown by increased phospho-BAD) in the PTEN− cells (Fig. 3.11D) and that overexpression of BAD in the PTEN− cells enhanced PLX4720-mediated apoptosis (Fig. 3.11D). PLX4720 treatment also increased total BAD expression in both the PTEN+ and PTEN− cell lines. Little PLX4720-induced changes in Mcl-1 expression were observed in the PTEN+ and PTEN− cell lines (Fig. 3.13).

**Fig. 3.12.** BIM expression is higher in PTEN+ (WM164, WM983A) compared to PTEN− (M233, WM9, WM793) following treatment with PLX4720. Cells were treated for 0, 8, 24 or 48 hrs with 3µM PLX4720.

**Fig. 3.13.** Following treatment with PLX4720, no significant change is seen within the PTEN+ and PTEN− cells in the expression of Bcl-2 and Mcl-1. PTEN+ (WM164) and PTEN− (WM793) were treated for 0, 8, 24 and 48 hours with 10µM PLX4720 prior to protein extraction.
**PTEN is required for efficient BIM upregulation following BRAF inhibition**

We next explored the link between PTEN expression status and PLX4720-mediated induction of BIM. siRNA knockdown of PTEN using 2 siRNA sequences led to the inhibition of PLX4720-induced BIM expression in PTEN+ cells (Fig. 3.14A). We next determined whether reintroduction of wild-type PTEN (PTEN-wt) or lipid phosphatase mutated PTEN (PTEN-G129E) into a PTEN− cell line enhanced BIM expression when BRAF was inhibited. In these studies, we used an isogenic pair of WM793 melanoma cell lines that expressed either doxycycline inducible PTEN-wt or PTEN-G129E mutant. Control studies showed that doxycycline (100 ng/mL, 48 hours) increased expression of PTEN in both cell lines (Fig. 3.14B). The impaired lipid phosphatase function of the G129E mutant was confirmed by the fact that only the induction of PTEN-wt suppressed pAKT activation (Fig. 3.14B). The role of PTEN in the PLX4720-mediated induction of BIM was confirmed by the enhanced expression of BIM seen when PTEN-wt was induced compared to when PTEN-G129E was induced (Fig. 3.14B) and was paralleled by a significant increase in PLX4720-mediated apoptosis (P < 0.05; not shown). Interestingly, the addition of PLX4720 decreased the expression of PTEN through mechanisms that are not currently clear. The effects of PI3K/AKT signaling upon the suppression of BIM were mostly mediated through AKT3, with siRNA knockdown of AKT3 found to increase BIM expression when BRAF was inhibited (Fig. 3.15). As a final test of the relevance of BIM induction in the PLX4720-induced apoptotic response, we showed that siRNA knockdown of BIM led to an impairment of PLX4720 (3 and 10 µmol/L) induced apoptosis (Fig. 3.14C).
Fig. 3.14. PTEN is required for efficient upregulation of BIM following BRAF inhibition. A, WM164 cells (PTEN+) transfected with nontargeting siRNA (NT) or 2 PTEN-specific siRNAs (I and II) before treatment with PLX4720 (3 or 10 µmol/L, 48 hours). Proteins were probed for expression of BIM, GAPDH, and PTEN. B, induction of PTEN-wt but not PTEN-G129E in WM793 (PTEN−) cells was sufficient to increase BIM expression when BRAF was inhibited. Left, Western blot shows induction of PTEN-wt and PTEN-G129E following doxycycline treatment. Right, induction of PTEN-wt + PLX4720 induces BIM more efficiently than PTEN-G129E + PLX4720. C, BIM is required for PLX4720-induced apoptosis in PTEN+ cells. WM164 and WM983A cells were incubated with nontargeting siRNA (NT) or transfected with 2 BIM-specific siRNAs (BIM I and BIM II) before treatment with PLX4720 (3 or 10 µmol/L, 48 hours). Flow cytometry studies showed a significant reduction in TMRM loss and Annexin V binding when cells were transfected with BIM siRNA compared with nontargeting control siRNA (*P < 0.05).
Fig. 3.15. Dual inhibition of BRAF and AKT3 leads to a significant increase in BIM expression in PTEN-cells. 1205Lu cells were incubated with lipofectamine alone (L) or transfected with 25nM non-targeting siRNA (NT), 25nM AKT1 siRNA or 25nM AKT3 siRNA for 72 hrs prior to treatment with DMSO or 3µM PLX4720 for 24hrs. Numbers below each band of the PLX treated groups represent the relative intensity of BIM normalized to the corresponding controls.

**Dual BRAF/PI3K inhibition enhances BIM expression and apoptosis in PTEN-cells**

One of the major effects of PTEN is to limit PIP3 levels through its lipid phosphatase activity. We next treated PTEN- cell lines with a PI3K inhibitor (GDC-0941, 3 µmol/L or LY294002 10 µmol/L), PLX4720 (3 and 10 µmol/L), or the 2 drugs in combination, and showed that combined PI3K and BRAF inhibition increased the level of BIM expression in both Western blot and immunofluorescence studies (Fig. 3.16A).
Fig. 3.16. Dual PI3K/BRAF inhibition upregulates BIM and enhances apoptosis in PTEN− cells. A, left, Western blot of 1205Lu cells treated with PLX4720 (3 µmol/L, 48 hours), GDC-0941 (3 µmol/L, 48 hours), or both (P+G); right, immunofluorescence staining of BIM (green) and DAPI (blue) in PTEN− cells after PLX4720 treatment (3 µmol/L, 48 hours), LY294002 (10 µmol/L, 48 hours), or both (PLX+LY). B, left, immunofluorescence staining of PTEN− 1205Lu following combined inhibition (3 µmol/L PLX4720 + 10 µmol/L LY294002, 48 hours) increases nuclear localization of FOXO3a (green). DAPI is shown in blue. 40x. Right, combined inhibition (3 µmol/L PLX4720 + 10 µmol/L LY294002, 48 hours) increases PTEN− WM793 BIM mRNA levels to those observed with single BRAF inhibition (3 µmol/L PLX4720, 48 hours) in PTEN+ WM35. C, PTEN− cells were treated with PLX4720 (3 µmol/L, 48 hours), GDC-0941 (3 µmol/L, 48 hours), or both (3P+3G). Annexin-V was analyzed by flow cytometry (*, P < 0.05 between combination and each inhibitor alone). D, BRAF/PI3K inhibitor treatment blocks the escape of 1205Lu cells (PTEN−) from therapy. 1205Lu spheroids were treated with PLX4720 alone (3 and 10 µmol/L, data shows 3 µmol/L), LY294002 (10 µmol/L) or both drugs for 72 hours. In other studies, spheroids were treated with drugs for 72 hours and then allowed to recover for 120 hours. Micrograph shows viability staining (green = live cells, red = dead cells). 10x.
Both the MAPK and PI3K/AKT pathways are known to regulate BIM RNA expression levels through the transcription factor FOXO3a. In agreement with this, PLX4720 treatment increased the nuclear accumulation of FOXO3a in the PTEN+ but not PTEN− melanoma cells (Fig. 3.16B; not shown). Consistent with a role for increased AKT signaling suppressing BIM expression in PTEN− cells, dual BRAF and PI3K inhibition increased nuclear FOXO3a localization in the PTEN− cell lines (Fig. 3.16B) and enhanced the level of BIM mRNA (Fig. 3.16B). siRNA knockdown of FOXO3a was further found to block PLX4720-mediated upregulation of BIM in PTEN+ cells (Fig. 3.17). The observation that PLX4720 treatment led to increased PI3K/AKT signaling in PTEN− melanoma cell lines suggested that dual BRAF/PI3K inhibition could be one strategy to overcome intrinsic resistance. In agreement with this, the combination of PLX4720 with the PI3K inhibitor GDC-0941 significantly enhanced the levels of apoptosis observed in PTEN− melanoma cell lines compared to either the BRAF or PI3K inhibitor alone (Fig. 3.16C). Similar results were also observed in a 3D spheroid assay, where combined PLX4720 (3 µmol/L) and LY294002 (10 µmol/L) treatment prevented the recovery of cell growth observed when melanoma spheroids were treated with either drug alone (Fig. 3.16D). The proposed mechanism for BIM regulation is shown in Fig. 3.18.

![Fig. 3.17. FOXO3a silencing prior to BRAF inhibition leads to a decrease in BIM expression. WM164 (PTEN+) cells were incubated with lipofectamine alone (L) or transfected with 25nM non-targeting siRNA (NT) or 25nM FoxO3a siRNA for 72 hrs prior to treatment with 3µM PLX4720 for 24hrs.](image-url)
Fig. 3.18. Schema illustrating the differences signaling in PTEN+ and PTEN- cells following inhibition of BRAF. In both the PTEN+ and PTEN+ cells, inhibition of BRAF decreases the MEK/ERK mediated phosphorylation of BIM leading to its stabilization. In PTEN- cells, BRAF inhibition is associated with increased AKT signaling leading to the phosphorylation of FOXO3a, an increase in its nuclear exclusion and a decrease in BIM mRNA levels. Overall this leads to a decrease in BIM expression in the PTEN- cells when BRAF is inhibited and an impaired apoptotic response.
Discussion

In this study we focused on the underlying mechanisms of intrinsic resistance observed that is observed in >50% of vemurafenib treated melanoma patients. Melanomas have constitutive activity in several oncogenic pathways to regulate cell cycle entry and survival. Of these, melanoma initiation and progression is known to be dependent on both the Ras/Raf/MEK/ERK and PI3K/AKT pathways \(^{98,174,266,267}\). The processes underlying this signaling activity differ according to the initiating oncogenic event. As such, melanomas with activating NRAS mutations rarely harbor concurrent alterations in either BRAF or PTEN/AKT as Ras stimulates both the Raf/MEK/ERK and PI3K/AKT pathways \(^{103,268}\). In contrast, melanomas with BRAF mutations require other mechanisms to activate their PI3K/AKT signaling and frequently show inactivation/deletion of PTEN or increased AKT3 expression \(^{97,269-271}\).

We began by investigating PTEN expression across a large sample (n=192) of melanocytic lesions and found that PTEN was lost in 10% to 27% of melanomas. Although PTEN loss overlapped with levels of pAKT staining, it was not always well correlated, agreeing with observations that other mechanisms may underlie the increased AKT activation associated with melanoma progression \(^{272}\). Our results agree with other published studies on smaller numbers of melanoma samples (n = 16–39), and confirm that reduced PTEN expression is a significant oncogenic event for a restricted subgroup of melanomas \(^{269,270,273}\). Although PTEN was retained in nonatypical nevi, a significant number (23%) of atypical nevi lacked expression, suggesting that this is an early event in melanoma development. This idea is supported by recent mouse
model studies showing that the conditional expression of the \textit{BRAF} V600E mutation leads to melanoma development only when PTEN is suppressed\textsuperscript{98}.

Though lack of PTEN expression did not predict for sensitivity of \textit{BRAF} V600E-mutated melanoma cell lines to the growth inhibitory effects of PLX4720, there were significant differences in PLX4720-mediated apoptosis between PTEN\textsuperscript{+} and PTEN\textsuperscript{−} melanoma cell lines. We hypothesized that PTEN\textsuperscript{−} melanoma cell lines would show higher levels of AKT activity and that this would mediate resistance to PLX4720. However, this was not the case. Instead we observed that increased AKT signaling only occurred in PTEN\textsuperscript{−} cell lines in the context of BRAF inhibition. The effects on AKT were recapitulated in PTEN\textsuperscript{+} melanoma cell lines when PTEN was knocked down, indicating that these effects were PTEN dependent. The increase in AKT signaling observed in the PTEN\textsuperscript{−} cell line panel was associated with PDK1 phosphorylation and increased expression of IGF-I. These effects were reversed following pretreatment with the IGF1R inhibitor NVD-ADW-742\textsuperscript{261}, suggesting a link between BRAF inhibition and enhanced IGF1R-mediated PI3K signaling. Similar findings, linking BRAF/MEK inhibition to increased IGF signaling, have also been reported by other groups\textsuperscript{173,212}.

AKT plays a critical role in cancer development and regulates cell survival through direct phosphorylation of BAD, stimulation of ribosomal S6 kinase, inhibition of FOXO, and inhibition of glycogen synthase 3-kinase\textsuperscript{272}. To determine the mechanism of PLX4720-induced apoptosis in the PTEN\textsuperscript{+} melanoma cell lines, LC-MRM analysis was used to quantify the relative expression of members of the Bcl-2 protein family\textsuperscript{274}. For the majority of apoptotic related proteins examined, PLX4720 treatment was associated with similar dynamics in both the PTEN\textsuperscript{+} and PTEN\textsuperscript{−} cell lines. These
findings agree with previous studies and show that BRAF inhibition leads to an increase in the expression in the proapoptotic protein BIM \(^{90,91,275}\). However, in contrast to previous investigations, our studies utilized highly selective and sensitive LC-MRM analysis, which allowed us to identify significant PTEN-dependent differences in the level of PLX4720-induced BIM expression. BIM is a proapoptotic BH3-only member of the Bcl-2 protein family that exists in 3 major splice forms; extra long (BIM-EL), long (BIM-L) and short (BIM-S) \(^{276,277}\). It exerts its cytotoxic activity by binding to and antagonizing the antiapoptotic proteins Bcl-2, Bcl-w, Bcl-XL, and Mcl-1 \(^{276,277}\). Expression of BIM is regulated both transcriptionally and post-transcriptionally by a number of signaling pathways, including BRAF/MEK/ERK, JNK, p38 MAPK, and PI3K/AKT \(^{90,276,278,279}\).

In melanoma, the *BRAF* V600E mutation regulates BIM expression through MEK/ERK pathway-mediated phosphorylation of the extra-long form of BIM (BIM-EL) at Serine 69, resulting in subsequent BIM degradation by the proteasome \(^{90,280}\). Our study was the first to demonstrate that the level of BIM expression following BRAF inhibition is also determined by PTEN status and that the differing levels of BIM induction can determine the extent of apoptosis induction when BRAF is inhibited. Apoptosis control in melanoma cells is complex and increased AKT signaling is likely to regulate survival at multiple levels. The cell death inducing protein, BAD, is a well-recognized target of AKT. AKT inactivates BAD via phosphorylation at Ser99, thereby preventing BAD from binding to Bax and relieving the antagonism of Bax on Bcl-2 and Bcl-XL \(^{91}\). A role for BAD inactivation in the escape of PTEN\(^{-}\) cells from PLX4720-induced apoptosis was suggested by the preferential inactivation of BAD when BRAF was inhibited. Further,
overexpression of BAD sensitized the same cell line to PLX4720-induced apoptosis. Another candidate proapoptotic factor upregulated in melanoma cells following BRAF/MEK/ERK inhibition is Bcl-2 modifying factor (BMF)\textsuperscript{275,281}. BMF, which is also regulated through the PI3K/AKT pathway, mediates its apoptotic effects by binding to Mcl-1. Although it is possible that BMF may also be differentially regulated in PTEN+/− cells, we, like other groups, were unable to confirm the selectivity of commercially available BMF antibodies\textsuperscript{275,282}.

In addition to regulating cytoplasmic PIP3 levels through its lipid phosphatase-dependent function, PTEN also localizes to the nucleus where it exerts its tumor suppressor function through lipid phosphatase-independent effects upon the regulation of chromosomal integrity, p53 acetylation and the expression of cyclin D1\textsuperscript{283}. As the lipid phosphatase-dependent and -independent functions of PTEN are likely to be very different, we re-expressed either wild-type PTEN or a PTEN mutant with impaired lipid phosphatase function (G129E) in melanoma cells that were PTEN−\textsuperscript{284}. These studies confirmed the requirement for the lipid phosphatase function of PTEN in the suppression of BIM expression, with PLX4720 treatment inducing only a weak upregulation of BIM protein when PTEN G129E was expressed. The importance of the lipid phosphatase function in the suppression of BIM expression was supported by experiments showing that combined BRAF/PI3K inhibition and siRNA knockdown of AKT3 both enhanced the level of BIM expression and increased the level of apoptosis in the PTEN− cells. In other systems, AKT downregulates BIM expression by phosphorylating and inactivating the transcription factor FOXO3a\textsuperscript{262,264}. In agreement with these reports, we confirmed that PLX4720 treatment led to increased nuclear
accumulation of FOXO3a in the PTEN+ cells only and demonstrated that siRNA knockdown of FOXO3a abrogated the increase in BIM expression.

In summary, we have identified an important role for PTEN loss in the intrinsic resistance of BRAF V600E mutated melanoma cells to the BRAF inhibitor PLX4720. These studies further suggest that increased BIM expression may be a useful biomarker in predicting clinical response to BRAF inhibition and demonstrates that LC-MRM is a powerful tool for monitoring BIM expression that could be translated to patient assessment. This work also provides a rationale for combining MAPK and PI3K/AKT pathway inhibitors or MAPK inhibitors with BCL-2 family member inhibitors (for example the combination of dabrafenib + trametinib with navitoclax, Table 1.4) for the management of melanomas that are BRAFV600E/PTEN−.

**Methods and Materials**

**Cell culture and MTT assay**

Melanoma cell lines were a gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA) and were grown as described in Chapter 2. MTT assays were performed as described in Chapter 2. The identity of the Wistar Institute cell lines was confirmed using the Coriell Institute (Camden, NJ) cell identity mapping kit. The M233 cell line was derived as described in 136 and its identity confirmed by Biosynthesis Inc (Lewisville, Tx) by STR validation analysis.

**Generation WM793TR-PTEN cell lines**

Wild-type and G129E PTEN (phosphatase deficient) human cDNAs were a gift from Dr. Bill Sellers (Dana-Farber Cancer Institute) 284. WM793TR-PTEN-wt, WM793TR-PTEN-G129E and WM793 cells overexpressing wild-type BAD were a kind gift from Dr Andrew
Aplin (Kimmel Cancer Center, Philadelphia, PA). Inducible expression of PTEN was obtained by treatment of cultures with doxycycline at a final concentration of 100ng/ml. The WM793 cells stably expressing wild-type BAD were generated as described in 275.

**Western blotting**

Proteins were blotted for as described in Chapter 2. The antibodies to phospho-AKT (Ser473 and T308), total AKT, phospho-BAD (S75 and S99), Bcl-2, BIM, BRAF, FOXO3a, phospho-PDK1, total PDK1, PTEN, phospho-S6 and total S6 were from Cell Signaling Technology (Beverly, MA).

**Flow cytometry**

Cells were treated with 3 or 10µM PLX4720 for 24 or 48 hr or treated with PLX4720 (3 or 10µM) in the absence or presence of GDC-0941 (3 µM, Selleck Chemical Co.) and harvested after 48 hr. Annexin-V/TMRM staining was performed as described in Chapter 2.

**RNA interference**

Cells were grown overnight in RPMI complete media. The following day, complete media was replaced with Opti-MEM (Invitrogen) and one of the following siRNA sequences in complex with Lipofectamine 2000 (Invitrogen): 50nM BRAF (Dharmacon), 20nM PTEN, 25nM BIM (Cell Signaling Technology). Scrambled siRNA's at each concentration were also added as non-targeting controls. A final concentration of 5% FBS in complete RPMI was added the next day. Cells were transfected for a total of 48-72 hr prior to treatment with PLX4720 (3-10µM).
**Quantitative real-time PCR**

Total RNA was isolated using Qiagen’s RNeasy mini kit. The following TaqMan® Gene Expression Assays primer/probes were used: Hs00197982_m1 (BIM), P/N 4319413E (18S) and Hs99999905_m1 (GAPDH). The 18S + GAPDH data were used for normalizing BIM. After a 2-min incubation at 50°C, AmpliTaq Gold was activated by a 10-min incubation at 95°C, followed by 40 PCR cycles consisting of 15 s of denaturation at 95°C and hybridization of probe and primers for 1 min at 60°C. All standards and samples were tested in triplicate wells and data were analyzed using SDS software version 2.3.

**Immunofluorescence staining**

Cells were plated onto coverslips and treated with PLX4720 for 48hrs before being fixed and permeabilized as previously described and imaged with a Leica confocal microscope at 40X magnification.

**3D spheroid assays**

Collagen implanted spheroids were prepared using the liquid overlay method as described in Chapter 2 and were treated with 3µM of PLX4720, 10µM LY294002 or both drugs in combination for 72hr before being analyzed by fluorescence microscopy as described in. In other studies, spheroids were treated for 72hrs, washed 3X in fresh media and allowed to recover for 120hrs before analysis.

**Liquid chromatography, multiple reaction monitoring mass spectrometry (LC-MRM) analysis**

Whole cell proteins extracts were separated by SDS-PAGE, visualized with Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA) and selected bands were excised.
Following digestion, the internal standard peptides were added in 2% acetonitrile. LC-MRM analysis was performed as described in with three replicate analyses for each peptide. Quantification was achieved by using the sum of the peak areas for all detected transitions using Xcalibur QuanBrowser (Thermo, San Jose, CA). Relative protein expression is determined using the ratio of peak area of the native peptide to corresponding internal standard (Table 3.2).

**Table 3.2. Labeled Internal Standards for LC-MRM Quantification of Apoptosis-Related Proteins.** Each protein is listed with its gel region and the target peptide sequence for quantitative monitoring as well as the stable isotope label or amino acid replacement used to synthesize the internal standard. After SDS-PAGE separation and proteolytic digestion, each peptide standard is spiked at the amount listed.

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<th>Target Protein</th>
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<th>IS (fmol)</th>
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</table>
**Immunohistochemical staining**

A melanoma tissue array was generated from de-identified formalin-fixed paraffin-embedded tissue samples from the Moffitt Pathology archives under a protocol approved by the Institutional Review Board of the University of South Florida. Slides were stained using the Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) as per manufacturer’s protocol. The PTEN antibody (1:200, #E4250, Spring Bioscience, Pleasanton, CA) was incubated for 32 min and the pAKT antibody (1:20, #4051, Cell Signaling, Danvers, MA) was incubated for 16 min. Slides were analyzed by two independent observers and consensus scored on a scale from (0 to +3).

**Statistical analysis**

Data show the mean of at least three independent experiments ± the S.E. mean, unless stated otherwise. GraphPad Prism 5 statistical software was used to perform the Student’s T-test where *indicates $P \leq 0.05$. 

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Chapter 4

The heat shock protein-90 inhibitor XL888 overcomes BRAF inhibitor resistance mediated through diverse mechanisms.

Note to Reader

Portions of this chapter have been previously published in Clin Cancer Res. 2012 May 1;18(9):2502-14 and have been reproduced with permission from the American Association for Cancer Research Publishing. Author contributions: Paraiso KH (designed/performed experiments, interpreted data, writing, figures); Haarberg HE, Rebecca VW (performed experiments); Wood E, Xiang Y, Koomen JM (designed and performed proteomic experiments); John JK, Sarnaik AA (patient specimens), Chen AY (statistics); Ribas A, Lo RS (cell lines); Weber JS, Sondak VK (data interpretation, wording); Smalley KS (study concept, designed experiments, interpreted data, writing, figures).

Introduction

In the previous chapters we discussed growing evidence that BRAF inhibitor resistance is complex, multifactorial, and results from intrinsic and acquired mechanisms. To date, the loss/inactivation of PTEN function, deletion of the retinoblastoma protein (RB), expression of the mitogen-activated protein kinase (MAPK) family member COT (MAP3K8) and amplification of cyclin D1 have each been shown to mediate de novo resistance by either diminishing the apoptotic response or allowing for cell-cycle entry when oncogenic BRAF is inhibited. Acquired resistance is
mediated through constitutive signaling by receptor tyrosine kinases (RTK; e.g. IGF1R and PDGFR-β), mutations in NRAS or MEK1, alterations in NF1, AKT1 and AKT3, increased expression of COT and as the result of BRAF amplifications or truncations. The apparent diversity of BRAF inhibitor resistance mechanisms, and the likelihood that others exist, is expected to complicate the design of future clinical trials (Tables 1.2 and 1.4). These observations have lead us to hypothesize that BRAF inhibitor resistance may be best managed through broadly targeted strategies that inhibit multiple pathways at once.

The HSP90 family of chaperones maintains the malignant potential of cancer cells by regulating the conformation, stability, and function of many RTKs and kinases required for oncogenic transformation. Many proteins required for melanoma initiation and progression, including mutated BRAF, CRAF, IGF1R, cyclin D1, CDK4, and AKT are known to be clients of HSP90. The role of HSP90 in the stabilization of so many cancer-related proteins has made it an attractive target for therapeutic intervention. Currently, more than 13 small molecule inhibitors of HSP90 are at various stages of preclinical and clinical development (Table 1.5).

Although HSP90 inhibitors have shown only limited single-agent activity, more promising clinical efficacy has been shown when HSP90 inhibitors are combined with other agents. There is now good evidence that HSP90 inhibitors can overcome trastuzumab resistance in breast cancer and potentiate the effects of bortezomib in treatment-refractory myeloma. In this study, we show that all of the signaling proteins implicated thus far in the escape from BRAF inhibitor therapy are clients of HSP90, and that the pharmacologic inhibition of HSP90 abrogates both acquired and
intrinsic vemurafenib resistance by restoring the apoptotic response. These studies support the use of HSP90 inhibitors in overcoming BRAF inhibitor resistance.

**Translational Relevance**

The impressive clinical response of melanoma patients to the BRAF inhibitor vemurafenib is limited by the onset of resistance. Resistance can be intrinsic or acquired and is mediated through an array of mechanisms including acquired mutations in NRAS and MEK1, truncated BRAF, overexpression of COT, CRAF, PDGFR-β, cyclin D1, and IGF1R. This apparent diversity of resistance mechanisms, coupled with the phenotypic and cell signaling plasticity of melanoma cells, represents a considerable clinical challenge for which no management strategies currently exist. Here, we show that all of the signaling proteins implicated thus far in the escape from vemurafenib therapy are clients of HSP90. Inhibition of HSP90 using XL888 overcomes both acquired and intrinsic vemurafenib resistance by restoring the apoptotic response, which suggests that the combination of vemurafenib and an HSP90 inhibitor may be a strategy to delay and/or overcome BRAF inhibitor resistance.

**Results**

*Inhibition of HSP90 overcomes resistance to vemurafenib resistance mediated through diverse mechanisms*

We first assembled a panel of BRAF V600E mutant melanoma cell lines with different mechanisms of intrinsic resistance and acquired vemurafenib resistance (Table 4.1). Treatment of matched BRAF inhibitor–naive and BRAF inhibitor–resistant melanoma cell lines with vemurafenib showed a statistically significant difference in the extent of growth inhibition ($P = 0.02$; $t = -4.38$; Fig. 4.1 and Fig 4.2) when resistance
was mediated through increased PDGFRβ expression (M229R) and an acquired NRAS mutation (M249R), as well as 2 lines with uncharacterized mechanisms of resistance (WM164R and 1205LuR) (Fig. 4.2A). Cell lines with amplification of cyclin D1 (WM39) and overexpression of COT (RPMI 7951) showed signs of intrinsic resistance to vemurafenib (IC50 > 3 μmol/L). By contrast, treatment with the HSP90 inhibitor XL888 led to dose-dependent decreases in the growth of all the cell lines with no significant difference in IC50 values observed between the naive and resistance pairs of cell lines (t = 0.25, P = 0.82; Fig. 4.2A). The growth inhibitory effects of XL888 were associated with induction of either a G1-phase cell-cycle arrest (WM164, M229, M229R, M249, M249R, 1205Lu, and WM39) or a G2-M phase cell-cycle arrest (WM164R, 1205LuR, and RPMI 7951; Fig. 4.2B). Treatment of all of the vemurafenib-resistant melanoma cell lines with XL888 (300 nmol) induced high levels (>66%) of apoptosis as shown by Annexin V binding, caspase-3 cleavage, and loss of mitochondrial membrane potential (TMRM) in every cell line tested (Fig. 4.2C and Fig. 4.3). The cytotoxic effects of XL888 were durable with no signs of colony formation observed in any of the cell lines (up to 4 weeks: Fig. 4.2D and Fig. 4.4).

Table 4.1: List of cell lines used in this study with mechanisms of resistance outlined.

<table>
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<td>1205LuR</td>
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<tr>
<td>RPMI7951</td>
<td>COT overexpression</td>
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</tr>
</tbody>
</table>

References:


**Fig. 4.1.** Analysis of GI50 values in the naïve and resistant cell line panel shows a statistical significance difference between the two groups (P=0.02).
Fig. 4.2. The HSP90 inhibitor XL888 blocks the growth and survival of melanoma cell lines with diverse mechanisms of vemurafenib resistance. A, growth assay of vemurafenib-naive and vemurafenib-resistant melanoma cell lines and melanoma cell lines with intrinsic resistance. Left, cells treated with vemurafenib (1 nmol/L–10 µmol/L: 72 hours) before being subject to the MTT assay. Right, cell growth assay of the cell line panel from (A) to the HSP90 inhibitor (1 nmol/L–10 µmol/L: 72 hours). B, cell-cycle effects of XL888 (300 nmol/L: 24 hours) on vemurafenib-sensitive and vemurafenib-naive cell lines. Cells were fixed, stained with propidium iodide, and distributions analyzed by flow cytometry. C, XL888 induces apoptosis in every model of acquired vemurafenib resistance tested. Cells were treated for either 72 or 144 hours with XL888 (300 nmol/L). Apoptosis measured by Annexin V (flow cytometry). D, top: colony formation assay showing the long-term effectiveness of XL888. Cell lines were treated with 300 nmol/L XL888 for 4 weeks before being fixed and stained with crystal violet. Bottom: quantification (absorbance) of colony formation.
Fig. 4.3. XL888 induces apoptosis in every model of vemurafenib resistance tested. Figure shows loss of mitochondrial membrane potential (TMRM) following 72 and 144 hr of XL888 treatment (300 nM). Blot shows cleavage of caspase-3 following XL888 treatment (300 nM, 48 hrs).

Fig. 4.4. High power view of the control plates from the colony formation assay shown in Figure 4.2D.

**Inhibition of HSP90 degrades all of the proteins identified as being critical for vemurafenib resistance**

We next asked whether XL888 treatment induced the degradation of all the signaling mediators implicated in acquired and intrinsic resistance (Fig 4.5 summarizes melanoma-relevant HSP90 clients).
XL888 treatment (300 nmol/L, 48 hours) led to the degradation of IGF1R, PDGFRβ, ARAF, CRAF, and cyclin D1 and the inhibition of AKT, ERK, and S6 signaling in all of the cell lines with acquired BRAF inhibitor resistance (Fig. 4.6A). These effects were found to be time dependent with some sensitive proteins, such as pAKT being downregulated at 8 hours or earlier (Fig. 4.6A). In the intrinsically vemurafenib-resistant melanoma cell lines RPMI7951 and WM39, XL888 treatment was found to degrade both COT and cyclin D1, respectively (Fig. 4.6A).
Fig. 4.6. XL888 degrades proteins involved in BRAF inhibitor resistance leading to apoptosis induction. A, Left: XL888 degrades IGF1R, PDGFRβ, ARAF, CRAF, and cyclin D1 and inhibits pAKT, pERK, and pS6 signaling in 4 cell lines with acquired BRAF inhibitor resistance. XL888 degrades the expression of COT and cyclin D1 in cell lines with intrinsic resistance to vemurafenib. Right: time dependency of the XL888-mediated effects on pAKT, pERK, pS6, COT, and BIM. RPMI7951 cells were treated with XL888 for 0 to 48 hours. B, XL888 (1 µmol/L, 144 hours) blocks growth and survival of vemurafenib-resistant melanoma cell lines grown as 3D collagen-implanted spheroids. Staining shows cell viability, green corresponds to live cells and red to dead cells, 4x. C, XL888 + vemurafenib enhances apoptosis in RPMI7951, WM793 and 1205 Lu. Cells treated with vemurafenib (3 µmol/L), XL888 (300 nmol/L), or both for 48 hours. Apoptosis was measured by Annexin V staining and flow cytometry. D, XL888 + vemurafenib reduces the survival of intrinsically resistant melanoma cell lines grown as 3D collagen-implanted spheroids. RPMI7951 and WM793 were treated with XL888 (1 µmol/L), vemurafenib (3 µmol/L), or both for 48 hours. Viability measured as above.
Because the microenvironment modulates the response of melanoma cells to targeted therapies, we next grew the panel of vemurafenib-resistant cell lines as collagen implanted 3-dimensional (3D) spheroids and noted that XL888 was effective at inducing cell death (Fig. 4.6B). In line with the observation that COT mediates resistance to vemurafenib, the combination of XL888 with vemurafenib significantly enhanced the level of apoptosis/cytotoxicity in 3D culture in RPMI7951 cells, compared with XL888 alone (Fig. 4.6C and D). A similar enhancement was noted when the vemurafenib ± XL888 combination was applied to 2 melanoma cell lines in which the primary resistance was mediated through PTEN loss (WM793 and 1205Lu; Fig. 4.6C and D).

**Development of a quantitative pharmacodynamic assay of HSP90 inhibition**

The clinical development of HSP90 inhibitors has been hampered by the lack of a good pharmacodynamic assay for quantifying target inhibition within the tumor. As inhibition of HSP90 typically leads to the increased expression of other HSP family members, which can be used as a surrogate for HSP90 inhibition, we developed a highly sensitive quantitative LC-MRM assay for the quantification of 11 HSP family members (ref. 200; Fig. 4.7A). Treatment of cell lines that were naive, intrinsically resistant, and with acquired vemurafenib resistance with XL888 (300 nmol/L) led to robust time-dependent increases in the expression of HSP70 isoform 1 (HSP71; Fig. 4.7B). Western blot experiments confirmed the XL888-dependent increases in HSP70 expression in every cell line evaluated (Fig. 4.7C). The potential clinical relevance of the LC-MRM assay was shown by the successful quantification of HSP70 and other chaperone proteins from fine needle aspirates (~2,000 cells) taken from 2 melanoma specimens (Fig. 4.7D).
Fig. 4.7. Development of a quantitative pharmacodynamic assay for HSP90 inhibition. A, workflow of the LC-MRM experiment to measure HSP chaperone levels. After reversed-phase HPLC separation, peptides are selected by their mass-to-charge ratio and dissociated by collisions with background gas before the fragment ions are mass selected to enable specific detection and quantification of individual peptides in complex mixtures. B, heatmap showing XL888-induced (0–48 hours, 300 nmol/L) HSP70 expression in all of the melanoma cell lines irrespective of vemurafenib resistance mechanism. C, Western blot confirmation of HSP70 upregulation following XL888 treatment (300 nmol/L, 48 hours). D, quantification of absolute (fmol/µg) expression of the HSP chaperone protein expression in fine needle aspirates from 2 melanoma specimens.
**XL888 treatment causes the regression of vemurafenib-resistant xenografts in vivo associated with increased intratumoral HSP70 expression**

The relevance of HSP90 inhibition as a strategy to overcome BRAF inhibitor resistance *in vivo* was shown by the ability of XL888 (100 mg/kg, orally, 3 times a week) to significantly induce the regression of, or growth inhibition of established M229R and 1205LuR xenografts in SCID mice (Fig. 4.8A and Fig. 4.9).

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**Fig. 4.8.** XL888 induces the regression of established M229R xenografts and is associated with increased intratumoral HSP70 expression. A, XL888 leads to regression of M229R melanoma xenografts. M229R cells were grown until a palpable tumor had formed before being treated with XL888 thrice per week (100 mg/kg) by oral gavage. Data shows growth curves normalized to starting volumes; bar graph shows mean tumor volumes after 15 days of XL888 treatment. XL888 treatment led to significant levels of tumor regression (*P* = 0.003). B, heatmap showing the increase in HSP70 isoform 1 (HSP71) expression in XL888-treated (15 days, 100 mg per kg) xenograft samples compared with vehicle controls. C, XL888 treatment (10 days) led to the induction of apoptosis in established M229R xenografts as measured by increased TUNEL staining (green) and was associated with the induction of BIM expression and the suppression of Mcl-1 expression.
It was noted that the XL888 was well tolerated by the mice, with no significant alterations in body weigh observed over the study period (Fig. 4.10A). XL888 was also noted to be tumor specific in *in vitro* studies, with minimal growth inhibitory effects observed upon 2 primary human skin fibroblast cell lines (Fig. 4.10B and C). LC-MRM–mediated analysis of xenograft samples following 15 days of XL888 treatment showed a robust (8.6-fold) increase in intratumoral HSP70 expression compared with controls (Fig. 4.8B). XL888 treatment was noted to be proapoptotic *in vivo* and led to increased TUNEL staining in M229R xenografts associated with increased expression of BIM and decreased expression of Mcl-1 (Fig. 4C.8).

![Graph showing tumor growth inhibition](image)

**Fig. 4.9.** XL888 significantly inhibits the growth established 1205LuR xenografts relative to vehicle control (* P=0.0036, ** P>0.05). 1205LuR cells were grown until a palpable tumor had formed before being treated with XL888 (100 mg/kg) three times per week by oral gavage. Data shows increases in tumor volume normalized to starting volumes and mean tumor volume at the end of the experiment.
Fig. 4.10. XL888 treatment has minimal effects upon mouse weights and the growth and survival of two normal human skin fibroblast cell lines. A: Mean mouse weights of the vehicle and XL888 treated animal groups following day 15 of treatment. B: Minimal apoptosis is induced in two primary human skin fibroblast cell lines (FF2504, FF2407) after XL888 treatment (72 hrs, 300 nM). Apoptosis was measured by Annexin-V staining and flow cytometry. C: MTT assay for XL888 upon two human skin fibroblast cell lines.
**HSP90 inhibition restores nuclear localization of FOXO3a, upregulates BIM expression, and inhibits Mcl-1 expression in vemurafenib-resistant cell lines**

To determine the mechanism of XL888-induced apoptosis in the vemurafenib-resistant melanoma cell lines, we first focused upon BIM. Whereas vemurafenib treatment increased expression of BIM in melanoma cell lines that were drug naive, the resistant cell lines suppressed their expression of BIM even in the continuous presence of vemurafenib (Fig. 4.11A). XL888 treatment reversed this and increased BIM expression, irrespective of resistance mechanism (Fig. 4.11A). It was noted that XL888 treatment increased the expression of BIM-EL, BIM-L, and BIM-S expression in the M229R, 1205LuR, RPMI7951, and WM39 cell lines, induced expression of BIM-L and BIM-S in the WM164R cell line, and BIM-EL in the M249R cell line (Fig. 4.11A). These effects were mediated in part through increased BIM protein stability as noted by decreased BIM phosphorylation at Ser69 in all of the cell lines tested apart from M249R (Fig. 4.11A). We next asked whether HSP90 inhibition also affected BIM expression at the mRNA level. In vemurafenib-naive cells, inhibition of BRAF leads to the nuclear accumulation of the transcription factor FOXO3a and increased BIM expression. In contrast, cell lines with acquired resistance to vemurafenib excluded FOXO3a from the nucleus and suppressed BIM protein and mRNA expression even in the continuous presence of vemurafenib (Fig. 4.11A and Fig. 4.12). XL888 treatment reversed these effects and led to the nuclear accumulation of FOXO3a and an increase in BIM mRNA and protein expression (Fig. 4.11A and Fig. 4.12). An increase in nuclear size following XL888 treatment was also noted. The importance of BIM expression in the XL888-
mediated cell death response was shown by the significant inhibition of apoptosis observed when BIM expression was knocked down by siRNA (Fig. 4.11B).

Fig. 4.11. HSP90 inhibition increased BIM, decreases Mcl-1, and restores apoptosis in vemurafenib-resistant melanoma cell lines. A, Left: Western blot showing that XL888 (48 hours, 300 nmol/L) decreases BIM phosphorylation (Ser69) and increases BIM expression. Right: qRT-PCR experiment showing that treatment with XL888 (300 nmol/L, 48 hours) increases the expression of BIM at the mRNA level. B, siRNA knockdown of BIM significantly decreases XL888 (300 nmol/L, 48 hours) mediated apoptosis in 2 vemurafenib-resistant melanoma cell lines (M229R and 1205LuR). C, left, Western blot of Mcl-1 expression in vemurafenib-resistant melanoma cell lines treated with XL888 (300 nmol/L) for 48 hours. Right: qRT-PCR showing that XL888 (300 nmol/L, 48 hours) treatment downregulates Mcl-1 expression at the mRNA level. D, induction of Mcl-1 reduces the magnitude of XL888-induced apoptosis. Western blot shows the induction of Mcl-1 following doxycycline treatment. Induction of Mcl-1 (DOX+XL) significantly reduces the level of XL888-induced apoptosis compared with XL888 (XL: 300 nmol/L, 72 hours) alone. *, P < 0.05.
Mcl-1 is prosurvival BH3 family protein member that antagonizes the activity of BIM\textsuperscript{92}. Treatment of melanoma cell lines in which vemurafenib resistance was mediated through PDGFRβ, COT overexpression, and 2 melanoma cell lines with unknown resistance mechanisms with XL888 (300 nmol/L, 48 hours) led to a marked decrease in the expression of Mcl-1 (Fig. 4.11C). qRT-PCR experiments showed that XL888 treatment also blocked Mcl-1 expression at the mRNA level (Fig. 4.11C). The importance of Mcl-1 expression for the survival of vemurafenib-resistant melanoma cell lines was confirmed by the significant induction of apoptosis observed following siRNA knockdown of Mcl-1 expression (Fig. 4.13). Further evidence for the role of Mcl-1 expression in the drug resistance phenotype came from overexpression studies in which induction of Mcl-1 expression following doxycycline treatment led to a significant reduction in the magnitude of XL888-induced apoptotic response (Fig. 4.11D).
Fig. 4.13. siRNA knockdown of Mcl-1 induces apoptosis in vemurafenib resistant melanoma cell lines. 1205LuR and M229R cells were treated with either non-targeting (NT) or Mcl-1 siRNA (Mcl-1 si) for 96 hrs. Protein was resolved and probed for Mcl-1 and GAPDH expression by Western blot. Levels of apoptosis were quantified by Annexin-V staining and flow cytometry.

**HSP90 inhibition is more effective at inducing BIM expression and apoptosis than combined MEK+PI3K inhibition**

The simultaneous targeting of mitogen-activated protein/extracellular signal–regulated kinase (MEK/ERK) and phosphoinositide 3-kinase (PI3K)/AKT signaling is being explored as a strategy for overcoming vemurafenib resistance. We next asked whether HSP90 inhibition was more effective than the MEK+PI3K inhibitor combination at restoring apoptosis in vemurafenib-resistant melanoma cells. Although both XL888 and the PI3K inhibitor GDC-0941 were highly efficient at increasing nuclear accumulation of FOXO3a (Fig. 4.14A), XL888 treatment led to a greater induction of BIM expression at both the protein and mRNA levels and significantly restored the apoptotic response (Fig. 4.14B and C). Similarly, XL888 treatment was also more effective than the MEK or PI3K
inhibitor, alone or in combination, at downregulating the expression of Mcl-1 at both the mRNA and protein levels (Fig. 4.14B and C). This was in marked contrast to the responses observed in the parental M229 and 1205Lu cell lines, in which the MEK ± PI3K inhibitor combination was equally effective as XL888 at inducing BIM expression (Fig. 4.15). Although there is evidence that the BH3 protein family member BMF plays a role in the apoptotic response to BRAF inhibition XL888 treatment only weakly induced BMF mRNA expression (Fig. 4.16). In contrast, treatment of 2 vemurafenib-resistant cell lines with either the MEK inhibitor (M229R) or the MEK+PI3K inhibitor (1205LuR) led to a robust induction of BMF expression but induced less apoptosis than following XL888 treatment (Fig. 4.14D and Fig. 4.16). As the phosphorylation of BIM by MEK/ERK leads to its proteasomal degradation and the 26S proteasome is an HSP90 client protein, we next determined the contribution of proteasome inhibition to the cytotoxic effects of XL888. Although XL888 treatment was observed to partly degrade the 26S proteasome, HSP90 inhibition had a considerably weaker effect upon proteasomal activity than either the MEK+PI3K inhibitor combination or the proteasome inhibitor (MG-132; Fig. 4.17). In agreement with the marked effects of HSP90 inhibition on BIM and Mcl-1 expression compared with the MEK, PI3K, and MEK+PI3K inhibitor combination, XL888 was observed to induce significantly higher levels of apoptosis than each of the other drug combinations in cell lines in which resistance was mediated through amplification of COT, PDGFRβ overexpression and in 2 other models where the resistance mechanism is as yet unknown (Fig. 4.14D). The level of apoptosis induced by the MEK+PI3K inhibitor combination was equivalent to that of the HSP90 inhibitor when resistance was mediated through NRAS mutation or cyclin D1 amplification (Fig. 4.17).
4.14D).

Fig 4.14. HSP90 inhibition is more effective at restoring the apoptotic response than combined MEK+PI3K inhibition. A, immunofluorescence staining of 1205LuR and M229R cells for BIM and FOXO3a after treatment with vehicle, XL888 (300 nmol/L), AZD6244 (3 µmol/L), GDC-0941 (3 µmol/L), or both AZD6244 + GDC-0941 (each at 3 µmol/L). B, XL888 is more effective than MEK+PI3K inhibitors at increasing BIM and decreasing Mcl-1 mRNA expression in 1205LuR and M229R cell lines. BIM and Mcl-1qRT-PCR of cells treated as above. C, XL888 is more effective than MEK+PI3K inhibitors at increasing BIM and decreasing Mcl-1 protein expression in 1205LuR and M229R cell lines. BIM and Mcl-1Western blot of cells treated as above. D, XL888 is more effective at inducing apoptosis of melanoma cell lines in which resistance is mediated through COT and PDGFRβ expression and in 2 models in which the resistance mechanism is unknown. Measurement of apoptosis for cells treated as above.
Fig. 4.15. The combination of the MEK + PI3K inhibitor is more effective at inducing BIM expression in the vemurafenib naïve compared to vemurafenib resistant melanoma cell lines. M229 and M229R and 1205Lu and 1205LuR cell lines treated with vehicle, XL888 (300 nM), AZD6244 (3 µM), GDC-0941 (3 µM) or both (3 µM each) for 48 hrs and Western blotting was performed for BIM.

Fig. 4.16. XL888 (HSPi) is less effective than either an inhibitor of MEK (M229R) or the MEK + PI3K inhibitor (M+P) at inducing BMF mRNA in vemurafenib resistant melanoma cell lines. Cell cultures were treated with either vehicle (CT), HSPi (XL888, 300nM), MEKi (AZD6244, 3µM), PI3Ki (GDC-0941,3µM) or the MEK + PI3K inhibitor combination for 48 hrs, before being analyzed by q-RT-PCR.

Fig. 4.17. Although XL888 degrades the 26S proteasome, it is less effective than the MEK + PI3K inhibitor combination at inhibiting chymotrypsin like proteasome activity. A: Western blot showing the degradation of 26S proteasome following XL888 treatment (300 nM, 48 hrs). Numbers indicate fold decrease in proteasome activity. B: Quantification of cellular chymotrypsin-like proteasome activity following treatment with either vehicle (CT), XL888 (XL, 300nM), AZD6244 (AZD, 3µM), GDC-0941 (GDC, 3µM), the MEK + PI3K inhibitor combination (A+G) or the proteasome inhibitor MG-132 (1 and 3 µM).
Discussion

This study addressed whether targeting multiple signaling pathways through the inhibition of HSP90 is sufficient to overcome intrinsic and acquired resistance to the BRAF inhibitor vemurafenib (PLX4032). XL888 is a novel, orally available HSP90 inhibitor with high selectivity for HSP90α and HSP90β (IC_{50} = 22, 44 nmol/L, respectively) and little activity against a panel of 29 other diverse kinases (IC_{50} all >3,600 nmol/L; ref. 292. XL888 induced growth arrest and apoptosis in melanoma cell lines where vemurafenib resistance was mediated through NRAS mutations, PDGFRβ overexpression, COT overexpression, and cyclin D1 amplification. It was also proapoptotic in 2 melanoma cell lines with acquired vemurafenib resistance mediated through as yet unknown means. In all of the vemurafenib-sensitive cell lines, XL888 induced a G_1-phase cell-cycle arrest and reduced the percentage of cells in S-phase. In some of the resistance models, XL888 treatment instead induced cell-cycle arrest in G_2–M, suggesting an altered signaling dependency following the acquisition of drug resistance. In all cases, the responses to XL888 were highly durable with no resistant colonies emerging after 4 weeks of continual drug treatment. The prolonged growth inhibition associated with XL888 was in marked contrast to that observed following vemurafenib (or PLX4720) treatment in which resistant colonies emerged in every case^{133,206,293}. We next confirmed that XL888 decreased the expression of all the client proteins implicated so far in acquired and intrinsic vemurafenib resistance (IGF1R, cyclin D1, PDGFR-β, AKT, COT, ARAF, and CRAF)^{134,206,209-215}. In each case, not only did XL888 reduce the levels of the RTK/kinases implicated in BRAF inhibitor resistance, it also blocked the signaling activity of the pathways (MEK, AKT, and mTOR/S6).
involved in therapeutic escape$^{133,135,212,220}$. Although IGF1R, COT, AKT, ARAF, MEK, and CRAF have previously been reported to be HSP90 clients and subject to proteasome-mediated degradation following HSP inhibition, this is the first report to potentially identify PDGFR-β as a client of HSP90 (a current list of HSP90 clients is maintained at http://www.picard.ch/downloads/Hsp90interactors.pdf).

The potential use of HSP90 inhibitors to overcome vemurafenib resistance was illustrated by the ability of XL888 to inhibit multiple, nonoverlapping resistance pathways in the same cell line model, for example, the inhibition of PDGFRβ, IGF1R, and COT in the COT-amplified cell line and PDGFRβ, IGF1R, and COT in the PDGFRβ-overexpressing cell line. The fact that melanomas express multiple RTKs and can flexibly switch between multiple signaling pathways suggests that individual melanoma cells may have a number of escape mechanisms at their disposal, underscoring the need to concurrently target multiple oncogenic pathways. There is already evidence from other cancers that HSP90 inhibitors can overcome multiple drug resistance mechanisms. In preclinical studies of breast cancer, inhibitors of HSP90 abrogate diverse trastuzumab resistance mechanisms, including those mediated by PI3K mutations, truncation mutants of p95-HER2, and the upregulation of membrane-associated mucin-4$^{294-296}$. In NSCLC, inhibition of HSP90 prevents drug resistance associated with the oncogenic switch from EGFR to c-MET$^{296}$. HSP90 inhibitors have also been proven effective at managing drug resistance in the clinic, with activity being reported against trastuzumab-resistant HER2$^+$ breast cancer and bortezomib-resistant multiple myeloma$^{290,291,297,298}$. 
The measurement of HSP90 inhibition in vivo has proven to be challenging. Though HSP90 inhibition is well correlated with the increased expression of the co-chaperone, HSP70 (which can be quantified in peripheral blood mononuclear cells), HSP70 levels do not correlate well with either intratumoral HSP90 inhibition or clinical activity. The high abundance of heat shock chaperone proteins makes them amenable to direct quantification by mass spectrometry with minimal processing. As patients with advanced melanoma typically present with accessible cutaneous lesions that can be biopsied or undergo fine needle aspiration, we developed a novel quantitative pharmacodynamic mass spectrometry–based assay for the quantification of HSP90 and its co-chaperones. In agreement with previously published studies on HSP90 inhibitors, XL888 treatment lead to upregulation of HSP70 isoform 1 expression in every vemurafenib-sensitive and resistant cell line tested. Although there is evidence that increased HSP70 expression limits apoptosis in leukemic cells, the therapeutic relevance of this observation in melanoma is still unclear. The in vivo use of the LC-MRM technique was shown by the robust increases in HSP70 expression observed in xenografts following XL888 treatment and the ability to quantify levels of HSP90 and its key co-chaperones in small needle biopsies (fine needle aspirates) taken from fresh melanoma specimens. These results demonstrate that LC-MRM–based pharmacodynamic assays can be utilized to measure expression levels of intratumoral proteins and therefore is a technique that could be adopted for the direct quantification and monitoring of HSP90 client proteins during the course of therapy.

Inhibition of BRAF, either by siRNA knockdown or small molecule inhibitors of BRAF or MEK, induces apoptosis in BRAF V600E mutant melanoma cells through the
proapoptotic proteins BIM, BMF, and BAD. BIM is a BH3 family protein member that plays a pivotal role in the induction of cell death by binding to and antagonizing the prosurvival proteins Bcl-2, Bcl-w, Bcl-XL, and Mcl-1.

Vemurafenib resistance (both intrinsic and acquired) is characterized by a diminished apoptotic response and impaired BIM expression in the continuous presence of drug.

The observation that BIM is regulated both transcriptionally and post-transcriptionally through many pathways including ERK, AKT, JNK, and p38 MAPK, led us to hypothesize that XL888 (through the simultaneous targeting of multiple signaling pathways) may overcome vemurafenib resistance by upregulating BIM expression at both the mRNA and protein levels. Regulation of BIM mRNA is mediated by the transcription factor FOXO3a. AKT phosphorylates FOXO3a at T32, S253, and S315, leading to FOXO3a’s nuclear exclusion and localization to the cytoplasm. BIM levels are also controlled posttranslationally through phosphorylation at a number of sites (including S69) by MEK/ERK signaling, with the phosphorylation of BIM leading to its polyubiquitination and proteasomal degradation.

Our previous studies showed that vemurafenib increased nuclear FOXO3a localization and BIM expression in drug-naive cells leading to increased apoptosis. Here we noted that vemurafenib resistance was associated with suppression of nuclear FOXO3a and BIM expression in the continued presence of drug and was reversed upon addition of XL888. Interestingly, XL888 treatment was more effective at restoring the expression of BIM at the mRNA and protein levels and inducing apoptosis than dual inhibition of MEK and PI3K, perhaps suggesting the involvement of other (as yet unidentified) pathways that are also HSP90 clients. Although expression of BIM is
regulated both through 26S ubiquitin-dependent and 20S polyubiquitin–independent proteasomal mechanisms and the 26S proteasome is a known HSP90 client, we were unable to show a role for downregulation of the 26S proteasome in the recovery of BIM expression following HSP90 inhibition \(^{303,304}\). A number of recent studies have suggested a role for increased BMF (Bcl-2 modifying factor) expression in mediating the apoptotic response of melanoma cells treated with inhibitors of BRAF and MEK \(^{275,281}\). Here, we observed that XL888 treatment was a relatively weak inducer of BMF expression in the vemurafenib-resistant melanoma cell lines compared with that seen following single-agent MEK or dual PI3K + MEK inhibition, suggesting that BMF is relatively dispensable in overcoming BRAF inhibitor resistance in our models.

The decision between survival and apoptosis is regulated through the balance of pro- and antiapoptotic Bcl-2 family proteins. Survival of melanoma cells is controlled in part by the antiapoptotic protein, Mcl-1, whose stability is regulated by the BRAF/MEK/ERK pathway \(^{92}\). A potential role for Mcl-1 in the tolerance of BRAF inhibition was suggested by the studies showing that acquired vemurafenib resistance led to the recovery of MAPK signaling, whereas resistant cells maintained their Mcl-1 expression in the presence of vemurafenib, and that the forced overexpression of Mcl-1 decreased the vemurafenib-induced apoptotic response \(^{92,133}\). Inhibition of HSP90 led to the degradation of Mcl-1 protein and reduced Mcl-1 expression at the mRNA level. XL888 was also more effective at reducing Mcl-1 mRNA levels than inhibitors of MEK, PI3K, and the MEK+PI3K inhibitor combination. It therefore seems likely that the induction of BIM in together with Mcl-1 downregulation plays a key role in the induction of XL888-mediated apoptosis.
Preclinical and clinical strategies for managing vemurafenib resistance in melanoma have been centered upon combining vemurafenib with inhibitors of the MEK and PI3K/AKT/mTOR pathways. Although our study supports use of the MEK+PI3K inhibitor combination when resistance is mediated through NRAS mutations or cyclin D1 amplification, it appears suboptimal when resistance is mediated by increased COT expression, PDGFRβ overexpression, and in 2 other cell lines models with undetermined resistance mechanisms. These findings suggest that other pathways (that also happen to be HSP90 clients) are required for therapeutic escape or that vertical inhibition of the same pathway at multiple points (e.g., ARAF/CRAF/MEK or IGF1R/AKT/S6) may be a more effective way of shutting down a signal transduction pathway.

Another strategy for targeting MAPK dependent BRAF inhibitor resistance is simultaneous inhibition of BRAF and MEK. However, data from recent phase I/II clinical trial (NCT01072175) of 71 patients failing on vemurafenib, dabrafenib or experimental BRAF inhibitors indicated that vertical inhibition only minimally improved clinical outcomes (OR = 13-15%, PFS = 1.8-3.9 months). Though it is possible that a triple combination of BRAF/MEK/ERK inhibitors could successfully block the MAPK pathway, ERK inhibitors have yet to be proven clinically efficacious. Further, due to the signaling plasticity of solid tumors such as melanoma, it is likely that complete inhibition of the MAPK pathway prior to adaptation would be required to eradicate the nidus of potentially treatment refractory cancer cells.

In summary, in this study we showed for the very first time that all of the signaling proteins implicated thus far in intrinsic and acquired BRAF inhibitor resistance are
clients of HSP90 and that inhibition of HSP90 can restore sensitivity to vemurafenib-mediated cell death through BIM upregulation Mcl-1 inhibition. Further, these observations have provided the rationale for targeting HSP90 and BRAF in $BRAF_{V600E}$ melanoma as a strategy to limit the therapeutic escape. A strategy that is currently in phase I clinical trial (NCT01657591). Thus far 15 patients have been enrolled (median age = 60 years, 73% male, majority stage IV). Four X888 dose escalations were evaluated in combination with a single dose of vemurafenib (960 mg bid vemurafenib + 30mg $^{\text{cohort 1}}$, 45mg $^{\text{cohort 2}}$, 90mg $^{\text{cohort 3}}$, or 135mg $^{\text{cohort 4}}$ XL888 twice weekly). Preliminary reports have been encouraging (OR = 85%) with 2 complete responders and a partially responding patient whose residual tumor was resected and shown to have no pathological evidence of viable melanoma cells. The most common side effects were typically associated with vemurafenib (anorexia, fatigue, arthralgia, and rash), with dose limiting toxicities occurring in only in cohort 4 (grade 3 diarrhea and pancreatitis). Of note, similar to BRAF + MEK combinations, secondary cutaneous events occurred less frequently in higher dose vemurafenib + XL888 cohorts (cohort 3 and 4)$^{140,306}$.

**Materials and Methods**

**Cell culture and generation of BRAF inhibitor resistance**

The parental 1205Lu, WM39, and WM164 melanoma cells lines were a gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA) and were genotyped as being $BRAF_{V600E}$ mutant in Smalley and colleagues $^{168}$. The M229, M229R, M249, and M249R were described in Nazarian and colleagues $^{206}$. The RPMI7951 melanoma cell line was purchased from American Type Culture Collection. The identities of all cell
lines were confirmed by Biosynthesis Inc., through short tandem repeat validation analysis. Naive and intrinsically resistant lines were cultured in 5% FBS, RPMI. For all studies, all acquired resistant cell lines were maintained in 5% media with the addition of vemurafenib at the following concentrations: 1 µmol/L for M229R and M249R, 2 µmol/L for WM164R, and 3 µmol/L for 1205LuR.

**Growth inhibition**

Cells were plated at a density of $2.5 \times 10^4$ cells per mL and left to grow overnight before being treated with increasing concentrations of vemurafenib or XL888 as described in Smalley and colleagues. Data show the mean of at least 3 independent experiments ± the SEM.

**Western blotting**

Proteins were extracted and blotted for as described in Smalley and colleagues. After analysis, Western blots were stripped once and reprobed for β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to show even protein loading. The antibodies to IGF1R, PDGFRβ, ARAF, CRAF, phospho-AKT Ser473, total AKT, phospho-ERK (extracellular signal–regulated kinase), total ERK, cyclin D1, phospho-S6, total S6, phospho-BIM (Bcl-2 interacting mediator of cell death; Ser69), total BIM, HSP70, and MCL-1 were from Cell Signaling Technology. Anti-26S was purchased from Abcam whereas the antibody against COT was from Santa Cruz Biotechnology. For mouse xenograft studies, tumor samples were harvested and immediately placed into RNAlater solution (Invitrogen) before protein extraction.
Flow cytometry
Cells were plated into 6-well tissue culture plates at 60% confluency and left to grow overnight before being treated with either 300 nmol/L XL888, 3 µmol/L AZD6244, 3 µmol/L GDC-0941 (Selleck), or the combination of 3 µmol/L AZD6244 and 3 µmol/L GDC-0941 for 72 or 144 hours. In some studies, RPMI7951, WM793, and 1205Lu cells were treated with 300 nmol/L XL888 in the presence or absence of 3 µmol/L vemurafenib and harvested after 48 hours. Annexin V and TMRM staining was done as described in Paraiso and colleagues\textsuperscript{133}.

Three-dimensional spheroid assays
Melanoma spheroids were prepared using the liquid overlay method\textsuperscript{174}. Spheroids were either treated for 144 hours with vehicle or 1 µmol/L XL888 or for 48 hours with vehicle, 1 µmol/L XL888, 3 µmol/L vemurafenib, or a combination of the 2 drugs before being washed and analyzed as described in Smalley and colleagues\textsuperscript{174}.

RNA interference
M229R and 1205LuR were plated at 1 × 10\textsuperscript{5} and left to grow overnight in RPMI complete media. Complete media was replaced with Opti-MEM (Invitrogen) and Mcl-1 or BIM (both 25 nmol/L; Cell Signaling Technologies) siRNAs in complex with Lipofectamine 2000 (Invitrogen) were added. In addition, scrambled siRNAs were added as nontargeting controls. A final concentration of 5% FBS in complete RPMI was added the next day. In the BIM studies, cells were transfected for a total of 48 hours before a 48-hour treatment with 300 nmol/L XL888. In the Mcl-1 studies, cells were treated for 96 hours.
**Immunofluorescent staining**

M229R and 1205LuR cells were seeded at 50% confluency before being treated with 300 nmol/L XL888, 3 µmol/L AZD6244, 3 µmol/L GDC-0941, or AZD6244 + GDC-0941 in combination as previously described \(^ {150}\). Cells were stained with antibodies against BIM and FOXO3a followed by staining with secondary anti-rabbit AF488 and imaged with a Leica confocal microscope.

**Proteomics sample preparation**

Proteins were extracted as described for Western blotting and processed as described in Remily-Wood and colleagues \(^ {299}\).

**Liquid chromatography, multiple reactions monitoring mass spectrometry analysis**

Liquid chromatography, multiple reactions monitoring mass spectrometry (LC-MRM) was done as described in Remily-Wood and colleagues \(^ {299}\). Protein expression was determined using the ratio of peak area of the native peptide to corresponding internal standard; normalization of tissue results was done using GAPDH to control for cellularity (Table 4.2). Data were then normalized to the pretreatment (cell lines) or vehicle controls (tissue) and plotted to show the changes in expression after drug treatment.

**Table 4.2.** Endogenous peptides, corresponding internal standards, and selected transitions for the quantification of heat shock proteins. Underlined amino acid residues are labeled with 13C and 15N in the corresponding synthetic standards.

<table>
<thead>
<tr>
<th>Protein (UniProt Identifier)</th>
<th>Endogenous Peptide</th>
<th>Transitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS90A</td>
<td>ALLFVIPR</td>
<td>y₂ - y₆</td>
</tr>
<tr>
<td>HS90B</td>
<td>ALLFIPR</td>
<td>y₃ - y₆</td>
</tr>
<tr>
<td>H90B2</td>
<td>HSQFLGYPITLYLEK</td>
<td>y₅ - y₁₂</td>
</tr>
<tr>
<td>CDC37</td>
<td>LQAEAQQLR</td>
<td>y₁ - y₉</td>
</tr>
<tr>
<td>HSPB3</td>
<td>ADLNNLIR</td>
<td>y₃ - y₇</td>
</tr>
<tr>
<td>HSP71</td>
<td>NQVALNPQNTVFDAK</td>
<td>y₉, y₄, y₅ - y₁₂</td>
</tr>
<tr>
<td>HSP72</td>
<td>EIAYLGGK</td>
<td>y₅ - y₆</td>
</tr>
<tr>
<td>HSP7C</td>
<td>GTLPVVEK</td>
<td>y₃ - y₇</td>
</tr>
<tr>
<td>HSP74</td>
<td>AFSDPFVEAEK</td>
<td>y₅, y₆ - y₉</td>
</tr>
<tr>
<td>GRP78</td>
<td>VEIIANDQGRN</td>
<td>y₄ - y₉</td>
</tr>
</tbody>
</table>
Human specimen procurement

Patients scheduled to undergo surgical resection for metastatic melanoma were prospectively consented and accrued to an existing melanoma tissue procurement protocol approved by the Moffitt Cancer Center Scientific Review Committee and The University of South Florida Institutional Review Board. Following excision of the specimen in the operating room, fine needle tumor aspirates were taken using a 22-gauge needle for proteomic analysis of the resulting tumor homogenate.

MCL-1-inducible cell line

WM793TR MCL-1 cells were a kind gift from Dr. Andrew Aplin (Kimmel Cancer Center, Philadelphia, PA; ref. ^2^). Mcl-1 expression was induced by the addition of 100 ng/mL doxycycline for 72 hours before treatment with 300 nmol/L XL888 for an additional 72 hours.

Quantitative real-time PCR

Cells were treated for 48 hours with 300 nmol/L XL888, 3 µmol/L AZD6244, 3 µmol/L GDC-0941, or AZD6244 and GDC-0941 in combination before RNA isolation. Total RNA was isolated using Qiagen's RNeasy mini kit. The following TaqMan Gene Expression Assays primer/probes were used: Hs00197982_m1 (BIM), Hs01050896_m1 (MCL-1), Hs00372937_m1 (BMF), P/N 4319413E (18S), and Hs99999905_m1 (GAPDH). The 18S + GAPDH data were used for normalizing BIM. Quantitative reverse transcriptase PCR (qRT-PCR) reactions were carried out as previously described ^15^.

Colony formation

Cells (1 × 10^4^ per mL) were grown overnight before being treated with vehicle (dimethyl sulfoxide) or XL888 (300 nmol/L) for 4 weeks as described in Paraiso and colleagues
and relative colony density was determined by solubilizing the crystal violet dye in 10% acetic acid followed by measurement of absorbance at 450 nm.

**Xenograft experiments**

BALB severe combined immunodeficient (SCID) mice (The Jackson Laboratory) were subcutaneously injected with $2.5 \times 10^6$ cells per mouse and grown to approximately 100 mm$^3$ before dosing. Mice were treated with either 100 mg XL888/kg ($n = 5$) or an equivalent volume of vehicle (10 mmol/L HCl), 3× per week by oral gavage. Mouse weights and tumor volumes ($L \times W^2/2$) were measured 3× per week. Upon completion of the experiment, vehicle- and drug-treated tumor biopsies were processed for LC-MRM analysis (as above). Detection of apoptosis by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (Millipore) was carried out according to the manufacturer's instructions.

**Statistical analysis**

Data show the mean of at least 3 independent experiments ± the SEM, unless stated otherwise. Statistically significant results were considered in which $P \leq 0.05$.

**Statistical analysis of GI50 values**

Triplicate experiments were performed for each cell line under each drug treatment. To estimate the IC50 values for each cell line for each treatment, a 4-parameter Hill equation was used to model the nonlinear sigmoid relationship between the drug concentration and % cell survival. Briefly, $y = \frac{(E - B) \cdot \left(\frac{x}{Gi50}\right)^m}{1 + \left(\frac{x}{Gi50}\right)^m} + B + \epsilon$, where $x$ is the drug concentration, $y$ is the % survival, $\epsilon$ is the error term. The four parameters in the model to estimate are: 1) the control-level effect (E); 2) the background-level effect (B);
3) the median effect concentration (GI50); and 4) the strength of the inhibition (m). The constraint of non-negative minimum background effect (B) is imposed so the model is biologically meaningful. Levenberg-Marquardt algorithm for nonlinear least squares was used to fit the model and estimate all four parameters for each cell line under each treatment. Estimated concentration at 50% cell survival, i.e., IC50, is estimated using the plugged-in parameters after model fitting. Note that when the control and background effects are 100% and 0%, respectively, the estimated GI50 and IC50 values would be identical. To compare the estimated IC50 values between the resistance and naïve paired of cell lines, after log transformation, a paired t-test was performed for each drug.

**Measurement of proteasome activity**

M229R and 1205LuR cells were harvested and plated at 7,500 cells per well in a white-walled 96 well plate. Cells were allowed to grow overnight prior to treatment with either 0.3µM XL888, 3µM AZD6244, 3µM GDC-0941 or the combination of 3µM AZD6244 and 3µM GD C-0941 (48hrs) or MG-132 (0.3, 1 or 3µM for 24hrs). Proteasome activity was assessed using the Proteasome-Glo Chymotrypsin-Like Cell Based Assay (Promega, Madison, WI) and was measured in relative luminescence units (RLU).
Chapter 5

Inhibition of HSP90 by AT13387 Delays the Emergence of Resistance to BRAF Inhibitors and Overcomes Resistance to Dual BRAF and MEK Inhibition in Melanoma Models.

Note to Reader

Portions of this chapter have been previously published in Molecular Cancer Therapeutics. 2014 Dec;13(12):2793-804 and have been reproduced with permission from the American Association for Cancer Research Publishing. Author contributions: *Smyth T, *Paraiso KH (*contributed equally, designed/performed experiments, interpreted data, writing, figures); Hearn K, Rodriguez-Lopez AM, Munck JM, Haarberg HE, Thompson NT, Azab M (performed experiments); Sondak VK, Lyons JF (data interpretation, wording); #Smalley KS, #Wallis NG (#contributed equally, study concept, designed experiments, interpreted data, writing, figures).

Introduction

A number of different drug combinations have been investigated in an attempt to overcome or delay BRAF inhibitor resistance. Clinically the combined inhibition of BRAF and MEK with dabrafenib and trametinib appears to successfully increase progression-free survival (PFS) but ultimately, even with this combination, most patients relapse. Resistance mechanisms observed for the combination are similar to those seen for the monotherapy, and resistance to BRAF inhibition often confers cross-resistance to subsequent MEK inhibition. Other proposed combinations, including combining
BRAF inhibitors with phosphoinositide 3-kinase (PI3K), mTOR, c-MET or cyclin dependent kinase (CDK) 4 inhibitors \textsuperscript{311-313}, may address individual resistance mechanisms but are unlikely to target them all. In addition, multiple mechanisms of resistance have been observed in tumors from individual patients \textsuperscript{213,310,314}, further underscoring the need for therapeutics with broad-spectrum activity.

Mutant \textit{BRAFV600E} protein is an HSP90 client, relying upon the chaperone machinery for its correct folding and stability \textsuperscript{287,315}. In addition to \textit{BRAFV600E}, HSP90 clients include key components of cellular signalling pathways involved in BRAF inhibitor resistance and as such, HSP90 inhibition has been proposed as a potential approach to concurrently target multiple BRAF inhibitor resistance mechanisms in melanoma \textsuperscript{135,197,316}. Unlike the first generation ansamycin class of HSP90 inhibitors, such as 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), AT13387 is a second generation, fragment-derived inhibitor, which is active in a number of \textit{in vitro} and \textit{in vivo} tumor models \textsuperscript{317}. Preclinically, AT13387 has been shown to be effective in kinase inhibitor-resistant diseases such as imatinib-resistant gastrointestinal stromal tumor (GIST) models \textsuperscript{318}. Currently, AT13387 is in three Phase II clinical trials in combination with targeted agents (NCT01294202: GIST; NCT01712217: Anaplastic Lymphoma Kinase (ALK)-positive lung cancer and prostate cancer).

Here, we demonstrated that AT13387 can overcome acquired resistance generated to BRAF inhibitors alone or to a BRAF/MEK inhibitor combination. In addition, combining AT13387 with a BRAF inhibitor in a sensitive \textit{in vivo} model significantly delayed the emergence of BRAF inhibitor resistance. These data further support the
clinical testing of a frontline combination of an HSP90 inhibitor with a BRAF inhibitor alone or as a triple combination of HSP90/BRAF/MEK inhibitors.

**Results**

*AT13387 prevents the emergence of vemurafenib resistance in vitro*

AT13387 is a potent \( K_d \) 0.71 nM inhibitor of HSP90 (30;32) with broad spectrum activity in tumor models. AT13387 potently inhibited the proliferation of a panel of melanoma cell lines, with different genetic backgrounds and sensitivity to the BRAF inhibitor, vemurafenib (Table. 5.1) and depleted relevant client proteins (Fig. 5.1). Since HSP90 inhibitors, including AT13387, can overcome a variety of resistance mechanisms to BRAF inhibition once established, we investigated if HSP90 inhibition could also have a delaying effect on the emergence of this resistance in models initially sensitive to vemurafenib.

**Table. 5.1.** Effects of AT13387 treatment on melanoma cell lines and xenograft models. Inhibition of proliferation was measured by Alamar blue detection and IC50 values generated as described in Materials and Methods. IC50 values shown are the mean of at least 3 independent experiments (SEM), except for SK-MEL-5 (vemurafenib) which shows the two independent IC50 determinations.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Genetic Background</th>
<th>Inhibition of proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC(_{50}) AT13387 (nM)</td>
</tr>
<tr>
<td>A375</td>
<td>BRAF(^{V600E})</td>
<td>22</td>
</tr>
<tr>
<td>SK-MEL-28</td>
<td>BRAF(^{V600E})</td>
<td>73</td>
</tr>
<tr>
<td>SK-MEL-5</td>
<td>BRAF(^{V600E})</td>
<td>190</td>
</tr>
<tr>
<td>A2058</td>
<td>BRAF(^{V600E}), PTEN null</td>
<td>34</td>
</tr>
<tr>
<td>WM266.4</td>
<td>BRAF(^{V600D}), PTEN null</td>
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<tr>
<td>RPMI-7951</td>
<td>BRAF(^{V600E}), COT↑</td>
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<tr>
<td>SK-MEL-2</td>
<td>NRAS(^{Q61R})</td>
<td>45</td>
</tr>
</tbody>
</table>

A375 and SK-MEL-28 melanoma cell lines are both initially sensitive to vemurafenib treatment and their proliferation is also inhibited by AT13387 (Table. 5.1).
To investigate effects on the emergence of vemurafenib resistance, A375 and SK-MEL-28 cells were exposed to 2 μM vemurafenib in vitro. After two weeks of treatment, vemurafenib-resistant colonies had appeared (Fig. 5.2A). However, significantly fewer colonies were generated when vemurafenib was combined with 18 nM AT13387 (Fig. 5.2B), suggesting that the combination delays the emergence of resistant clones.

**Fig. 5.1.** Melanoma cells were treated with the indicated concentrations of AT13387 or vemurafenib for 24 h. Proteins were resolved by SDS PAGE and then immunoblotted with the indicated antibodies.
AT13387 treatment delays the emergence of vemurafenib resistance in vitro. A375 (A) or SK-MEL-28 (B) cells treated with 2 μmol/L vemurafenib and 18 nmol/L AT13387 alone or in combination. After 2 weeks, colonies were visualized by staining with 0.1% crystal violet and quantitated. Graphs, average of three individual experiments for each cell line; *, **, *** and **** indicate a significant difference from vemurafenib or AT13387 ($P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$).

**AT13387 delays the emergence of vemurafenib resistance in vivo**

AT13387 inhibits the growth of both vemurafenib-sensitive and -resistant melanoma xenograft mouse models (Fig. 5.3 and Fig. 5.4A). To further investigate the delay on emergence of resistance seen in vitro we carried out a comparable experiment in a SK-MEL-28 in vivo model (Fig. 5.4A and B). AT13387 significantly inhibited the growth of SK-MEL-28 xenografts when dosed at 70 mg/kg ip once weekly. As expected, oral administration of vemurafenib at 50 mg/kg twice a day caused significant regression of the SK-MEL-28 tumors over the initial period of dosing and combination of vemurafenib and AT13387 showed similar initial regression, with no significant difference observed between the two treatment groups (Fig. 5.4A).
Fig. 5.3. Mice bearing A2058 xenograft tumors received AT13387 at 70 mg/kg or its vehicle, 17.5% (w/v) 2-HPb-cyclodextrin, twice (Days 1, 4, 8 and 11) a week respectively. Vemurafenib at 50 mg/kg or its vehicle, 5% DMSO in 1% methylcellulose, was orally administered twice a day on Days 1-5 and 8-12, and once a day on Days 6, 7, 13 and 14. Mean tumor volumes were plotted with error bars representing standard error of the mean of N=8 (vehicles) or 7 (AT13387, vemurafenib and combination groups). Vehicle control (●) AT13387 single-agent (■), vemurafenib single-agent (x), AT13387 and vemurafenib combination (○). * indicates statistically significant difference from the control group (P<0.05).

However, over an extended dosing period (150 days) 2 tumors in the vemurafenib + vehicle group regrew (Tumor #8 and #7, PFS of 94 and 147 days, respectively), while no such regrowth was seen in the combination group over the same time period (Fig. 5.4B). Combined with our in vitro data this further suggests that a combination of AT13387 and vemurafenib in a vemurafenib-sensitive model can delay the emergence of resistance that arises to vemurafenib treatment alone. The combination of AT13387 and vemurafenib was well tolerated with no significant increase in toxicity observed (Fig. 5.5).
Fig. 5.4. AT13387 treatment delays the emergence of vemurafenib resistance in vivo. (A) Mice bearing SK-MEL-28 xenograft tumors received AT13387 at 70 mg/kg or its vehicle, 17.5% (w/v) 2-HPβ-cyclodextrin, i.p. once a week. Vemurafenib at 50 mg/kg or its vehicle, 5% DMSO in 1% methylcellulose, was orally administered twice a day on days 1 to 5 and 8 to 12, and once a day on days 6, 7, 13, and 14. Mean tumor volumes were plotted with error bars representing SEM of n = 8 (vehicles and SK-MEL-28 vemurafenib groups) or 7 (AT13387 and combination groups). Vehicle control (●) AT13387 single-agent (●, blue), vemurafenib single-agent (▲, red), AT13387, and vemurafenib combination (●, green). *, statistically significant difference from the control group (P < 0.05). (B) Vemurafenib monotherapy (▲, red) and vemurafenib and AT13387 combination (●, green) groups of SK-MEL-28 tumor-bearing mice continued to be dosed for 150 days as described above to allow resistance to develop to the therapies. (C) Levels of proteins were compared between one of the SK-MEL-28 vemurafenib-relapsed tumors (tumor #7) and a vemurafenib-naive SK-MEL-28 tumor. Tumors were lysed, protein extracted, resolved by SDS-PAGE, and immunoblotted with the indicated antibodies.

**Body weights of animals in SK-MEL-28 xenograft study**

Fig. 5.5. Effects of AT13387 and vemurafenib treatment on body weight of mice bearing SK-MEL-28 xenografts.

**AT13387 is active in models of acquired vemurafenib resistance**

Having demonstrated that AT13387 could delay the emergence of acquired vemurafenib resistance, we then continued to investigate the effect of AT13387 treatment once resistance had been acquired in these models. Two cell lines (A375R,
SK-MEL-28R) with acquired resistance to vemurafenib were generated using different methods. Acquired resistance in the A375R cell line was produced by culturing A375 cells in vitro with 2 µM vemurafenib for 3 weeks, while the SK-MEL-28R cell line was created from cells recovered from one of the SK-MEL-28 xenograft tumors, which became resistant to vemurafenib after the extensive dosing described above (Tumor #7). The proliferation of these cell lines was no longer inhibited by vemurafenib in vitro (IC$_{50}$ values > 10 µM, Fig. 5.7) but was still potently inhibited by AT13387 with IC$_{50}$ values in a similar range to those of the parental cell lines (22 nM vs. 19 nM for A375 vs. A375R; 73 nM vs. 16 nM for SK-MEL-28 vs. SK-MEL-28R) (Table 5.1, Fig. 5.6). Treatment with AT13387 still brought about the depletion of client proteins and inhibition of signalling as in the parental lines (Fig. 5.7A, Fig. 5.1). Levels of PDGFRβ and EGFR, which were again upregulated in the SK-MEL-28R cell line, were also depleted by 24 h treatment with 1 µM of AT13387 in vitro (Fig. 5.1). Levels of cleaved PARP (Fig. 5.7A) and Annexin V (Fig. 5.7B) were increased on treatment of the SK-MEL-28R cell line with 0.1 or 1 µM AT13387 indicating apoptosis was being induced. Cell death and inhibition of growth was also observed when collagen implanted 3D spheroids of the SK-MEL-28R cell line were treated with AT13387 (Fig. 5.7C).
Fig. 5.6. Effect of AT13387 on proliferation of resistant cell lines. The melanoma lines that had acquired resistance to vemurafenib alone (A375R and SK-MEL-28R) or to vemurafenib and selumetinib combination (SKMEL-28RR, WM164RR and 1205LuRR) were incubated in various concentrations of AT13387 and viability determined using Alamar Blue. A375R and SK-MEL-28R were treated with AT13387 or vemurafenib alone. 1205LuRR, WM164RR and SK-MEL-28RR were incubated with various concentrations of AT13387 in presence of 1 µM each of vemurafenib and selumetinib. Each graph is representative of 2-5 independent experiments performed in triplicate or quadruplicate. The table shows the mean IC50.

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<th>Cell line</th>
<th>Inhibition of proliferation IC50 AT13387 (nM)</th>
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<td>1205LuRR</td>
<td>76</td>
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<td>35</td>
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Fig. 5.7. AT13387 treatment is still effective in *in vitro* models of acquired vemurafenib resistance. Cells with acquired vemurafenib resistance (A375R and SK-MEL-28R) were treated with varying concentrations of AT13387 or vemurafenib. Effects on cellular protein levels were measured by Western blotting with the indicated antibodies after 24 hours (A). SK-MEL-28R cells were treated with the indicated concentrations of AT13387 for 5 days in the presence of 1 µmol/L vemurafenib and apoptosis measured by Annexin V staining. Data, mean of three independent experiments each performed in duplicate (B). 3D spheroids were treated with the indicated concentrations of AT13387 for 6 days in the presence of 1 µmol/L vemurafenib. Red, dead cells; green, live cells; scale bar, 100 µm; bar graphs, average spheroid size as quantified by ImageJ analysis; *, significant difference in size compared with control (P < 0.05; C).
Finally, the *in vivo* activity of AT13387 was investigated in a SK-MEL-28R xenograft model. Mice were subcutaneously injected with the vemurafenib-resistant SK-MEL-28R cells to form tumor xenografts and immediately treated with vemurafenib (50 mg/kg po bidx5/qdx2), to maintain resistance. As expected, vemurafenib treatment did not inhibit tumorigenicity of these cells and xenograft tumors grew during dosing. When tumors reached a mean volume of approximately 100 mm$^3$, AT13387 (70 mg/kg once weekly) was added to the vemurafenib treatment. This significantly (*P*<0.01 from Day 3 onwards) inhibited the tumor growth (26 % T/C on Day 10), indicating these vemurafenib-resistant tumors retained sensitivity to AT13387 treatment (Fig. 5.8A), although growth was not completely suppressed as it was in the upfront combination. Single agent treatment with AT13387 also significantly (*P*<0.01 from Day 3 onwards, 43% T/C on Day 10) inhibited tumor growth compared to vehicle control (Fig. 5.8B).

**Fig. 5.8.** AT13387 treatment is effective in *in vivo* models of acquired vemurafenib resistance. SK-MEL-28R tumor xenografts were established in SCID mice and continuously treated with 50 mg/kg vemurafenib orally twice daily to maintain resistance. When the mean tumor volume reached approximately 110 mm$^3$, once-weekly treatment with AT13387 (○, 70 mg/kg i.p.) or vehicle (▲, cyclodextrin solution i.p.) was added (A) or vemurafenib treatment was stopped and tumors treated with vehicle (●, cyclodextrin solution i.p.) or AT13387 (■, 70 mg/kg i.p.) alone (B). *, **, ***, and **** indicate a significant difference between the two treatments (*P* < 0.05, *P* < 0.01, *P* < 0.001, and *P* < 0.0001, respectively).
**AT13387 is active in cells with dual resistance to BRAF and MEK inhibitors**

Combined BRAF and MEK inhibition has been shown to extend PFS in the clinic but still ultimately leads to resistance in almost all cases. We therefore investigated whether HSP90 inhibition could also overcome resistance to a dual BRAF and MEK inhibitor combination. Three melanoma cell lines were generated with resistance to both BRAF and MEK inhibitors by prolonged (4-5 months, depending upon cell line) incubation with vemurafenib and selumetinib. The resulting cell lines (SK-MEL-28RR, WM164RR, 1205LuRR) were found to be resistant to the vemurafenib and selumetinib combination as well as dabrafenib and trametinib (Fig. 5.9A and B). All the resistant cell lines showed increased levels of phospho-ERK compared to the parental cell lines and, although there was a decrease in phospho-ERK in all of the resistant cell lines in the presence of vemurafenib and selumetinib, significant levels of signalling comparable to untreated parental cell lines were maintained (Fig. 5.10A). BRAF and MEK inhibitors also had little effect on the growth of collagen-implanted 3D spheroids of these cell lines (Fig. 5.11). In contrast, treatment of these cell lines with AT13387 inhibited MAPK signalling as well as AKT signalling (Fig. 5.10B), induced apoptosis (Fig. 5.10C) and inhibited the growth and survival of the resistant cell lines in a 3D spheroid assay (Fig. 5.10D).

Similar to the vemurafenib resistant SK-MEL-28R cell line, western blot analysis of the 1205LuRR, SK-MEL-28RR and WM164RR cell lines demonstrated increased EGFR and/or PDGFRb expression in the dual resistant cell lines (Fig. 5.13). We investigated whether other RTKs were upregulated in these cell lines using a phospho-
RTK array (Fig. 5.14). As RTKs are known to signal largely through the phosphorylation of tyrosine, this analysis also allowed us to measure relative changes in RTK activity.

**Fig. 5.9.** Characterization of BRAF/MEK inhibitor-resistant cell lines. Effect of BRAF and MEK inhibitors on proliferation of SK-MEL-28 naïve vs. RR. SK-MEL-28 (●) and SK-MEL-28RR cell line derived by prolonged incubation with vemurafenib and selumetinib (■) were incubated in various combinations of dabrafenib and trametinib, or vemurafenib and selumetinib and viability determined using Alamar Blue. Each graph is representative of 3 independent experiments performed in quadruplicate.

**Fig. 5.10.** Effect of BRAF and MEK inhibitors on SK-MEL-28RR. Both BRAF inhibitor and MEK inhibitor combination treatments caused little increase in Annexin V-positive or TMRM-negative cells when analyzed by FACS, indicating no increase in apoptosis.
Fig. 5.11. AT13387 overcomes acquired resistance to the BRAF and MEK inhibitor combination. Cell lines, parental (N) and those with acquired resistance to the BRAF inhibitor (vemurafenib) and the MEK inhibitor (selumetinib; RR) were treated with 1 µmol/L of each of vemurafenib and selumetinib for 2 hours. Proteins were resolved by SDS-PAGE and immunoblotted with antibodies for ERK and phospho-ERK (A). Cell lines resistant to the BRAF and MEK inhibitor combination were treated with the indicated concentrations of AT13387. Proteins were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Increasing concentrations of AT13387 blocked the expression of CRAF and inhibited phospho-ERK and phospho-AKT signaling (B). AT13387 induced apoptosis in melanoma cell lines with resistance to the BRAF and MEK inhibitor combination. Cultures were treated with AT13387 (1 µmol/L, 5 days) before being stained with Annexin-V and analyzed by flow cytometry; ****, significant difference from control (P < 0.0001; C). The addition of AT13387 induces cell death in three cell line models of BRAF and MEK inhibitor resistance. Top, spheroids were grown in 1 µmol/L vemurafenib and 1 µmol/L selumetinib in the absence or presence of AT13387 for 6 days (1 or 3 µmol/L); red, dead cells; green, live cells; scale bar, 100 µmol/L. Bottom, bar graphs show average spheroid area as quantified by ImageJ analysis at the indicated concentrations of AT13387 in the presence of 1 µmol/L vemurafenib and 1 µmol/L selumetinib; *, significant difference in size compared with control (P < 0.05; D).

Fig. 5.12. Effect of BRAF and MEK inhibitors on 3D spheroid formation of resistant cell lines. 3D spheroid cultures of SK-MEL-28RR, WM164RR and 1205LuRR were treated with vemurafenib + selumetinib or dabrafenib + trametinib. These cultures show positive invasion and little cell death in the presence of these drugs. Red = dead cells, green = live cells.
Fig. 5.13. Upregulation of EGFR and PDGFRβ in BRAF/MEK inhibitor resistant cell lines. Levels of EGFR and PDGFRβ in naïve and resistant cell lines were assessed by western blotting.

Fig. 5.14. HSP90 inhibition blocks RTK activation. WM164RR and 1205LuRR cell lines were treated for 24 hours with 1 µmol/L AT13387 before probing for phosphorylation. RTK array dot blots showing global changes in RTK tyrosine phosphorylation between naive, resistant, and AT13387-treated resistant cells. Bar graphs show relative intensities of each RTK with a positive phospho-signal as quantified by densitometry.

Comparison of the overall level of tyrosine phosphorylation of RTKs between the WM164RR and 1205LuRR cell lines and their respective parental lines revealed that
overall tyrosine phosphorylation appeared to increase following acquired BRAF plus MEK inhibitor resistance (Fig. 5.14) and that this increased level of phosphorylation was again decreased on treatment with AT13387 (1mM, 24hrs). In particular, the phosphorylated levels of PDGFRb increased substantially in both resistant cell lines compared to very low levels in parental lines, and were depleted again after treatment with AT13387 (Fig. 5.14).

Discussion

Combinatorial BRAF plus MEK inhibitor regimens have proven successful at increasing overall responses and progression free survival when compared to targeted BRAF monotherapy \(^{127,183,184}\). However, even with this combination, most patients eventually relapse with resistance mechanisms observed for the combination being similar to those seen for single agent BRAF or MEK inhibitor resistance \(^{213,307-310}\). In previous studies, HSP90 inhibitors such as 17-AAG, PF-4470296, XL888 and ganetespib have been effective in a number of kinase-inhibitor resistant models including those with BRAF inhibitor resistance \(^{197,319-322}\). Here we demonstrated for the first time that the HSP90 inhibitor, AT13387, is also effective in overcoming acquired resistance to dual BRAF and MEK inhibition where treatment of dual agent resistant cell lines in both 2D and 3D culture with AT13387 lead to significant growth arrest and cell death. In long-term xenograft studies, the frontline combination of AT13387 plus vemurafenib was also found to be well tolerated and significantly delayed the resistance onset. These data suggest that HSP90 inhibitors, as a single agent or in combination, have potential use as both a front-line therapy for treatment naïve melanomas and as a second-line therapy for BRAF and BRAF plus MEK refractory disease.
Current strategies for preventing and overcoming vemurafenib resistance are focused on concurrent BRAF and MEK or BRAF and PI3K/AKT/mTOR inhibition of signaling pathways. Though our previous studies support the use of such combinations when resistance is mediated through NRAS or cyclin D1 mutations, these studies also showed that inhibitor combinations that target the MAPK and PI3K/AKT were not as effective as single agent HSP90 inhibition when resistance is mediated through increased COT expression, PDGFRβ overexpression or in two cell line models with unknown resistance mechanisms (both of which had significant levels of IGF1R and one of which had significant levels of PDGFR and IGF1R)\(^{197}\). Mechanistically, RTK upregulation emerged as a potential resistance mechanism for both BRAF and BRAF plus MEK inhibitor resistant models with increased levels of both PDGFRβ and EGFR being observed in relapsed vemurafenib treated xenograft tumors and significant increases in phospho-PDGFRβ being detected in dual BRAFi/MEKi resistant cell lines. Importantly, AT13387 treatment lead to decreases in overall phospho-RTK levels in the dual resistant cell lines as well as total PDGFRβ and EGFR protein levels in single agent resistant cell lines. Similar to the results seen with XL888, AT13387 also lead to marked decreases in the expression of key HSP90 client proteins which was associated with inhibition of ERK and AKT signaling.

While early evaluations of HSP90 inhibitors (e.g. 17-AAG and IPI-504) showed some evidence of clinical activity in melanoma, investigations utilizing first-generation, ansamycin class inhibitors, were terminated due to limiting toxicities and low bioavailability\(^{323,324}\). However, second-generation formulations of HSP90 inhibitors have much lessened toxicity profiles and significantly improved potency. As such this new
class of HSP90 inhibitors, which include AT13387, ganetespib and XL888, are being investigated for the treatment of melanoma as single agents or in combination with vemurafenib or dabrafenib plus trametinib (Table 1.3) with thus far promising preliminary therapeutic responses being seen for the combination of vemurafenib plus XL888.

Materials and Methods

Small molecule inhibitors
AT13387 was synthesized at Astex Pharmaceuticals (Cambridge, UK) as described by Woodhead et al. and stored as a lyophilized powder. Vemurafenib (PLX4032) was purchased from Sequoia Research Products Ltd (Pangbourne, UK) or Selleck Chemicals (Houston, TX, USA). Selumetinib (AZD6244) was purchased from Selleck Chemicals. Dabrafenib and trametinib were from Chemie Tek (Indianapolis, IN, USA). All other reagents were purchased from Sigma unless otherwise stated.

Cell culture and reagents
The human cell lines A375, SK-MEL-28, SK-MEL-2, SK-MEL-5 and WM266-4 were purchased from the American Type Culture Collection, Teddington, UK. The A2058 human cell line was purchased from the European Collection of Cell Cultures, Porton Down, UK. The RPMI-7951 human cell line was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschwieq, Germany. These cell lines were not passaged for more than 6 months since authentication by the cell banks (DNA fingerprinting and cytogenetic analysis or short-tandem repeat (STR) PCR). The 1205Lu and WM164 melanoma cell lines were a kind gift from Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). Their identity was validated by STR analysis.
(Biosynthesis, Lewisville, TX) at six monthly intervals. All cells were grown in their recommended culture medium, supplemented with 10 % FBS and maintained at 37 °C in an atmosphere of 5 % CO₂ unless otherwise stated. All cell culture reagents were purchased from Invitrogen unless otherwise stated. A375R cells were generated by culturing A375 cells in the presence of 2 µM vemurafenib for 3 weeks. The resultant vemurafenib-resistant cell line was maintained in 2 µM vemurafenib. SK-MEL-28R cells were derived as follows: A SK-MEL-28 xenograft tumor that had relapsed on chronic vemurafenib treatment (50 mg/kg po bidx5/qdx2 for 150 days, see below) was excised out of the SCID mouse on Day 151 (Tumor #7). The tumor was cut into small pieces and collagenase IV-digested for 45 min at 37 °C. The digested tumor cell suspension was passed through a 100 µm filter, washed and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS, penicillin/streptomycin and 1 µM vemurafenib. All assays were performed in antibiotic-free medium at passage numbers 2 to 8. Dual BRAF and MEK inhibitor resistant cell lines were established by chronically treating SK-MEL-28, WM164 and 1205Lu for 4-5 months with 1 µM each of vemurafenib and selumetinib. Cell lines were maintained in 5% FBS in RPMI 1640 media with 1 µM vemurafenib and 1 µM selumetinib.

**Viability assays**

Viability studies were carried out using Alamar Blue (Invitrogen) as described previously with slight modifications. Briefly, 5x10³ cells were seeded in 200 µl of complete culture medium per well into flat-bottomed 96-well plates one day before the drug treatment. Cells were incubated with compound in 0.1% (v/v) dimethyl sulfoxide (DMSO) for 3 or 6 days before viability was assessed by using Alamar blue. IC₅₀ values
Flow cytometry

Melanoma cell lines were plated into 6-well tissue culture plates at 60% confluency and left to grow overnight. Vemurafenib-resistant cells were treated with 1 mM vemurafenib alone or in combination with AT13387 while dual inhibitor-resistant lines were treated with 1 mM vemurafenib and 1 mM selumetinib alone or in combination with AT13387. After the indicated treatment time, cells were harvested and stained for Annexin V as described previously.

Western Blotting

Cells were seeded at 10^6 cells per well and incubated overnight at 37 °C followed by treatment with AT13387 or other compounds. At the indicated time points, cells were harvested and lysed in ice-cold TG lysis buffer or RIPA buffer. Xenograft tumor samples were prepared by homogenizing snap-frozen tumors in ice-cold TG lysis buffer using a Precellys 24 homogenizer and incubating on ice for at least 15 min. After a freeze-thaw, all lysates were centrifuged at 14,000 rpm for 5 min at 4 °C to remove debris. Protein concentrations were determined using BCA protein assay (Pierce) and normalised. For western blot analysis, samples were resolved by SDS PAGE on NuPage® Novex Bis-Tris or Tris-Glycine gel systems, transferred to nitrocellulose or PVDF membranes and incubated with primary antibodies specific for: HSP70 (Enzo Life Sciences), BRAF, COT (Santa Cruz), BRAF^{V600E} (Spring Biosciences), CRAF (BD Biosciences), phospho-AKT^{S473}, total AKT, phospho-ERK^{T202/Y204}, total ERK, phospho-S6^{S235/236}, total S6, cleaved PARP, PDGFRβ, cKIT, IGF1R, EGFR (Cell
Signaling Technology), actin (Abcam), or GAPDH (Sigma) (Table S1). Blots were then incubated with either infrared-dye-labeled secondary antibodies and fluorescence detected on the Odyssey infrared imaging system (LI-COR Bioscience) or incubated with horse-radish-peroxidase-conjugated secondary antibodies (Amersham) followed by detection with ECL (Perkin Elmer).

**Table 5.2.** Further details of the suppliers of antibodies listed in Materials and Methods.

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<th>Company</th>
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**Colony formation assays**

A375 and SK-MEL-28 cells were seeded in 6-well plates at a density of 500 cells per well. Both cell lines were maintained in culture by feeding with 2 ml fresh complete culture medium. Cells were treated for two weeks with AT13387 (18 nM) and
vemurafenib (2 µM) as single agents or in combination; fresh compound was added every three days. Colonies were fixed and stained with 0.1% crystal violet solution. Plates were scanned and colonies quantified using the GelCount software (Oxford Optronix).

**Three-dimensional spheroid assays**

Melanoma three-dimensional (3D) spheroids were prepared as described previously. Briefly, 50 mL of a 1.5% solution of agarose was added to each well of a 96-well tissue culture plate and allowed to solidify. Into each well, 5×10^3 cells in 200 mL of media (containing the respective compound that the cells are resistant to) were overlayed on the agarose bed and allowed to grow over 5 days. The resultant spheroids were implanted into rat tail collagen I and treated for 6 days with either 1 mM vemurafenib alone or in combination with AT13387 (single agent resistant cell lines) or 1 mM vemurafenib and 1 mM selumetinib alone or in combination with AT13387 (dual agent resistant cell lines). Spheroids were then washed three times in media and stained with calcein-AM and ethidium bromide (Molecular Probes) for 1 hour at 37 °C, according to the manufacturer’s instructions. Pictures of the invading spheroids were taken with a 5x objective using a Nikon 300 inverted fluorescence microscope.

**Receptor Tyrosine Kinase (RTK) array**

Dual inhibitor resistant WM164RR and 1205LuRR cell lines were seeded at 50% confluency and grown overnight. Cells were then treated for 24 hours with 1 µM vemurafenib and 1 µM selumetinib (control) or 1 µM AT13387, 1 µM vemurafenib and 1 µM selumetinib. Vehicle (DMSO) treated naïve WM164 and 1205Lu cell lines were similarly plated and harvested. Global levels of RTK tyrosine phosphorylation were
determined using R&D Systems (Minneapolis, MN, USA) Proteome Profiler Human Phospho-RTK Array Kit. Briefly, cells were lysed and proteins extracted with Lysis Buffer 17 supplemented with Cell Signaling Technology’s (Beverly, MA, USA) protease/phosphatase inhibitor cocktail. RTK array membranes were then incubated with 700 µg of protein lysate. All remaining steps were conducted per vendor instructions. Densitometric values of all positive signals on the RTK array were measured with Adobe Photoshop CS3. Corresponding levels of tyrosine phosphorylation were calculated by subtracting the average background values from the average values of duplicate RTK spots.

**Xenograft Studies**

The care and treatment of experimental animals were in accordance with the United Kingdom Coordinating Committee for Cancer Research guidelines and with United Kingdom Animals (Scientific Procedures) Act 1986. All animals were purchased from Harlan, UK. SK-MEL-28 and SK-MEL-28R xenografts were prepared by subcutaneously injecting 5x10^6 cells suspended in serum-free DMEM mixed 1:1 with Matrigel (BD Biosciences, ~10 mg/ml protein concentration) into the flank of each male BALB/c SCID mouse. A2058 xenografts were prepared similarly in BALB/c nude mice. Tumor burden was estimated from caliper measurements and by applying the formula for an ellipsoid. Mice were randomised into different treatment groups when the mean of the tumors reached 100 mm³. AT13387 was suspended in an aqueous solution of 17.5% (w/v) (2-hydroxypropyl)-β-cyclodextrin and administered intraperitoneally (ip) at 70 mg/kg, once (SK-MEL-28 and SK-MEL-28R) or twice (A2058) a week. Vemurafenib was suspended in 5% (v/v) DMSO and 95% (v/v) of an aqueous solution of 1% (w/v)
methylcellulose, and administered at 50 mg/kg twice daily on weekdays and once daily on weekends (bidx5/qdx2) by oral gavage (po). All drugs were given at a dose volume of 10 ml/kg. T/C was calculated as 100×mean treated tumor volume over mean control volume. Tolerability was estimated by monitoring body weight loss and survival over the course of each study.

**Statistical analyses**

All statistical analyses were performed using GraphPad Prism version 6.01. Effects of various drug treatments were compared using one-way ANOVA for comparing 3 or more groups, or unpaired t-test for 2 groups. Differences were deemed statistically significant when $P<0.05$. 
Chapter 6

**Ligand independent EphA2 signaling drives the adoption of a targeted therapy-mediated metastatic melanoma phenotype.**

**Note to Reader**

Portions of this chapter have been previously published in Cancer Discovery. 2014 Dec 26 and have been reproduced with permission from the American Association for Cancer Research Publishing. Author contributions: Paraiso KH (designed/performed experiments, interpreted data, writing, figures); Das Thakur M, Stuart D (designed/performed PDX experiments); Sloot S, Gibney GT (interpreted patient chart data); Fang B, Koomen JM (performed proteomic experiments); Pasquale EB (data interpretation, wording, plasmids); Villagra A (data interpretation HDAC experiments, inhibitors); Flaherty KT, Sondak VK (data interpretation, wording); Tsao H (plasmids) Fedorenko IV, John J (performed experiments); Meier FE (patient specimens); Messina JL, Rao U, Tawbi HA, Kirkwood JM (performed/interpreted pathology experiments); Smalley K (study concept, designed experiments, interpreted data, writing, figures).

**Introduction**

Though BRAF/MEK inhibition has improved progression-free survival compared to BRAF inhibitor alone, resistance is still prevalent. Currently, most of the clinically validated mechanisms of BRAF and BRAF/MEK inhibitor resistance are genetic and include acquired mutations that reactivate the MAPK pathway (BRAF splice mutants, NRAS mutations, MEK1/2 mutations) as well as genetic changes that increase
PI3K/AKT signaling (NF1, PTEN, PI3KCA and AKT1)\textsuperscript{134,150,206,213,309}. It has also been shown through RTK array-based platforms that increased receptor tyrosine kinase (RTK) signaling can also enable escape from BRAF and MEK inhibition\textsuperscript{222,328}. While gene- and phospho-array based approaches provide valuable information, they do not address dynamic global signaling changes that occur following acquired resistance.

To address how global rewiring of melanoma signaling promotes resistance, we utilized mass spectrometry based phosphoproteomic and network analyses to identify signaling changes in tyrosine, threonine and serine that occur prior to and following acquired BRAF inhibitor resistance. This comprehensive bioinformatics approach uncovered a “resistance interactome” consisting of ~150 nodes that was marked by significant alterations and the emergence of signaling nodes such as ITGB1, EphA2, EphB4, FAK1, STAT3 and PXN. GeneGo pathway enrichment analysis revealed enhanced cytoskeletal rearrangement, adhesion, integrin signaling, cell migration and extracellular matrix remodeling. Consistent with our bioinformatic predictions, melanoma cell lines with acquired vemurafenib resistance were considerably more invasive than their drug naïve counterparts.

There is evidence from other cancer types that therapeutic intervention drives the adoption of phenotypes e.g. epithelial-to-mesenchymal transition (EMT), whereby epithelial cells can acquire the invasive characteristics of mesenchymal cells\textsuperscript{329}. For melanoma, the patterns of disease recurrence seen at progression are complex, with 50% of individuals progressing at only new sites of metastasis, 44% at existing sites of metastasis and 6% at both new and existing sites\textsuperscript{330}. Though it is known that melanoma cells with intrinsic BRAF inhibitor resistance frequently show increased
invasive potential, the role of chronic BRAF inhibitor treatment in mediating the dissemination of melanoma cells has been little explored. Ephrins constitute the largest family of RTKs and play diverse roles in cell migration, development and maintenance of the stem cell niche. Under physiological conditions, EphA2 kinase binds to its membrane bound ligand EphrinA1, leading to the inhibition of AKT and reduced cell migration. In cancer, EphA2 is often overexpressed and, following phosphorylation by AKT at S897, can signal in a ligand-independent manner to drive tumorigenic behavior and increased cell migration.

In the present study we utilized a label-free phosphoproteomic approach to quantify all of the phosphorylation events associated with long-term inhibition of BRAF signaling. These analyses revealed a previously unsuspected link between BRAF and BRAF/MEK inhibition and the adoption of an aggressive, invasive phenotype driven through ligand-independent EphA2 signaling.

Results

**Phosphoproteomics identifies BRAF inhibitor resistance to be associated with an invasive signature**

We began by asking how chronic vemurafenib treatment rewired the signaling network of BRAF mutant melanoma at a systems level through the use of a mass spectrometry-based phosphoproteomic platform (Fig. 6.1). The goal of these studies was to uncover phenotypic adaptations to chronic BRAF inhibition. Our approach offers advantages over other proteomic methods such as reverse phase protein array (RPPA) in being comprehensive, unbiased and not limited by antibody availability. In brief, tyrosine phosphorylated peptides were retrieved by immunoprecipitation and the
threonine and serine phosphorylated peptides isolated by subjecting the resulting flow-through to strong cation exchange (SCX) and immobilized metal affinity chromatography (IMAC). Liquid chromatography tandem mass spectrometry (LC-MS/MS) (Fig. 6.1) was used to quantify all of the phospho-peptides from isogenic vemurafenib naïve and resistant 1205Lu melanoma cells. Levels of peptide phosphorylation were quantified using MaxQuant and the protein-protein interactions characterized using GeneGO. Cytoscape mapping of the global signaling changes showed an increase in both the number of nodes and the number of edges in vemurafenib resistant 1205Lu cells (Naïve: 544 nodes, 1208 edges. Resistant: 552 nodes, 1288 edges) (Fig. 6.2).

![Workflow of the phosphoproteomic experiment](image)

**Fig. 6.1.** Workflow of the phosphoproteomic experiment.
Fig. 6.2. Comprehensive phosphoproteomic overviews of BRAF inhibitor naïve (L) and BRAF inhibitor resistant (R) melanoma. Proteins identified from the 1205Lu naïve and inhibitor resistant proteomes were analyzed using MetaCore (GeneGo) software. Direct protein-protein interactions emerging from this analysis were visualized with Cytoscape software.

Chronic BRAF inhibition was associated with an enrichment of phospho-proteins involved in adhesion, cytoskeletal remodeling, FAK and integrin signaling (Fig. 6.3) as well as the emergence of a highly interconnected resistance interactome involving EphA2, EGFR, EphB4, FAK1, HDAC1, integrins (ITGA3, ITGA5, ITGAV, ITGB1, ITGB5), nucleolin, p130CAS, paxillin, SHC1, Tensin-3 and Zyxin (Fig. 6.4A and B). As this suggested the adoption of a migratory/invasive phenotype, we next characterized a panel of BRAF (designated “R” cell lines) and BRAF/MEK inhibitor resistant cell lines (designated “RR”), which were generated through chronic drug treatment for >6 months (Fig. 6.5).
Fig. 6.3 Comprehensive phosphoproteomics identifies an invasive, motile signaling signature associated with BRAF inhibitor resistance. GeneGo enrichment analysis revealed several highly significant pathways (-log p-value >2) appearing within the resistance interactome.

Fig. 6.4. A: Further enrichment analysis of the resistance network showed that several proteins were not only found be increased (log 10 relative peak intensity) following acquired resistance but also recurred across several of the resistance pathways. B: As these proteins would broadly contribute to resistance we conducted a connectivity analysis that revealed that the redundant nodes formed a highly connected sub-network.
Fig. 6.5. Characterization of BRAF/MEK inhibitor resistant melanoma cell lines. Resistance was confirmed through Alamar Blue experiments in which sub-confluent cultures were treated with increasing concentrations of dabrafenib (SK-MEL-28 and WM164: 0.001 - 3 µM, 1205Lu: 0.001 - 10µM), in the continual presence of trametinib (1 µM), increasing concentrations of trametinib (SK-MEL-28 and WM164: 0.001 - 3 µM, 1205Lu: 0.001 - 10 µM), in the continuous presence of dabrafenib (1 µM), increasing concentrations of vemurafenib (SK-MEL - 28 and WM164: 0.001 - 3 µM, 1205Lu: 0.001 -10 µM) in the presence of selumetinib (1 µM) or increasing concentrations of selumetinib (SK-MEL-28 and WM164: 0.001 - 3µM,1205Lu: 0.001 - 10 µM) in the continuous presence of vemurafenib (1 µM). *Figure shows a representative plot of WM164 naïve and resistant cell lines.

It was noted that the resistant cell lines had increased motile behavior in both transendothelial migration assays (where melanoma cells were allowed to migrate through confluent endothelial cell monolayers), and 3D spheroid assays (in which melanoma cells migrated into the surrounding collagen matrix) (Fig. 6.6, Fig. 6.7, and not shown). One potential candidate identified from the screen was EphA2, a cell surface receptor tyrosine kinase implicated in development, stem cell niche maintenance and cancer progression.332
Acquired resistance to MAPK inhibition leads to increased invasion and migration. Left, human vascular endothelial cells (HUVECs) were plated onto transwell inserts and grown to confluency. Dil labeled (red fluorescence) naïve or resistant melanoma cells were then plated on top of the HUVEC layer and allowed to invade. Non-migratory cells were removed and the remaining cells were imaged by fluorescence microscopy.

**Fig. 6.6.** Quantification of invasive of BRAF and BRAF/MEK inhibitor resistant cell lines Figure 6.6.

**Ligand-independent EphA2 signaling drives the adoption of an invasive phenotype**

Despite EphA2 being implicated in the suppression of cell adhesion and migration following stimulation with ephrin-A1, it can also function in a forward signaling,
ligand-independent manner following phosphorylation by AKT at S897 \(^{334}\) (See Model in Fig. 6.8). Validation of the phosphoproteomic screen through Western blotting showed increased EphA2, phospho-EphA2 (S897), phospho-FAK (Y397), phospho-Paxillin (Y118) and total Paxillin expression in BRAF inhibitor resistant cell lines (Fig 6.9A). Increased S897-EphA2 expression was also observed in other BRAF inhibitor resistant (R) cell lines as well as those with acquired BRAF/MEK inhibitor resistance (RR) (Fig 6.9B). An analysis of drug naïve 1205Lu and A375 melanoma cells (which expressed low-levels of EphA2 basally) did not reveal vemurafenib to increase EphA2 phosphorylation at S897, with the addition of drug instead reducing phosphorylation at this site (Fig. 6.10). This, along with two previous studies showing that vemurafenib and its analogue PLX4720 did not alter EphA2 kinase activity in in vitro assays, suggested that the induction of ligand-independent EphA2 signaling was not a direct consequence of BRAF inhibitor treatment \(^{147,336}\). In epithelial cancers, unrestricted forward EphA signaling is accompanied by decreased ephrin ligand expression \(^{334}\). The loss of bidirectional Ephrin-Eph signaling in cell lines with acquired BRAF and BRAF/MEK inhibitor resistance was suggested by the reduction of ephrin-A1 ligand expression (Fig. 6.9C) and the ability of exogenous ephrin-A1 ligand to suppress the invasion of vemurafenib resistant 1205LuR cells in a matrigel assay, in which cells migrated towards serum (Fig. 6.9D). In drug resistant melanoma cells, siRNA knockdown of EphA2 led to a significant (P<0.005) abrogation of invasiveness in a matrigel invasion assay in both the presence and absence of drug, but did not resensitize the cells to vemurafenib-mediated apoptosis (3 µM, 48 hrs) (Fig. 6.9E, Fig. 6.11, Fig. 6.12). It therefore appeared that EphA2 expression mediated the adoption of a drug-induced
phenotypic switch but not vemurafenib resistance per se. Conversely, the introduction of EphA2 into a melanoma cell line that lacked its expression increased cell invasion through matrigel (Fig. 6.9F). To rule out that the inhibition of invasion was due to cell death, we also performed Annexin V/DAPI staining and found no differences between non-targeting and EphA2 siRNA treated cohorts (data not shown).

**Fig. 6.8.** Schema depicting ligand dependent and ligand independent ephrin signaling. EphA2 can proceed in a ligand independent way following phosphorylation at S897 by AKT leading to increased migration and invasion.

*Ligand-independent EphA2 signaling is AKT-dependent*

In all cases, S897 phosphorylation of EphA2 was mediated through PI3K/AKT, with Western blotting showing the resistance phenotype to be associated with increased phospho-AKT and phospho-GSK3β levels (Fig. 6.13A and Fig. 6.14) and the ability of a PI3K (GDC-0941, 3 µM) and an AKT inhibitor (MK-2206, 3 µM) to reverse receptor phosphorylation (Fig. 6.13B). Further studies showed PI3K inhibition (PI-103, 1 µM) to prevent melanoma cell invasion in a 3D spheroid assay (Fig. 6.13C).
Fig. 6.9. BRAF and BRAF/MEK inhibition induces an invasive phenotype driven through ligand-independent ephrin-A2 signaling. A: Western Blot of increased total EphA2, phospho-EphA2 (S897), phospho-FAK (Y397), phospho-Paxillin (Y118) and paxillin expression in BRAF inhibitor resistant (R) vs. naïve (N) 1205Lu melanoma cells. B: Increased EphA2 and S897-EphA2 expression is observed in multiple models of BRAF (R) and BRAF/MEK (RR) inhibitor resistance. C: Ephrin-A1 ligand expression is decreased in cell lines with BRAF (R) and BRAF/MEK inhibitor (RR) resistance. D: Ephrin-A1 ligand prevents invasion of 1205LuR cells. Cells were treated with Ephrin-A1 ligand (1 µg/ml, 24 hrs) E: siRNA EphA2 reverses the invasive phenotype of BRAF (R) and BRAF/MEK (RR) resistant cell lines. (Upper) siRNA knockdown of EphA2 and representative images of reduced matrigel invasion. (Lower) Quantification of invasion following treatment with non-targeting control or siRNA against EphA2. F: EphA2 enhances invasion of SK-MEL-28 cells into collagen. SK-MEL-28 cells were transfected with a plasmid containing EphA2 and selected for 14 days. After this time cells were plated on top of matrigel and allowed to invade. Image shows a Z-stack of phalloludin-stained melanoma cells through matrigel invading towards serum. Bar graph shows the quantification of invasion relative to control.
Fig. 6.10. Acute vemurafenib treatment does not induce ligand independent EphA2 signaling. Treatment naïve A375 and 1205Lu cells lines were treated with BRAFi (3µM PLX4032/vemurafenib, 1hr) prior to probing for ligand-independent (S897) and ligand-dependent (Y772) EphA2 phosphorylations.

<table>
<thead>
<tr>
<th></th>
<th>A375</th>
<th>1205Lu</th>
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<tbody>
<tr>
<td>CT PLX</td>
<td>CT PLX</td>
<td>EphA2, Y772</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EphA2, S897</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EphA2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAPDH</td>
</tr>
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Fig. 6.11. Knockdown of EphA2 expression does not resensitize melanoma cells to the pro-apoptotic effects of BRAF inhibition. Vemurafenib resistant 1205LuR cells were transfected with either 25nM non-targeting siRNA (NTsi) or 25nM EphA2 siRNA for 24hrs in the absence of vemurafenib. After 24 hours, fresh serum supplemented media with 3µM vemurafenib (white bars) or DMSO (black bars) was added. Cells were then analyzed for Annexin V binding/DAPI uptake 72 hours after transfection.
Knockdown of EphA2 reduces invasion in the absence and presence of vemurafenib. Vemurafenib resistant 1205LuR cells were transfected with 25nM non-targeting siRNA (NTsi) or 25nM EphA2 siRNA in the absence of vemurafenib. After 24 hours, fresh media with 3µM vemurafenib (black bars) or DMSO (white bars) was added. Cells were then treated with vemurafenib or DMSO for an additional 48 hours after transfection. The resistant 1205LuR were then allowed to invade and migrate across a matrigel barrier in the presence or absence of vemurafenib for 48 hours prior to confocal microscopy imaging.

Treatment with the ephrin-A1 ligand restored EphA2 tyrosine phosphorylation (Y772), and kinase activity, inhibited AKT phosphorylation on S473, AKT-dependent EphA2 phosphorylation at S897 and FAK phosphorylation at Y397 (Fig. 6.13D, Fig. 6.15, Fig. 6.16). The potential role of FAK signaling in the drug-mediated invasive phenotype was suggested by the ability of the FAK inhibitor PF-228 to inhibit invasion in a 3D spheroid assay (Fig. 6.16). Introduction of an EphA2 S897A mutant that is refractory to AKT mediated phosphorylation prevented its interaction with an AKT substrate (Fig. 6.13E) and reversed the invasion of BRAF inhibitor resistant cells through matrigel and their migration in a scratch assay (Fig. 6.13F).
Fig. 6.13. Ligand-independent phospho-EphA2 (S897) signaling is PI3K/AKT dependent. A: Western blot of pAKT, AKT, PTEN and GAPDH in melanoma cells that are drug naïve (N), BRAF inhibitor resistant (R) and BRAF/MEK inhibitor resistant (RR). B: The PI3K inhibitor GDC-0941 (3 µM, 24 hr) or the AKT 24 inhibitor MK-2206 (3 µM, 1 hr) decreases the phosphorylation of AKT (S473) and EphA2 (S897). C: PI3K inhibition reduces the invasion of BRAF inhibitor resistant melanoma cell lines. (Top panel) 1205LuR cells were plated on top of Matrigel and treated with either vehicle or PI-103 (1µM) for 24 hrs before being stained with phalloidin and imaged used confocal microscopy. (Bottom panel) 1205LuR cells were grown as 3D spheroids, implanted into collagen and treated with vehicle or 1µM PI-103 prior to staining with calcein AM. Invading cells were imaged with an inverted fluorescence microscope and levels of invasion were calculated with ImageJ software. D: Ephrin-A1 ligand reduces EphA2 (S897) phosphorylation levels in 1205LuR cells. Cells were treated with ephrin-A1 ligand (1 µg/mL, 24 hr) before being analyzed by Western Blot. E: Transfection of the EphA2 serine to alanine substitution at position 897 mutant plasmid (S897A) prevents S897 phosphorylation of EphA2. 1205LuR cells were transfected with either WT (control) or mutant S897A EphA2 plasmid. Lysates from transfected cells were immunoprecipitated with antibodies against S897 and total EphA2 and immunoblotted with anti-phospho-serine/threonine AKT substrate antibody. Total, non-immunoprecipitated protein was also probed for EphA2 and GAPDH as loading controls. F: Mutant S897A expression reduces 1205LuR cell invasion and scratch wound closure. 1205LuR cells transfected with WT (control) or S897A mutant EphA2 plasmid were either allowed to invade through matrigel or plated into 6 well tissue culture plates and grown to confluency before being scratched with a p20 pipet tip after which the wound was allowed to close over 24 hours.
Fig. 6.14. PI3K and AKT inhibitors inhibit AKT phosphorylation and the AKT substrate GSK3β. Vemurafenib resistant 1205LuR cells were treated for 1 hr with 3µM vemurafenib alone (CT), 3µM GDC-0941 + 3µM vemurafenib (GDC) or 3µM MK-2206 + 3µM vemurafenib (MK) prior to Western Blotting.

Fig. 6.15. EphrinA1 ligation leads to increased EphA2 tyrosine phosphorylation. 1205LuR cells were treated for 1 hr with 1µg/mL IgG-Fc or 1µg/mL ephrinA1-Fc and probed with anti-Y772-EphA2 antibody.

Fig. 6.16. Ligation of EphA2 by ephrin A1 is associated with decreased signaling through FAK. (Left panel) Vemurafenib resistant 1205LuR cells were treated for 24 hrs with 1µg/mL ephrinA1 ligand or Fc-IgG control prior to Western Blotting. (Right panel) 1205LuR cells were treated for 5 days with 5µM FAK inhibitor (PF228) or an equivalent volume of DMSO control prior to inverted microscopy imaging and scoring of invasion.
Although increased phosphorylation by AKT explained the ligand-independent EphA2 signaling, it did not explain the increased EphA2 expression in BRAF inhibitor-resistant melanoma cells. We therefore asked whether EphA2 expression in BRAF inhibitor resistant cell lines was dependent upon continuous drug selection pressure. Removal of BRAF or BRAF/MEK inhibition led to an initial decrease in the fitness of the resistant cells as seen by an increased doubling time, followed by the partial reversal of resistance (Fig. 6.17, Fig. 6.18), an effect associated with decreased phosphorylation of both AKT and EphA2 S897 (Fig. 6.19A). Phenotypically, these effects were paralleled by a marked reduction in the invasive capacity of the cells (Fig. 6.19B, C).

Fig. 6.17. Removal of BRAF or BRAF/MEK inhibitors increases cell doubling time. Treatment naïve (1205Lu, WM164), chronically treated (1205LuR: 3µM vemurafenib, WM164RR: 1µM vemurafenib + 1µM selumetinib) and resistant cell lines given a 4 week drug holiday (1205LuR no drug, WM164RR no drug) were plated at a density of 25,000 cells per 2 mL of a 6 well tissue culture dish. Cells were counted on days 1, 3, 5 and 7 and doubling times calculated.
Fig. 6.18. Removal of drug leads to a reversal of the drug resistance phenotype. WM164RR cells were grown in drug free media for 4 weeks before being treated with increasing concentrations of dabrafenib and trametinib (0.001-1 µM of each drug combined). Data shows the comparison of naïve WM164 cells, WM164RR maintained in the presence of BRAF/MEK inhibitor and WM164RR following 4 weeks of reversion.

Fig. 6.19. Removal of drug reverses EphA2 signaling and the invasive phenotype of melanoma cells. A: Reversion of phospho-EphA2, EphA2, pAKT and total AKT expression following removal of BRAF inhibitor (R) or BRAF/MEK (RR) inhibitors for 0-4 weeks. B: Removal of drug reverses the invasive phenotype of BRAF and BRAF/MEK inhibitor resistant cell lines through an endothelial monolayer. C: Quantification of data shown in B.
There is evidence that acquired BRAF inhibitor resistance leads to epigenetic changes that impair the apoptotic response, and that these effects can be reversed through HDAC inhibition. We next treated the resistant melanoma cultures with the pan-HDAC inhibitor LBH-589 and observed decreases in EphA2 protein expression, its phosphorylation at S897 and an inhibition of AKT phosphorylation (Fig. 6.20A,B). In agreement with the role of phosphorylated EphA2 in melanoma cell migration, treatment with the HDAC inhibitor LBH-589 significantly reduced the invasion of melanoma cells in a 3D spheroid assay (Fig. 6.21).

**Fig. 6.20.** HDAC inhibition reverses EphA2 phosphorylation, EphA2 expression and PI3K/AKT signaling. A: EphA2 phosphorylation is PI3K/AKT-dependent and epigenetically regulated. The HDAC inhibitor LBH589 (100nM, 24 hrs) decreases the phosphorylation of AKT and EphA2. B: LBH589 reduces EphA2 and AKT phosphorylation across multiple resistant cell lines.
Fig. 6.21. The pan-HDAC inhibitor LBH589 reverses the invasive phenotype of A375R and 1205LuR cells. Spheroids of either A375R or 1205LuR were implanted into collagen and treated with either BRAF inhibitor (vemurafenib, 3 µM) or LBH589 (100nM) for 3 days before being imaged by confocal microscopy. Spheroids were stained with calcein-AM (green, live cells) or propidium iodide (red, dead cells). Magnification x5.
**Ligand-independent EphA2 signaling is induced in vivo following BRAF inhibition and is associated with metastatic dissemination.**

The in vivo relevance of ligand-independent EphA2 signaling and its link to metastasis was explored in melanoma patient specimens. It was found that whereas 0% of primary (stage I/II) melanoma lesions (n=12) stained strongly (+2/3) for EphA2, 21% of metastatic lesions (stage III/IV) (n=19) showed strong staining (Fig. 6.22 A,B: Table 6.1), with 57.9% of metastatic lesions showing some strong focal S897-EphA2 staining. We next established an in vivo resistance model where fragments of treatment naïve human melanoma specimens were implanted subcutaneously into nude mice (patient-derived xenografts, PDX)\(^{339}\). Vemurafenib dosing was commenced over 100-200 days, until resistance emerged (Fig. 6.23)\(^{339}\).

**Table 6.1.** Levels of EphA2 staining between primary (n=12) and metastatic melanoma lesions (n=19). Table shows breakdown of IHC staining by total EphA2 staining, phospho-EphA2 staining and focal phospho-EphA2 staining.

<table>
<thead>
<tr>
<th>stage</th>
<th>S897-EphA2 (+2/3)</th>
<th>focal staining S897-EphA2</th>
<th>total EphA2 (+2/3)</th>
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<tr>
<td><strong>primary</strong> stage I/II, n=12</td>
<td>0.0%</td>
<td>25.0%</td>
<td>50.0%</td>
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<td><strong>metastasis</strong> stage III/IV, n=19</td>
<td>21.1%</td>
<td>57.9%</td>
<td>73.7%</td>
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Fig. 6.22. Increased total and phosphorylated (S897) EphA2 in clinical specimens is associated with metastatic dissemination. A: Representative images of sequentially sectioned primary (S897-EphA2: +1-2, total EphA2: +2) and metastatic (S897-EphA2: +2, total EphA2: +3) IHC stained patient tumor specimens. B: Metastasis is associated with higher tumor-wide positive staining (+2/3) for pEphA2, increased focal S897-EphA2 staining and greater tumor-wide total EphA2 staining. Representative images of pEphA2 staining of the advancing edge of 2 separate metastatic tumor specimens. Arrows indicate focal S897-EphA2 staining at the leading edge. C: Images from 3 matched pairs of primary and metastatic vemurafenib-treated patient derived melanoma mouse xenografts stained for total EphA2 showing greatly increased levels of total EphA2 expression in the matched metastatic vs. primary lesion. Images show paired samples from 3 independent mice. D: Vemurafenib treatment increases EphA2 expression in melanoma patient specimens. Representative images of staining for EphA2 and S897-EphA2 in matched melanoma lesions (from patient E) pre-treatment and collected on vemurafenib therapy. See Table 6.2 for details. E: Vemurafenib resistance is associated with increased EphA2 expression in specimens derived from a patient with matched primary melanoma and metastatic lesions that emerged on therapy. Images show IHC staining for EphA2, S897 EphA2 and pAKT on a matched pair of primary (pre-vemurafenib treatment) and subcutaneous metastatic (post-vemurafenib treatment) lesions.
Local or distant metastases were frequently observed in vemurafenib resistant PDXs, but not in the vehicle treated animals (as these were sacrificed at much earlier time points) (Das Thakur et al., In preparation). Staining of matched pairs of subcutaneous and metastatic specimens from the same drug-treated animals at necropsy showed high levels of immunohistochemical staining for EphA2 in the metastatic lesions that were absent from the matched primary subcutaneous lesions (Fig. 6.22C). In common with other studies, a heterogeneous pattern of EphA2 staining was noted, with both membranous and cytoplasmic/nuclear staining observed \(^{335}\). Analysis of specimens from a cohort of 6 melanoma patients receiving vemurafenib therapy (14-370 days) revealed increased EphA2 and S897-EphA2 expression in the majority of on-treatment and post-relapse samples that were lacking in the pretreatment lesions (5/6) (Table 6.2: Fig. 6.22D shows pre and post treatment specimens from patient E).

**Table 6.2.** Relative EphA2 expression from matched pre and post-vemurafenib treatment biopsies. Table details time of sample procurement and results of IHC staining for phospho-EphA2 staining (S897).

<table>
<thead>
<tr>
<th>patient</th>
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<th>time of sample procurement</th>
<th>S897-EphA2 pre vs post treatment</th>
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<tr>
<td>A</td>
<td>14</td>
<td>on therapy</td>
<td>increase</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>on therapy</td>
<td>increase</td>
</tr>
<tr>
<td>C</td>
<td>21</td>
<td>on therapy</td>
<td>increase</td>
</tr>
<tr>
<td>D</td>
<td>86</td>
<td>post-failure</td>
<td>decrease</td>
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<td>E</td>
<td>176</td>
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<tr>
<td>F</td>
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Chronic treatment of A375 cells with vemurafenib (1 µM, 1-4 weeks) further supported the rapid induction of EphA2 and S897-EphA2 expression following BRAF inhibition, with increased EphA2 protein expression observed by 3 weeks and increased EphA2 and S897-EphA2 expression seen after 4 weeks of drug treatment (Fig. 6.24). It thus seemed that induction of EphA2 expression was a relatively rapid adaptation to BRAF inhibition, which may precede the development of acquired drug resistance.

Fig. 6.24. Time-course of EphA2 and S897-EphA2 induction following chronic vemurafenib (PLX4032) treatment. Drug naïve A375 cells were treated with vemurafenib (1 µM) for 1-4 weeks and probed for the expression of EphA2 and S897-EphA2. Blots were stripped and reprobed for expression of GAPDH to confirm even protein loading.

A potential link between acquired resistance and the role of BRAF inhibition in the emergence of new metastases was suggested by the observation patients failing vemurafenib therapy (n=28) had significantly higher incidence of tumor growth at new, distant disease sites (68%) rather than the regrowth of existing lesions, compared to patients receiving dacarbazine (n=20, 35%, Table 6.3).

Table 6.3. Incidence of new metastases emerging in patients receiving either BRAF inhibitor (vemurafenib) or chemotherapy (dacarbazine).
These differences were seen despite the time on therapy being similar (147.5 days vs. 101.5 days for vemurafenib and dacarbazine, respectively, \( p=0.1379 \)) (Table 6.3). We next determined whether EphA2 expression increased in new metastatic lesions that arose on BRAF inhibitor therapy. The IHC staining of a pair of matched pre-treatment and therapy-derived metastases from a patient failing vemurafenib therapy showed high levels of EphA2, phospho-EphA2 and phospho-AKT expression in the treatment-derived metastatic lesions (Figure 4E), with much less EphA2 expression observed in the pre-treatment primary lesion.

Discussion

Hostile microenvironments such as hypoxia, metabolic changes and nutrient deprivation favor the detachment of cancer cells and their migration to more favorable niches\(^\text{340,341}\). We here demonstrate that chronic BRAF inhibition induces a migratory, invasive phenotype in melanoma cells. In epithelial tumors, therapeutic adaptation leads to an EMT, which is sometimes associated with increased tumor invasiveness and metastatic spread\(^\text{329,342}\). Phosphoproteomic screening identified ligand-independent EphA2 signaling as a key driver of the metastatic phenotype in melanoma cells with acquired BRAF and BRAF/MEK inhibitor resistance. In agreement with this, increased S897 phosphorylated EphA2 was also identified in melanoma metastases from patients on treatment. Our findings mirror those in astrocytoma, where tumor aggressiveness was correlated with high expression of S897 phosphorylated EphA2 as well as phospho-AKT\(^\text{334}\). In prostate cancer and glioma cell lines, ligand-independent EphA2 signaling is known to promote invasion in an AKT-dependent manner and was reversed following treatment with Ephrin-A1 ligand\(^\text{334}\).
Previous modeling studies have demonstrated BRAF inhibitor resistance to be dependent upon continuous drug administration, with treatment withdrawal leading to tumor regression. It was further observed that ligand-independent EphA2-mediated invasion also required constant BRAF and BRAF/MEK inhibitor-mediated selection pressure. The reversibility of the invasive phenotype upon drug removal suggested that ligand-independent EphA2 signaling could be abrogated through alternate dosing schedules. There is currently some debate as to whether intermittent BRAF/MEK inhibitor dosing can forestall resistance better than continuous dosing, with evidence being provided for each scenario. There is also clinical evidence that continuation of drug beyond the time of progression can prolong clinical benefit. Our data support the notion that continuous drug dosing can increase the fitness and metastatic potential of melanoma cells which suggests that the development of new metastases may be limited through intermittent drug dosing. Based on the current data, tyrosine kinase inhibitors directed against EphA2 are unlikely to reverse the migration driven through the EphA2/AKT axis. Instead, direct targeting of the EphA2 receptor with agonists should inhibit both ligand-independent EphA2 signaling and AKT signaling to limit metastatic dissemination.

Previously, the HSP90 inhibitor, AT13387, was shown to be effective at inducing cell death in both BRAF and BRAF + MEK inhibitor resistant cell line models through simultaneous degradation or inhibition of several key client proteins that are necessary for survival and proliferation. As whole exome sequencing did not reveal any genetic alterations that would account for drug insensitivity (data not shown), the mechanism of resistance for the BRAF (WM164R, 1205LuR) and BRAF + MEK (WM164RR,
1205LuRR) resistant cell lines is thought to be concurrent upregulation of multiple RTKs (e.g. PDGFRb, IGF1R, AXL and Tie-1). These findings have caused us to shift our focus from inhibitors that target one or two kinases to broad-spectrum inhibitors that target multiple hubs that exist within the BRAFV600E resistance interactome. To date, several proteins that are important for melanoma signaling, including EphA2, have been identified as HSP90 clients\textsuperscript{189}. Based on our current findings, it is likely that an agonist of EphA2 would be the most effective at inhibiting deregulated EphA2 ligand-independent signaling. In previous studies it was shown that combining an HSP90 inhibitor with the EphA2 agonist, mAb208, lead to greater EphA2 degradation than for either agent alone\textsuperscript{344}. In addition, other groups have also found that HSP90 inhibitor-mediated degradation of EphA2 protein increased the ability of cytotoxic CD8+ T-cells to recognize EphA2 expressing tumor cells and that combining an HSP90 inhibitor with an EphA2 peptide vaccine greatly increased immunotherapeutic efficacy\textsuperscript{344,345}. Taken together, our current findings underscore the importance of targeting multiple escape routes at once in order to achieve long lasting therapeutic benefit, where the addition of an EphA2 targeting arm would increase response duration by limiting the development of new disease in patients on BRAF and BRAF/MEK inhibitor therapies.

**Materials and Methods**

**Cell culture and generation of BRAF inhibitor resistance**

Cells were cultured in 5% FBS, RPMI 1640 media. Parental 1205Lu, SK-MEL-28, and WM164 melanoma cells lines were a gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). Parental A375 cell line was purchased from American Type Culture Collection on April 18th 2012. Identities of all cell lines were confirmed by
Biosynthesis Inc., through short tandem repeat validation analysis at 6 monthly intervals. The date of last validation for these studies was December 20th 2013. Dual BRAF and MEK inhibitor resistant (RR) lines were established by chronically treating 1205Lu, SK-MEL-28, and WM164 for >6 months with 1 µM each vemurafenib and selumetinib. Unless otherwise noted, single-agent vemurafenib (R) cell lines were maintained in 5% media with the addition of vemurafenib at the following concentrations: 1 µM for A375R, 2 µM for WM164R, and 3 µM for 1205LuR. Dual agent RR inhibitor resistant lines were maintained in 5% FBS, RPMI 1640 with 1 µM vemurafenib and 1 µM selumetinib.

**Proliferation assay**

Assays were performed as described in (18). Briefly, 4,000 cells were seeded into each well of a 96-well plate prior to drug treatment and allowed to attach overnight. Media containing inhibitor solubilized in DMSO, or an equivalent volume of DMSO alone was added and cells incubated for 3 days prior to the addition of Alamar blue reagent (Invitrogen, Carlsbad, CA).

**Inhibitors**

Vemurafenib (PLX4032), selumetinib (AZD6244), GDC-0941, LBH589, PI-103 and MK-2206 were purchased from Selleck Chemicals (Houston, TX, USA). Dabrafenib and trametinib were from Chemie Tek (Indianapolis, IN, USA).

Phosphoproteomic analysis

**Sample Processing**

Naïve and vemurafenib resistant 1205Lu cell lines were each grown in ten 15 cm tissue culture dishes. Cells were grown to ~70% confluence prior to washing each dish with
10 ml of ice cold PBS + 1 mM orthovanadate (Sigma Aldrich, St. Louis, MO). Cells were then lysed according to the manufacturer’s instructions for the Phospho-Tyrosine Mouse mAb (P-Tyr-100) (Cell Signaling, Beverley, MA). Lysed proteins were reduced and alkylated prior to proteolytic digestion and phosphorylated tyrosine containing peptides generated from tryptic digestion were enriched with antibody-based (P-Tyr-100) immunoprecipitation (Figure 6.1). Flow through from the immunoprecipitation was then further enriched for phosphorylated serines and threonines by strong cation exchange peptide fractionation (SCX) and immobilized metal affinity chromatography (IMAC). The enriched phospho tyrosine, serine and threonine fractions were then subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS) and the resultant tandem mass spectra was searched against SEQUEST and MASCOT databases to identify phosphoproteins.

**Proteomic data analysis**

To calculate relative phospho-signal intensities, label-free protein quantification of the mass spectrometry data was analyzed using MaxQuant version 1.2.2.5 (www.maxquant.org). Carbamidomethylation of cysteine was set as a fixed modification; oxidation of methionine, N-terminal protein acetylation, phosphorylation of serine, threonine and tyrosine were included as variable modifications. “First search ppm” for the peptide mass tolerance was set to 20 ppm, 10 ppm for final search and fragment ion tolerance was set to 0.6 Da. Protein and peptide false discovery rates were set to 0.1 and 0.05, respectively, and minimum peptide length set to 6 amino acids. MS/MS data was searched against the Uni-Prot human database (Sprot_HUMAN_20111214.fasta). To filter out falsely identified peptide sequences, tandem mass-spec data were
combined with common contaminants and concatenated with the reversed versions (target decoy peptides) of all sequences using Andromeda search engine integrated into MaxQuant. Each cohort was run in duplicate and average intensities for each of the unique phosphopeptides identified was then calculated. In order to identify the significant phosphorylation changes that occurred following acquired vemurafenib resistance, the average intensities of the vemurafenib resistant cohort were divided by the average intensities of the naive cohort. The resulting ratio was log2 transformed and plotted to determine cut-off points (Fig. 6.25). Peptides that fell on the linear scale were removed as these were not significantly increased or decreased between the resistant and naive cohorts. Proteins with a positive log-scale value were highly phosphorylated in the resistant cell line while those with a negative log-scale value were not strongly phosphorylated in the resistant cell line. To perform pathway enrichment analyses, SwissProt protein labels corresponding to the phosphoproteins that were increased in the resistant cohort were then inputted into MetaCore’s GeneGo analysis software (thomsonreuters.com/metacore). Recurrent phospho-proteins found across the significantly enriched resistance pathways ((-log (p-value) < 2)) were identified. GeneGo software was further used to mine for direct protein-protein interaction within the recurrent phospho-proteins. The resulting subnetwork was then visualized with Cytoscape software (www.cytoscape.org) based on the connectivity metrics of degree, clustering coefficient, centrality and betweenness centrality. In order to model the individual signaling networks for the naïve and resistant cohorts, SwissProt protein labels corresponding to all naïve or resistant phospho-proteins were inputted into MetaCore’s GeneGo database and mined for direct protein-protein interactions.
Network connectivity metrics (degree, clustering coefficient, centrality and betweenness centrality) were again calculated with Cytoscape software and used to visualize the individual naïve and resistance interactomes.

**Fig. 6.25.** Plot of log2 transformed data from the proteomic analysis used to define cutoff points for increased and decreased phosphorylation following acquired vemurafenib resistance.

**EphA2 and S897A mutant plasmid transfection**

The human EphA2 plasmid was generated as described in \(^{225}\). The mutant S897A plasmid was a gift from Dr. Elena Pasquale. Naïve SK-MEL-28 cells were transfected with 4 µg of EphA2 or control plasmid while 1205LuR cells were transfected with 4 µg of S897A or WT EphA2 control plasmid with Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Stable transfectants were harvested at 14 days and protein expression levels were confirmed by Western blotting.
**Western blotting**

Proteins were extracted and blotted as described in 199. Antibodies against S897-EphA2, Y772-EphA2, EphA2, Y397-FAK, FAK, Y118-PXN, PXN, S9-GSK3, GSK3β, S473-AKT, AKT, phospho-S/T AKT substrate and PTEN were purchased from Cell Signaling Technology (Danvers, MA). Anti-ephrinA1 was from Santa Cruz Biotechnology (Santa Cruz, CA) while GAPDH was purchased from Sigma Aldrich. RNA interference Cells were transfected as described in 199 with 25nmol/L EphA2 and scrambled siRNA sequences (LifeTechnologies, Carlsbad, CA). Cells were transfected for 24 hours in the absence of inhibitor and an additional 48 hours in the presence of inhibitor prior to further experimentation.

**Quantitative real-time PCR**

Q-RT-PCR was performed as described in 199. TaqMan Gene Expression Assay Hs00171656_m1 primers/probe were used to quantify EphA2. The 18S (P/N4319413E) and GAPDH (Hs99999905_m1) used to normalize EphA2. All standards and samples were tested in triplicate and data were analyzed using SDS software version 2.3.

**3D spheroid assays**

Collagen implanted spheroids were prepared using the liquid overlay method as described in 199 and were treated with 100 nM LBH589 or 1µM PI-103 for 120 hours before being analyzed by fluorescence microscopy. Calculations of the areas of spheroid invasion into the collagen matrix were performed with ImageJ analysis software (Fig. 6.26).
**ImageJ 1.46r scoring of spheroid areas of invasion**

Images of calcein-AM stained spheroids (live cells, green fluorescence channel) were opened in ImageJ analysis software.

Spheroid images were then split into red, green and blue channels (ImageJ drop down menu>Image>Color>Split Channels) . The 8-bit, black and white images corresponding to the green fluorescence channel were then selected for further analyses.

Areas of invasion were defined by converting the 8-bit spheroid images to masked images (ImageJ drop down menu>Process>Binary>Convert to Mask). Total areas of spheroid invasion (in relative square pixel units) were calculated by selecting the masked image and choosing Analyze and Measure from the ImageJ drop down menu.

The relative percent reduction in invasion following drug treatment was calculated by subtracting the drug treated spheroid area from the untreated spheroid area and dividing by the untreated spheroid area.

**Fig. 6.26. ImageJ calculations of spheroid areas of invasion.**

**Transendothelial cell migration assay**

Migration assays were performed as described in \(^{346}\). Human umbilical vein endothelial cells (HUVECs) were plated into transwell inserts and grown to confluence. Dill labeled naïve or resistant melanoma cell lines were plated on top of the HUVEC layer and allowed to invade for 1–4 hours. Non-migratory cells were removed prior to imaging with an inverted Nikon Eclipse TS100 microscope. Calculations of the percent spheroid invasion into the collagen matrix were performed with ImageJ analysis software (Fig. 6.27).
**Transendothelial invasion assay**

Human umbilical vein embryonic cells (HUVEC) were seeded in the inner well of a Boyden chamber and grown to 100% confluency at which time the inner well EBM-2 media (Lonza, Walkersville, MD) was replaced with serum free RPMI 1640 media.

Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) red fluorescently labeled melanoma cells were then resuspended in serum free RPMI 1640 media and seeded on top of the HUVEC monolayer.

RPMI 1640 media containing 5% FBS was then added to the outer well. The Boyden chamber was next placed in a 37°C, 5% CO₂ incubator for 4 hours.

After 4 hours, non-invading melanoma cells were removed from the inner well. The remaining invasive melanoma cells were fixed, stained and imaged with an inverted fluorescence microscope.

**Fig. 6.27.** Transendothelial invasion assay.

**Scratch wound assay**

S897A and control plasmid transfected 1205LuR melanoma were grown to confluency prior to scratching with a p20 pipette tip. Wounds were imaged at 0 and 24 hours and percentage wound closure was calculated using ImageJ software 1.46r.

**Matrigel invasion assay**

Cells were overlayed onto transwell inserts coated with Matrigel (BD) and allowed to invade for 24-48 hours (Fig. 6.28). For ephrin A1 ligand experiments, cells were pretreated for 72 hours with 1µg/mL ephrinA1-Fc or IgG-Fc (R&D Systems). Cells were fixed and stained with phalloidin-AF594 and noninvasive cells removed prior to fluorescence imaging with an inverted Nikon Eclipse TS100 microscope. To quantify levels of invasion, fixed and stained cells were imaged with a Zeiss confocal microscope (20x) at 0 µm with 0.5 µm image slices taken throughout the distance of invasion.
**Matrigel invasion assay**

Matrigel was added to the inner well of a Boyden chamber and solidified by incubating for 30 minutes at 37°C.

The inner well was inverted and melanoma cells were seeded on top in RPMI 1640 media with FBS. To allow the melanoma cells to adhere to the matrigel, the inner wells were placed in a 37°C, 5% CO₂ incubator for 30 minutes.

After 30 minutes, the inner well was turned right side up and RPMI 1640 media containing FBS was added to the inner chamber while serum free RPMI 1640 media was added to the outer chamber.

Cells were then incubated for 48-72 hours at 37°C, 5% CO₂ prior to fixing, staining and confocal microscopy imaging.

**Fig. 6.28.** Matrigel invasion assay.

Rates of new metastasis for vemurafenib and dabrafenib treated patients

Patients managed at the Moffitt Cancer Center were selected from Moffitt medical records and archived melanoma specimens under the TCC/HRI, Moffitt pathology systems and written informed consent approved by the Institutional Review Board of the University of South Florida under the Declaration of Helsinki Protocols. For the vemurafenib and dabrafenib treated patients, the target population consisted of subjects with unresectable stage III or stage IV BRAF V600E mutant cutaneous melanoma treated with single-agent vemurafenib or dabrafenib as the first line of therapy. A similar patient cohort was also identified who received dacarbazine as their first line of therapy. Patients were selected for each treatment regimen who had similar numbers of restaging scans to eliminate sample bias. De-identified information pertaining to patient
demographics and clinical outcome during therapy was collected on subjects with exclusion of patients who completed less than 2 months of therapy or for whom follow-up information was not available.

**Xenograft studies**

Xenograft implantation of tumor pieces from the subcutaneous tissue of a vemurafenib treatment naïve 44 year-old male with recurrent BRAF V600E mutant melanoma (HMEX2613) was performed as described in 339. Established HMEX2613 tumors were dosed continuously with 45mg/kg vemurafenib. After 100-200 days, tumors became resistant to vemurafenib with 30% of mice being euthanized due to clinical signs. Upon necropsy, widespread metastasis was found. IHC was carried out on sub-cutaneous resistant tumors and matched with corresponding metastatic lesions.

**Immunohistochemistry: Xenograft and patient samples**

Slides were stained using the Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) per manufacturer's instructions with proprietary reagents. Slides were deparaffinized on the automated system with EZ Prep solution (Ventana). The retrieval method used was citrate buffer at pH 6.0. Rabbit antimouse polyclonal IgG primary antibody for EphA2 (Santa Cruz Biotechnology, Santa Cruz, CA, #sc-924) that reacts to human EphA2 was used at a 1:250 concentration in PSS antibody diluent (Ventana) and incubated for 12 min. Ventana Anti-mouse secondary antibody was used for 12 min and Ventana OmniMap kit used for detection. Slides were counterstained with Hematoxylin. De-identified formalin fixed paraffin-embedded tissue samples were obtained from the Moffitt Pathology, the University of Pittsburgh and the University of Tuebingen archives under a written informed consent protocol approved by
the Institutional Review Board of the University of South Florida under the Declaration of Helsinki Protocols and stained as above for the xenograft samples. Staining was visualized using the Ventana Chromomap Redkit. Slides were analyzed by two independent observers and consensus scored on a scale from (0 to +3).

**Statistical analysis**

Data show the mean of at least 3 independent experiments ± the SE mean, unless stated otherwise. GraphPad Prism 6 statistical software were used to perform the 2-tailed Student's t-test and for contingency analyses of patient data (2-tailed Fisher's exact test). For all statistical analyses, asterisks (*) indicates P ≤ 0.05.
Chapter 7

Summary

Chapter 1

The two major risk factors for developing melanoma are UV exposure and family history. Most diagnoses are made through histological criteria; however, there are subsets that are difficult to distinguish from benign melanocytic nevi. Melanomas frequently metastasize therefore proper diagnosis is critical. Among patients with stage IV disease, the anatomic site of metastases was the most significant predictor of survival with non-pulmonary visceral metastases and/or an elevated serum LDH having the worst one-year survival of 33% \(^\text{20}\). The four main genetic subtypes of melanoma are \(BRAF\), \(NRAS\), \(c-KIT\) mutant and \(BRAF/NRAS\) WT. Melanomas rely heavily on MAPK signaling for survival and growth making this pathway an attractive candidate for targeted kinase therapy. Inhibitors of \(BRAF\) and \(BRAF\) combined with \(MEK\) have been FDA approved for the treatment of \(V600\)-\(BRAF\) mutant melanomas. Other compounds currently under development for \(BRAF\) melanoma include \(ERK\), \(pan-RAF\), and \(HSP90\) inhibitors. Drug resistance is a major problem in maintaining durable responses with numerous and diverse melanoma host and microenvironmental factors playing a role.

Chapter 2

Vemurafenib and dabrafenib are clinical compounds that have been approved for the treatment of late stage, non-resectable and disseminated melanomas harboring a \(BRAFV600\) mutation. Here we investigated the effects that PLX4720, an analogue of
vemurafenib, would have on \textit{BRAF}V600E melanoma cell lines. We observed that BRAF inhibition caused a significant reduction in proliferation corresponding to G1 arrest, p27 stabilization, decreased RB phosphorylation and reduced cyclin D1 expression. Although short treatment time-point (<1 hr) resulted in a significant reduction in ERK activity and the induction of apoptosis, longer time-points showed that phospho ERK levels were restored quite rapidly (<48 hrs) in the presence of drug with chronic treatment eventually leading to the emergence of PLX4720 resistant clones. The resistant clones were characterized by reactivation of the MAPK pathway independent of an acquisition of a MEK1 mutation leading us to postulate that the resistant clones would be sensitive to MEK inhibition. Indeed, treatment with the UO126 MEK inhibitor resulted in decreased proliferation as measured by BrdU incorporation. Further, the combination of PLX4720 and UO126 prevented rebound ERK activation, resulted in marked levels of apoptosis and completely prevented the emergence of resistant clones. This study provided strong pre-clinical support for the clinical trial evaluating the combination of BRAF and MEK inhibitors to delay or overcome MAPK mediated resistance. In January of 2014 the combination of Tanfinlar™ (dabrafenib) + Mekinist™ (trametinib) was FDA approved while the combination of Zelboraf™ (vemurafenib) plus cobimetinib is in late stage clinical evaluation (coBRIM) with promising results.

\textbf{Chapter 3}

In this study we separated \textit{BRAF}V600E melanomas into 2 cohorts based on PTEN status. This stratification allowed us to observe that loss of PTEN confers intrinsic resistance to the BRAF inhibitor, vemurafenib. Utilizing LC-MRM (liquid chromatography
multiple reaction monitoring) base proteomic analysis, we quantified 17 members of the Bcl-2 protein family and found that upregulation of the pro-apoptotic BIM protein was important for the induction of apoptosis in PTEN expressing cell lines. Mechanistically, BRAF inhibition in the PTEN null cohort led to increased AKT activity corresponding to nuclear exclusion of FOXO3a and decreased BIM expression. Combining vemurafenib with an inhibitor of PI3K blocked the increase in AKT activity and increased nuclear FOXO3a accumulation. This led to increased BIM expression and significant increases in apoptosis. The significance of PTEN status on therapeutic outcome was validated in a study showing that dabrafenib treated melanoma patients with PTEN loss had shorter progression free survival rates than those with WT PTEN \textsuperscript{347}. These findings highlight the importance of further molecular subgrouping of V600E melanomas and have provide preclinical support to combining BRAF with PI3K/AKT inhibitors and BRAF with Bcl-2 family inhibitors.

**Chapters 4 and 5**

A number of vemurafenib resistance mechanisms have been reported for melanoma including upregulated receptor tyrosine kinase signaling (HER3, IGF1R, PDGFRb, EGFR, and c-MET), secondary mutations (NRAS and MEK1), amplifications (COT, BRAF, cyclin D1), loss of tumor suppressor (PTEN, NF1) and truncated BRAF expression \textsuperscript{134,176,210,223,226}. Many of these mechanisms converge upon the MAPK pathway and therefore lead to reactivation of ERK signaling. Due to increased PI3K/AKT/mTOR pathway transduction, AKT phosphorylation has also been observed in a smaller cohort of BRAFV600E melanomas \textsuperscript{348}. The ability of resistant cells to circumvent MAPK inhibition by switching to alternate survival mechanisms suggests that
resistance can only be completely constrained through strategies that target the MAPK pathway along with other key and non-redundant pathways. In melanoma, HSP90 stabilizes a multitude of client proteins known to be essential for growth, survival, differentiation and DNA damage response making it an attractive therapeutic target. Here we demonstrated that treatment of single and dual agent MAPK inhibitor resistant melanoma cells with the HSP90 inhibitors, XL888 and AT13387, led to the rapid degradation or inhibition of key HSP90 client proteins and resulted in marked inductions of apoptosis. Importantly, the combination of HSP90 and BRAF inhibitors was able to significantly forestall resistance in an in vivo model of BRAFV600E melanoma. These studies demonstrate that HSP90 inhibition is an effective therapeutic approach for managing the diverse array of resistance mechanisms and provided the ongoing clinical evaluation of combined BRAF and BRAF + MEK inhibition.

Chapter 6

In this study we used mass spectrometry-based phosphoproteomic screening to uncover key adaptive changes that lead to vemurafenib resistance. Based on significant global changes in phosphorylation events between naive and vemurafenib resistant cell lines (treated with 3mM vemurafenib >3 months), we have mapped the resistance interactome. Our unbiased analysis revealed novel signaling hubs that have been reported to contribute to metastasis. Consistent with our bioinformatics prediction, melanoma cell lines with acquired vemurafenib resistance were considerably more invasive than their drug naïve counterparts and showed increased trans-endothelial cell migration. Importantly, we found that resistance to dual BRAF + MEK inhibition (treated with 1mM vemurafenib + 1mM selumetinib, >4 months) also led to increased migration
and invasion. This was a significant finding as mortalities associated with melanoma are due in large part to disseminated disease, with worst prognoses in patients with metastasis to distant sites such as the brain, liver and bone. Our analysis revealed ligand-independent EphA2 signaling as an adaptation to BRAF inhibitor therapy leading to the adoption of a metastatic phenotype. EphA2-mediated invasion was ligand-independent and AKT-dependent and was reversible upon the removal of MAPK inhibition. In patient derived xenograft models, where resistance was established in vivo, BRAF inhibition lead to the development of EphA2 positive metastases. A retrospective analysis of melanoma patients on BRAF inhibitor therapy showed that 68% of those failing therapy develop metastases at new disease sites, compared to 38% of patients on dacarbazine. IHC staining of melanoma specimens taken from patients on BRAF inhibitor therapy as well as metastatic samples taken from patients failing therapy showed increased EphA2 staining. Here we suggested that the sequential dosing of targeted kinase inhibitors in combination with an agonist of EphA2 signaling could limit metastases associated with kinase inhibitor therapy.

Future Perspectives

At the onset of the studies described in this dissertation, our lab was keenly aware of the potential use of systems level platforms. Our experience with studying escape from targeted therapies suggested that kinase inhibition leads to rapid adaptive responses and rewiring of signaling networks, but while holistic approaches were greatly needed, the existing focus had been on individual genes or proteins. We also understood that studies that relied solely on genome-based data did not always translate into meaningful phenotypic changes. Therefore our goal was to utilize
sensitive and selective tandem mass spectrometry based proteomic analyses to understand the network of post-translational modifications that lead to drug resistance in melanoma. Armed with an arsenal of kinase inhibitors, we embarked on a journey into the unknown world of computational biology. In an eager spirit of discovery we set up large batch experiments to be interrogated for global serine, threonine and tyrosine phosphorylation changes and our collaborators in the Moffitt Proteomics Core quickly rewarded us with a return of thousands of identified phospho-peptides. Unfortunately, the technology of high throughput proteomics superseded our ability to analyze such large datasets. It was only through multiple meetings with experts in the fields of proteomics, bioinformatics and biostatistics, and the integration of concepts that I learned from these meetings, that I was finally able to untangle the messy hairball that represents the BRAF resistance interactome.

This integrated approach enabled me to evaluate the global changes that occur following the onset of acquired resistance and allowed us to make the important observation that BRAF inhibition can lead to increased metastatic potential of an already disseminated disease. Further, this phenotype change was not due to “hard wiring” of the genome but instead arose because of post-translational modifications that would not have been observed with genetic analysis alone. Improvements in high-throughput mass spectrometry based proteomics have made them even more sensitive and this type of analysis can now accurately quantify tens of thousands of unique peptide modifications which can potentially lead to functional changes. It is expected that the continuation of such interdisciplinary collaborations, such as the ones
I described here, will allow us to broaden our understanding of treatment refractory melanoma and thereby enable us to improve upon therapeutic strategies.

The work that is hoped will be achieved through such collaborations is summarized in Figure 7.1. In this model, additional DNA classifications of \(\text{BRAF}^\text{V600}\) mutated melanomas would allow for the further stratification of patients who would be likely to respond to MAPK inhibitor therapy (i.e. genetically homogenous tumors) vs. those who would be non-responders (i.e. genetically heterogeneous tumors). In this scenario, patients whose melanomas harbor additional mutations that are known to cause intrinsic resistance (e.g. \(\text{PTEN}\), \(\text{NF1}\), \(\text{AKT3}\), \(\text{cyclin D1}\), \(\text{MAPK38}\) mutations) would receive additional therapies (based upon the presence of a specific molecular target) in combination with inhibitors of \(\text{BRAF}\). As targeted and immunotherapies doses would be limited by their combined toxicities, frontline combinations that inhibit as many targets at once while maintaining acceptable levels of side effects would be selected first. Studies from our group and others have provided evidence that intermittent \(^{224,339}\) or sequential dosing (data not shown) would also be viable dosing options that might prevent kinase addiction and lower toxicities.

Late stage melanomas often metastasize to distant sites that cannot be surgically resected making it difficult to follow the molecular course of disease progression. As such, the role of circulating tumor cells (CTCs), which are thought of as liquid tumor biopsies, is of growing interest in the field of melanoma. In this model, diagrammed in Figure 7.1, patients would be monitored at regular intervals during the course of treatment and biospecimens, such as CTCs, or residual tumor if available, would be collected for the detection of early modifications (e.g. protein phosphorylation
(proteomic platform), DNA methylation and histone acetylation/deacetylation) that would be predictive of adaptive phenotypic changes. In this early intervention scenario, the patient’s therapeutic regimen would be redesigned to optimally target emergent resistance targets. Additional genetic testing during the course of therapy would also enable the detection of treatment refractory tumor cells and allow for the earliest possible intervention. Though this is an idealized model involving biomarkers and technologies that do not exist at present, it is hoped that continued efforts to understand the intrinsic and acquired nature of resistance, such as the ones discussed here, will lead to discoveries that will allow us to achieve durable therapeutic outcomes for patients with disseminated melanoma.

Fig. 7.1. Diagram of rationally designed therapeutic strategies. Based upon genetic subclassifications, BRAF patients would receive further rational combinations in addition to BRAF and/or MEK inhibitors. During the course of treatment, patients would be monitored for the protein, epigenetic and genetic changes predictive of relapse and their therapies redirected accordingly.
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Appendices
Apendix A: Springer Publishing Copyright Permission

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