Chapter 1

Overcoming Resistance to BRAF and MEK Inhibitors by Simultaneous Suppression of CDK4

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Additional information is available at the end of the chapter

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1. Introduction

Melanoma is one of the most prevalent malignancies and has a very poor prognosis. Mutations in v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) occur in approximately 50% of melanomas [4]. While the response to selective BRAF inhibitors (BRAFi) in *BRAF*-mutant melanoma is encouraging, virtually all patients rapidly develop secondary resistance [6, 7]. Based on the finding that the mitogen activated protein kinase/ERK kinase (MEK)-extracellular signal regulated kinase (ERK) pathway is frequently reactivated by various BRAFi resistance mechanisms, a combination trial of a selective mutant BRAF inhibitor (dabrafenib, GSK2118436) with a MEK inhibitor (trametinib, GSK1120212) is underway and has achieved clinical responses in 17% and disease control in 67% in patients who failed prior single-agent treatment with a BRAF inhibitor [9]. While these results are promising, there is a critical need to overcome resistance to BRAF and MEK inhibitors. The clinical efficacy of BRAFi and MEKi therapy is believed to rely on a functional retinoblastoma (RB) axis to inhibit cell proliferation. The inhibitor of cyclin-dependent kinase 4A (*INK4A*) gene encode the p16 protein, a critical cell cycle regulator that interacts with cyclin dependent kinase (CDK) 4, inhibiting its ability to phosphorylate and inactivate RB [12, 13]. Genetic disruption of *INK4A* occurs in approximately 50% of melanomas irrespective of *BRAF* mutation and has been detected in melanoma cells that developed resistance to BRAFi. Of note, cyclin D is still expressed even in the setting of maximum tolerance dosing of BRAF inhibitor [7]. We have reported that combination of BRAFi or MEKi with the expression of wild-type *INK4A* or a CDK4 inhibitor (CDK4i) significantly suppresses growth and enhances apoptosis in melanoma cells [1-3]. Currently, CDK4 inhibitors are in active clinical development (http://clinicaltrials.gov/). Based on our previous work and recent insights into molecular mechanisms of resistance to BRAF and MEK inhibitors, we hypothesize that simultaneous suppression of

CDK4 is an effective strategy to overcome resistance to BRAF and MEK inhibitors. BRAF mutation assays have been used to guide treatment with BRAF and MEK inhibitors, development of sensitive and specific INK4A/p16 assays may serve as predictive biomarkers for treatment with CDK4 inhibitors.

2. Body

Constitutive activation of RAS-RAF-MEK-ERK signaling pathway in melanomas.*NRAS* and *BRAF* mutations were found respectively in 10-20% and 60-80% melanomas [4]. NRAS and BRAF are components of the RAS-RAF-mitogen activated protein kinase/ERK kinase (MEK)-extracellular signal regulated kinase (ERK) signaling pathway (Fig. 1) [5]. This signaling pathway plays an essential role in cell proliferation, differentiation and survival [5, 14, 15]. Constitutive activation of the ERK pathway has been shown to mediate the transforming activity of mutant BRAF in melanoma cells [16-18]. Suppression of mutant *BRAF* expression has been shown to inhibit ERK pathway activation and subsequent suppression of melanoma cell proliferation and survival *in vitro* and *in vivo* [19-21]. Our previous data revealed that the inhibition of mutant BRAF decreased levels of phospho-ERK (p-ERK), a marker of ERK pathway activation in melanoma cells [5, 14, 15].

The high frequency of *BRAF* mutation in melanomas makes it an ideal target for therapy. Because normal cells require wild-type *BRAF* for survival [22], specifically inhibiting mutant, but not wild-type *BRAF* in tumor cells could avoid toxic side effects generated by targeting normal cells. The finding that mutations in v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) occur in approximately 50% of melanomas led to extensive investigation of targeting BRAF for melanoma treatment, resulting in the first approved mutant specific BRAF inhibitor for treatment of advanced melanoma.

Combine BRAF and MEK inhibitors with chemotherapeutic agents. Intrinsic therapy resistance is a major limitation in the treatment of malignant melanomas. The mechanisms involved in the resistance of melanomas are largely unknown [23, 24]. It is believed that apoptosis and cytostasis (growth arrest/differentiation) are two of the main cellular responses to anticancer agents and loss of either process promotes treatment failure [25-27]. Activating *BRAF* mutations could drive cell proliferation and increase the cell death threshold through ERK pathway or alternative mechanisms [28-30], resulting in the blockage of both cytotoxic and cytostatic effects of therapeutic drugs [14, 31, 32]. It has been shown that inhibition of ERK pathway sensitizes melanoma cells to apoptosis induced by DNA damaging agents including cisplatin and ultra-violate (UV) irradiation [32, 33]. Rational combination of BRAF and MEK inhibitors with selective chemotherapeutic agents, for example, dacarbazine (DTIC), may generate additive/synergistic therapeutic effects.

ERK pathway activation and p16 in melanocytic lesions. Melanocytic lesions can be grouped into two main categories: nevi and melanomas. Nevi are divided into several different types based on histology. These include acquired melanocytic nevi, congenital melanocytic nevi, blue nevi, Spitz nevi, and dysplastic nevi. Melanoma can be further divided based on

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clinical and traditional histological methods as superficial spreading melanoma, lentigo maligna melanoma, acral lentiginous melanoma, and nodular melanoma. In early stages of melanomas, neoplastic cells are confined to the epidermis or with microinvasion into the dermis. In advanced melanomas, cancer cells expand in the dermis and generate tumor nodules and have a high potential for metastatic spread. In the metastatic phase, cancer cells disseminate to lymph nodes or distant organs [34, 35]. For the early diagnosed and localized melanomas, surgery is the choice of treatment. But there is currently no effective treatment for invasive and metastatic melanomas. Patients with late stage melanomas have a high mortality rate and life expectancy averages approximately 6-8 months after diagnosis.

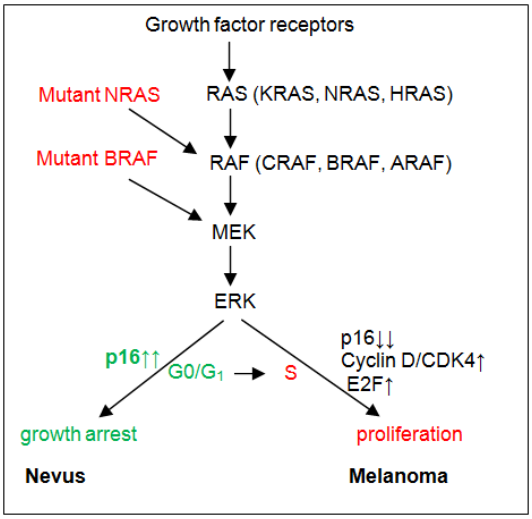

Figure 1. p16-cyclin D/CDK4 modifies the outcome of RAS/RAF/MEK/ERK signaling activation. RAF relays RAS signals through MEK to ERK. The activation of this pathway has multiple effects on cell proliferation, differentiation, and survival depending on the cellular contexts [5]. Constitutive activation of growth factor signaling pathways or NRAS and BRAF activating mutations can trigger over-expression of p16 leading to proliferative senescence, which manifest as benign nevus [10, 11]. Loss of p16 by genetic and epigenetic changes allows activation of cyclin D/CDK4 and inactivation of RB, leading to E2F activation, cell cycle progression from G1 to S phase, cell proliferation and tumor formation [12, 13]. Further genetic changes cause tumor progression to malignant melanoma. Of the three *RAS* and three *RAF* genes, *NRAS* and *BRAF* are mutated in melanoma [4].

Of note, in addition to melanomas, *BRAF* mutations are found at high frequencies (70-80%) in benign melanocytic nevi [36, 37]. There are a large numbers of melanocytic nevi in the general population compared to the relatively low incidence of melanomas [34, 35]. Clinically, it is known that nevi often regress over time. This suggests that BRAF mutations alone are insufficient to induce malignant transformation in nevus cells. The growth arrest of nevi is believed to result from oncogene-induced senescence, which is known as a protective mechanism against unlimited proliferation that could result from *BRAF* mutations and activation of the ERK signaling pathway (nevus in Fig. 1) [10, 11]. Tumor suppressor genes have been found to be involved in senescence process. For example, p16 is one tumor suppressor

found to be induced by ERK activation and telomere attrition involving cell senescence [8, 10, 11, 38]. The tumor suppressor p16 is encoded by *INK4A* (Fig. 2) and is often inactivated in a variety of human cancers, including 30-70% in melanomas [39, 40]. Most melanomas, but not nevi, have lost the expression of wild-type *INK4A*, either through DNA deletion/mutation or promoter hypermethylation [41-45]. It is possible that the loss-of-function of p16 in melanomas may make it possible to bypass the cellular senescence mechanism and function as an anti-tumor mechanism against ERK signal activation triggered by *NRAS* and *BRAF* oncogenic mutation (Fig. 1) [11, 46, 47].

Indirect evidence from cultured cells and animal models reveal that there may be a cooperative role between the constitutive activation of ERK pathway and the loss of p16 in tumor progression. Daniotti et al. [48] reported the co-existence of *BRAF* mutations and *INK4A* mutations/deletions/loss-of-expression in 26 of 41 (63%) short-term cell lines obtained from melanoma biopsies. Recent evidence suggests that growth arrest in benign nevi is due to cell senescence and that p16 at least partially contributes to the process of senescence in nevi [11, 46, 47].

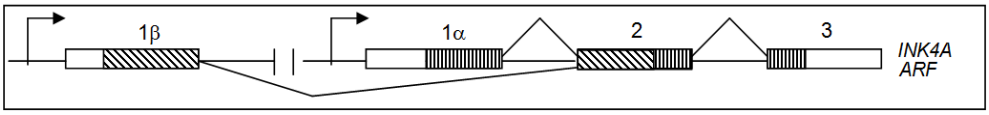

Figure 2. *INK4A* and *ARF* share sequences in the *CDKN2A* locus. Exons are shown as rectangles. Alternative first exons (1α and 1β) are transcribed from different promoters (arrows). Exons 1α and 1β are spliced to the same splicing acceptor site in exon 2 but are translated in alternative reading frames. *INK4A* coding sequences in exons 1α, 2, and 3 and *ARF* coding sequences in exons 1β and 2 are indicated by different shading patterns. Adopted from Sherr [8]. *INK4A* lesions detected by FISH and Sanger sequencing may also affect *ARF*.

Resistance of melanoma to BRAF and MEK inhibitors. The finding that mutations in *BRAF* occur in melanomas led to extensive investigation of targeting BRAF for melanoma treatment. While the response to selective mutant BRAF inhibitors (BRAFi) in *BRAF*-mutant melanoma is encouraging, virtually all patients rapidly develop secondary resistance. Based on the finding that the mitogen activated protein kinase/ERK kinase (MEK)-extracellular signal regulated kinase (ERK) pathway is frequently reactivated by various BRAFi resistance mechanisms, the first combination trial of a selective BRAF inhibitor (dabrafenib, GSK2118436) with a MEK inhibitor (trametinib, GSK1120212) is underway and has achieved clinical responses in 17% and disease control in 67% in patients who failed prior single-agent treatment with a BRAF inhibitor [9]. While these results are promising, again, the treatment response is short-lived; there is a critical need for additional strategies to overcome this deadly disease [49, 50].

There is evidence that treatment response to BRAFi and MEKi relies on a functional p16-cyclin D-CDK4-retinoblastoma (RB) axis. *INK4A* mutations/deletions occur in most of the melanoma cells that demonstrated resistance to BRAFi (e.g.; 451Lu, Mel1617, WM983, WM902, A375, M238, SKMEL28, and A2058) [51-57]. Over-expression of cyclin D and deletion of *RB* confer treatment resistance to BRAFi [56, 58]. Unlike other components of

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the p16-cyclin D-CDK4-RB axis that harbor genetic changes at low frequency in melanomas (e.g., *CDK4* and *RB* each approximately 3%) [59], and may not overlap with *BRAF* mutation (e.g., amplification of cyclin D1 gene *CCND1* and *CDK4*) [60], *INK4A* lesions are frequently detected in melanomas (~50%) irrespective of *BRAF* mutation [59-61]; therefore, abnormal p16 is a major mechanism of RB-axis attenuation in *BRAF*-mutant melanoma cells. p16 binds to and inhibits the catalytic activity of CDK4, representing a crucial gatekeeper at the G1>S checkpoint [62, 63]. The relative abundance of CDK4-cyclin D and p16 can determine the activity of the CDK4 kinase, thus regulate RB and cell-cycle progression [62, 63]. BRAF-MEK-ERK signaling pathway upregulates/activates the cyclin D-CDK4 enzyme, which phosphorylates and inactivates RB leading to cell cycle progression in melanoma cells; such an effect can be blocked by tumor suppressor p16 [2, 3, 61]. Several pathways that confer BRAFi resistance, including COT, RAF splicing variants, RAF dimerization, NRAS, IGF-1R, and RTK can reactivate cyclin D-CDK4 through signaling pathways including MEK-ERK as well as PI3K-AKT [51-53, 55, 56, 64]. Although the addition of MEKi to BRAFi may suppress reactivation of MEK-ERK-cyclin D-CDK4, alternative resistance mechanisms, including growth factor receptors and PI3K-AKT pathway can activate cyclin D-CDK4 [52, 55, 64-66] *in the absence of a functional p16*, adding CDK4 inhibitor may help overcoming resistance to BRAFi and MEKi (Fig. 3). *BRAF* mutation assays have been used to direct BRAFi treatment. There is significant genotypic heterogeneity of *INK4A* including bi- and mono-allelic deletions, nonsense and missense mutations, and also different levels of epigenetic modification by promoter hyper-methylation. Characterization of whether these *INK4A* changes correlate with different treatment resistance to BRAFi/MEKi/CDK4i may lead to companion molecular tests to better manage melanoma patients under BRAFi, MEKi, and CDK4i therapy.

As shown in Fig. 4, in addition to BRAF and MEK inhibitors, several drugs designed to inhibit the activity of CDK4 are in active clinical trials for melanoma and other cancers including LEE011 (Novartis Pharmaceuticals), LY2835219 (Eli Lilly and Company), PD-0332991 (Pfizer) (http://clinicaltrials.gov/).

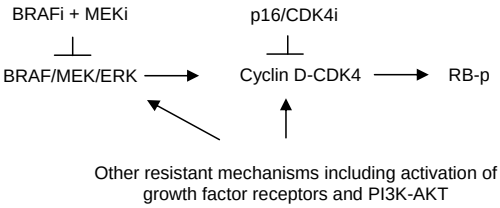

Figure 3. The presence of functional p16 may offset resistance mechanisms that lead to activation of cyclin D-CDK4 in melanomas that progressed under BRAFi/MEKi treatment, whereas abnormal p16 may predict treatment failure in melanomas that develop resistance mechanisms un-opposed by BRAFi + MEKi treatment.

Combined inhibition of CDK4 potentiate the effect of MEKi. In order to design better strategies for the treatment of this devastating disease a better understanding of melanoma biology is necessary. Multiple genetic and environmental factors have been linked to the de-

velopment and aggressive behavior of melanomas [49, 50]. *BRAF* mutations have been identified in approximately 60–80% of human melanomas, while *NRAS* mutations occur in about 10% of melanomas [4, 67]. Both NRAS and BRAF are components of the RAS-RAF-mitogen activated protein kinase/ERK kinase (MEK)-extracellular signal regulated kinase (ERK) signaling pathway. Apart from *NRAS* and *BRAF* mutation, other factors have been identified leading to constitutive activation of the ERK signaling, for example, amplification and somatic mutations of KIT and constitutive expression of hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) [49, 50]. ERK signaling pathway controls cell proliferation, differentiation, and survival, and has been shown to be a targetable pathway in melanoma treatment [5, 14, 15, 68].

Deregulation of the p16-cyclin D:cyclin-dependent kinases (CDK) 4/6-retinoblastoma (RB) pathway is a common paradigm in malignancy including melanoma [12, 13, 39] and represents another attractive target in melanoma treatment. The great majority of melanoma cells have lost or reduced expression of wild-type *INK4A* caused by genetic and epigenetic changes including mutation, deletion, and promoter hypermethylation [69, 70]. Loss of p16 leaves cyclin D:CDK4 unoppressed to phosphorylate and inactivate RB and cell cycle progression [8, 13, 49, 50, 69, 70]. Amplification of cyclin D1 and CDK4 genes have also been identified, mostly in melanomas that harbor wild-type *NRAS* and *BRAF* [58]. A germ-line Arg24Cys (R24C) mutation in CDK4 was identified in familial melanoma patients [40, 58]. This mutation abolishes CDK4 inhibition by p16 and thus is believed to be a functional equivalent to p16 loss. Both ERK signaling and CDK4 kinase have been shown to regulate RB protein and cell cycle progression [58, 61]. Activation of BRAF-MEK-ERK signaling pathway can cause upregulation of cyclin D resulting in the activation of CDK4 [61]. Activated CDK4 phosphorylates and inactivated RB proteins result in the liberation of E2F transcription factors and cell cycle progression. It has been shown that in advanced melanoma cells, RB is highly phosphorylated and inactive, and E2F transcriptional activity is constitutively high ([5, 12].

Various resistance mechanisms have been identified that contribute to treatment failure of melanoma patients to BRAFi and MEKi therapy. Loss of p16 may represent a common gateway permitting the phenotypic expression of several resistance mechanisms to BRAFi and MEKi (Figs. 1 and 3), a hypothesis that has not been and is waiting to be tested in clinical trials. We reported that simultaneous expression of *BRAF* siRNA and *INK4A* cDNA in melanoma cells leads to dramatically increased apoptosis (17), suggesting that correcting the two most common genetic lesions could be effective in melanoma treatment. It is unclear whether the effect is specific to *BRAF* and *INK4A* or can be generalized to other components of the ERK and RB pathways. It has been shown that *BRAF* and *INK4A* may have activities independent of the corresponding canonical ERK and RB pathways, and the two pathways also mediate cellular signals independent of aberrant *BRAF* and *INK4A*. For example, RAF can act through apoptosis signal-regulating kinase-1 (ASK1)/c-Jun-NH2-kinase or mammalian sterile 20- like-kinase 2 (MST2) pathways ([71]; cyclin D:CDK4 can be activated by enhanced phosphatidylinositol 3-kinase (PI3K) and wingless (WNT) signaling pathways in melano-

mas [27, 72]. Therefore, we tested PD98059 and 219476, commercially available inhibitors of MEK and CDK4, respectively, in human melanoma cells.

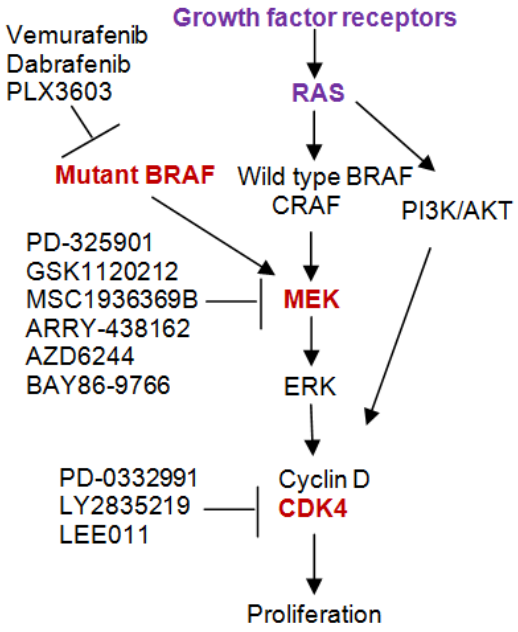

Figure 4. BRAF, MEK and CDK4 inhibitors are in active clinical development and may be used in combination to increase treatment efficacy. Melanoma cells acquire resistance to BRAF and MEK inhibitors by mechanisms including activation of growth factor receptors and RAS signaling pathways. Activation of growth factor receptors and RAS pathways can cause overexpression of cyclin D and activation CDK4 kinase, leading to cell cycle proliferation, which is believed to play major roles in the emergence of treatment resistance. Adding CDK4 inhibitors may overcome resistance to treatment targeting BRAF and MEK. Apart from Vemurafenib (PLX4032, RO5185426) (Hoffmann-La Roche) that has been U.S. Food and Drug Administration (FDA) approved for treatment of melanoma, other mutant BRAF inhibitors including PLX3603 (RO5212054) (Hoffmann-La Roche) and GSK2118436 (dabrafenib) (GlaxoSmithKline) are in active clinical trials. There are clinical trials of MEK inhibitors PD-325901 (Pfizer), GSK1120212 (GlaxoSmithKline), MSC1936369B (EMD Serono), ARRY-438162 (MEK162) (Array BioPharma), AZD6244 (AstraZeneca), and BAY86-9766 (Bayer). Several drugs designed to inhibit the activity of CDK4 are also in active clinical trials for melanoma and other cancers including PD-0332991 (Pfizer), LY2835219 (Eli Lilly and Company), LEE011 (Novartis Pharmaceuticals) (http://clinicaltrials.gov/).

MEK inhibitor PD98059 (Calbiochem, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO) as a 50 mM stock solution, aliquoted and stored at -20C. CDK4 inhibitor 219476 (Cat. # 219476, Calbiochem, San Diego, CA) was dissolved in DMSO as a 2 mM stock solution and stored at 4C. Human melanoma cell lines 624Mel, A101D, and OM431 were kindly provided by Dr. Stuart Aaronson (Mount Sinai School of Medicine, New York, NY). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and 50 units/mL

penicillin–streptomycin (Invitrogen, Carlsbad, CA) in a humidified incubator at 37C with 5% CO2. CellTiter 96® R AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega Corporation, Madison, WI) was used to measure dehydrogenase enzyme activity found in metabolically active cells. Melanoma cells were seeded in a 96 well plate at a density of 2 $\times 10^4$ cells/well in DMEM with 5% FBS. On the second day, the culture medium in each well was changed to 150 μL DMEM without phenol red and supplemented with 0.5% FBS. Cells were treated in triplicate for 24 and 48 hr with either vehicle solvent (control), 25 μM PD98059, 1 μM 219476, or their combination for 624Mel; control solvent, 50 μM PD98059, 1 μM 219476, or their combination for A101D; and control solvent, 50 μM PD98059, 2 μM 219476, or their combination for OM431 cells. CellTiter 96® AQueous One Solution Reagent (30 μL) was then added per well and cell cultures were returned to the incubator for another 4 hr. Subsequently, the absorbance of each well was measured at 450 nm with a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The absorbance of the well with only medium and CellTiter 96® AQueous One Solution Regent was background and subtracted from each sample well. The average and standard deviation of three wells with the same treatment were calculated.

Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling of DNA fragments (TUNEL) method using *in situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science, Indianapolis, IN). Melanoma cells were seeded in triplicate in a 6 well plate at a density of 2×10^5 cells/well in DMEM with 5% FBS and antibiotics. On the second day, cells were treated with PD98059 and 219476 under the same conditions as the MTS assay. After treatment with the respective chemicals for 48 hr, cells were harvested to detect apoptotic cells using the TUNEL assay according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Using a cytospin, cells were placed onto Polysine glass slides (Fisher Scientific, Fair lawn, NJ), fixed in 4% paraformaldehyde (Fisher Scientific, Fair lawn, NJ) at room temperature for 1 hr, then permeabilized with a fresh prepared mixture of 0.1% Triton X-100 (MP Biomedicals, Inc. Solon, OH) and 0.1% sodium citrate (Fisher Scientific, Fair lawn, NJ) for 5 min at room temperature. Slides were rinsed with phosphate buffered saline (PBS), air dried, and incubated with 50 μL of TUNEL reaction mixture, containing terminal deoxynucleotidyl transferase (TdT)- and fluorescein isothiocyanate (FITC)-labeled dUTP, in a dark humidified atmosphere at 37C for 2 hr. For nuclei counterstaining, slides were cover-slipped with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Fluoresce positive cells were viewed with a Nikon Eclipse TE 2000-U inverted microscope (Nikon Corp., Tokyo, Japan) equipped with a FITC filter and a DAPI filter. The percentage of apoptotic cells was determined for each sample in a blind fashion by counting the number of green fluorescent nuclei (TUNEL positive) among a total of 300 or more 4'-6-diamidino-2-phenylindole (DAPI)-stained blue nuclei in three random fields at magnification of 20/0.5 (objective) as described previously [1-3].

For Western blotting, 1×10^6 melanoma cells were seeded in a cell culture dish (10 cm) in DMEM containing 5% FBS and antibiotics. On the second day, cells were treated with PD98059 and 219476 at the same concentration as described in the MTS assay. For cell cycle

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regulators cyclin-dependent kinase inhibitor p27 kinase interacting protein 1 (KIP1) and RB, cells were treated with the chemicals in medium with 5% FBS for 24 hr and then harvested. For apoptosis-related protein B-cell chronic lymphocytic leukemia (CLL)/lymphoma 2 (BCL2), BCL2-like 1 (BCL2L1 or bcl-xL), inhibitor of apoptosis family (IAP) protein baculoviral IAP repeat-containing 5 (BIRC5 or survivin), apoptosis facilitator BCL2 interacting mediator (BIM), cysteine-aspartic acid protease (caspase) 3, and poly (ADP-ribose) polymerase (PARP), cells were treated with the various chemicals in DMEM with 5% FBS for 48 hr and then harvested. For phospho- and total-ERK, cells were treated with the chemicals in medium with 0.5% FBS for 18 hr and then harvested. Western blots were performed as described [1-3]. Briefly, harvested cells were lysed in Lysis Solution (Cell Signaling, Danvers, MA) supplemented with Complete Mini Protease Inhibitor Cocktail Tablets (Roche Diagnostics Corporation, Indianapolis, IN). Protein concentration of lysates was determined using the Quick Start Bradford 1 × Dye Reagent (Bio-Rad, Hercules, CA). Lysates were separated in either 12 or 15% SDS-polyacrylamide gel, electrophoretically transferred to Immobilon-P membrane (Millipore Corp, Billerica, MA), and probed with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The following antibodies were used: BCL2 and tubulin, beta (Sigma-Aldrich, St. Louis, MO); BCL2L1 and BIRC5 (Santa Cruz Biotechnology, Santa Cruz, CA); phosphor-ERK, total ERK, Caspase 3, PARP, and PhosphoPlus(R) RB (Ser780, Ser795, Ser807/811) Antibody Kit (Cell Signaling, Boston, MA); p27KIP1 (BD Biosciences, San Jose, CA); and peroxidase-conjugated antimouse and antirabbit secondary antibodies (Calbiochem, San Diego, CA). Immunoreactive bands were visualized with SuperSignal chemiluminescence substrate (Pierce, Rockford, IL). The blots were exposed to blue sensitive blue X-ray film (Phenix Research, Candler, NC) [1-3].

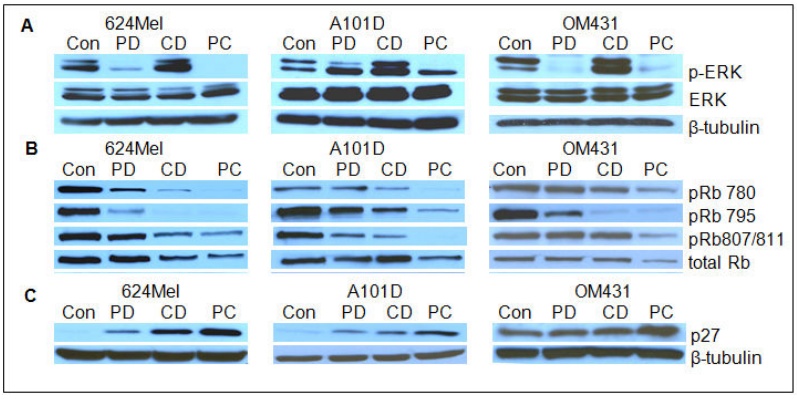

Figure 5. Regulation of ERK phosphorylation, RB phosphorylation, and p27KIP1 expression by PD98059 and 219476, alone and in combination. Human melanoma cell lines 624Mel, A101D, and OM431 were treated with either vehicle solvent (Con), PD98059 (PD), 219476 (CD), or PD98059 plus 219476 (PC) as described in Materials and methods. Western blot was performed using 20 µg total cell lysates, tubulin was used as loading control, as described previously [1-3].

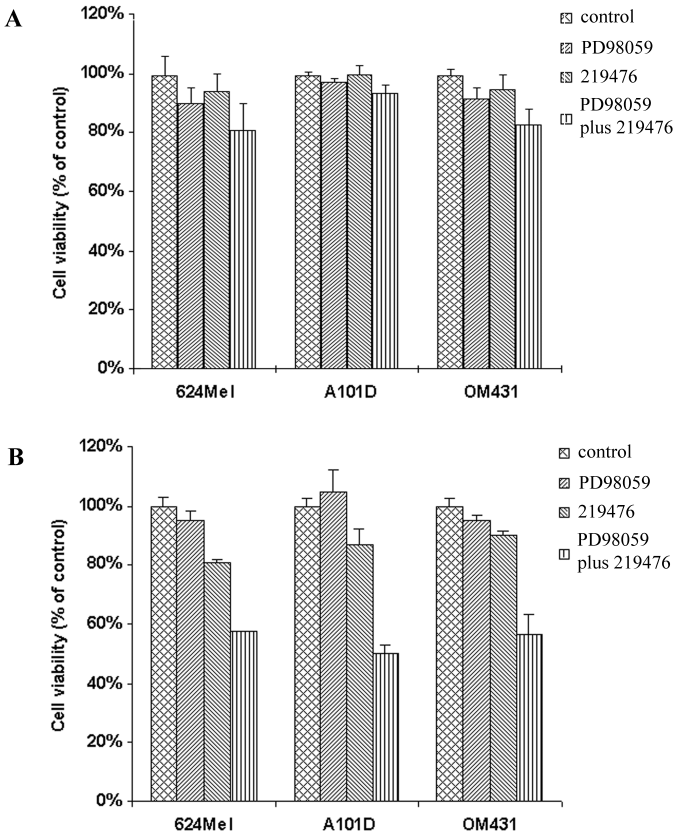

Figure 6. Cytotoxicity by PD98059, 219476, and combinatorial treatment. MTS cytotoxicity assay was performed in 624Mel, A101D, and OM431 cells after (A) 24-hr and (B) 48-hr treatment in medium supplemented with 0.5% FBS. The results are given as means ± SD from three independent tests, as described previously [1-3].

We have shown previously that human melanoma cell lines 624Mel, A101D, and OM431 cell lines harbor heterozygous *BRAF* T1799A mutation and loss of wild-type *INK4A* [1, 61]. Cells were treated, alone or in combination, with MEK inhibitor PD98059 (22) and CDK4 inhibitor 219476 (23). As anticipated, ERK phosphorylation was reduced in cells treated with PD98059, and PD98059 plus 219476 (Fig. 4A). Phosphorylation of S780, S795, and S807/811 of RB, known cyclin D:CDK4 and cyclin E:cyclin dependent kinase 2 (CDK2) targets (7),was decreased in cells treated with either PD98059 or 219476 (except S780 and S807/811 in OM431 cells), and further reduced in cells with combinatorial treatment (Fig. 4B). Of note, total RB was decreased under combinatorial treatment with PD98059 and 219476 in all three melanoma cells (Fig. 4B). Levels of p27KIP1, a negative regulator of cyclin E:CDK2, were increased in cells treated with either PD98059 or 219476, and further increased in cells with combinatorial treatment (Fig. 4C).

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PD98059 and 219476 inhibit tumor cell growth in a dose dependent manner [1, 2]. In order to make it possible to monitor the additional therapeutic effects of the combinatorial treatment, both chemicals were used at dosages lower than that which would lead to maximal effect by either agent. The cytotoxicity of PD98059 and 219476 was examined 24 and 48 hr after treatment using the MTS assay that measures the dehydrogenase enzyme activity found in metabolically active cells. After 24-hr treatment, there was no significant difference in cell viability between control, single, and combined treatment groups of 624Mel cells (p = .05, R-square 0.57320, ANOVA). Small but significant differences were observed in A101D and OM431 cells (p = .05, R-square 0.7136 and 0.8091 in A101D and OM431 cells, respectively, ANOVA); the differences were between the combined treatment vs. control and PD98059 in A101D cells, and between the combined treatment vs. control and single treatment of either PD98059 or 219476 in OM431 cells (Figure 2(a), HSD Test at 0.05 significance level). After 48-hr treatment, a significant difference in MTS counts existed for the control, PD98059, 219476, and PD98059 plus 219476 groups in all the three cell lines (p <.0001, R-square 0.981444, 0.956956, and 0.991102 in 624Mel, A101D, and OM431, respectively, ANOVA). Further analysis showed that simultaneous treatment with PD98059 and 219476 after 48-hr treatment resulted in significantly reduced numbers of cell survival than control-treatment or monotreatment as measured by MTS in all the three cell lines (Fig. 6B, HSD Test at 0.05 significance level).

Next, we performed the TUNEL DNA fragmentation assay to identify loss of viability due to programmed cell death after 48-hr treatment. As shown in Figure 3, at the drug concentrations used, significantly different levels of apoptosis exist among control for PD98059, 219476, and combinatorial treatment groups (p < .0001, R-square 0.973862, 0.990697, and 0.987900 in 624Mel, A101D, and OM431, respectively, ANOVA). Treatment with PD98059 alone resulted in no difference in apoptosis over controls in all three cell lines; 219476 enhanced apoptosis in OM431 but not in the other two cell lines; However, combined treatment dramatically increased apoptosis over that seen for the control-treatment and monotreatment (Fig. 7. HSD Test at 0.05 significance level).

As apoptosis was the major effect observed when melanoma cells were exposed simultaneously to MEK and CDK4 inhibitors, we examined the expression of several pro-apoptotic and anti-apoptotic proteins. Mono-treatment with PD98059 or 219476 caused a decreased or no change in the expression of anti-apoptotic proteins BCL2, BCL2L1, and BIRC5. While there were variations in the patterns of expression of BCL2, BCL2L1, and BIRC5 among the different cell lines (Fig. 8), combinatorial treatment caused a comprehensive down-regulation of the proteins in all three cell lines (Fig. 8). In addition, apoptosis facilitator BIM-EL was increased following treatment with PD98059 and PD98059 plus 219476 in all three cell lines. It was also increased in OM431 cells following treatment with 219476. Consistent with increased apoptosis, caspase 3 was activated by simultaneous treatment with PD98059 plus 219476 in all three cell lines, as shown by decreased procaspase 3, increased levels of the active form of caspase 3 (cleaved caspase 3), and degradation of PARP, a direct substrate of active caspase 3 (Fig. 8).

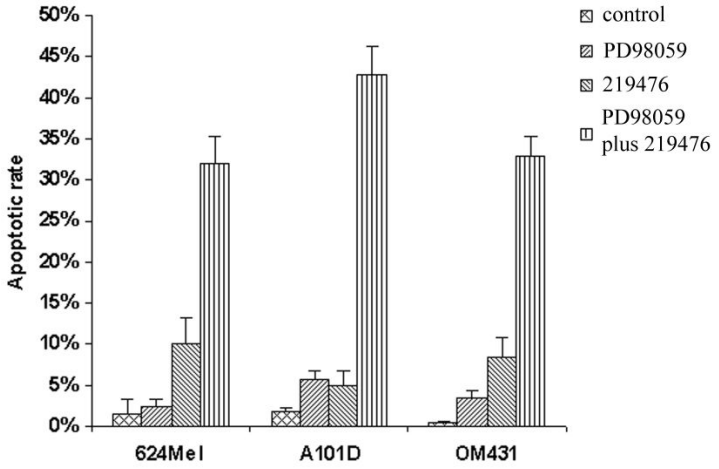

Figure 7. MEK and CDK4 inhibitors induce apoptosis of melanoma cells. TUNEL Assay was performed in 624Mel, A101D and OM431 cells after 48h treatment with vehicle solvent, PD98059, 219476, or PD98059 plus 219476 in medium with 0.5% FBS. The results were given as means ± SD from three independent assays, as described previously [1, 2].

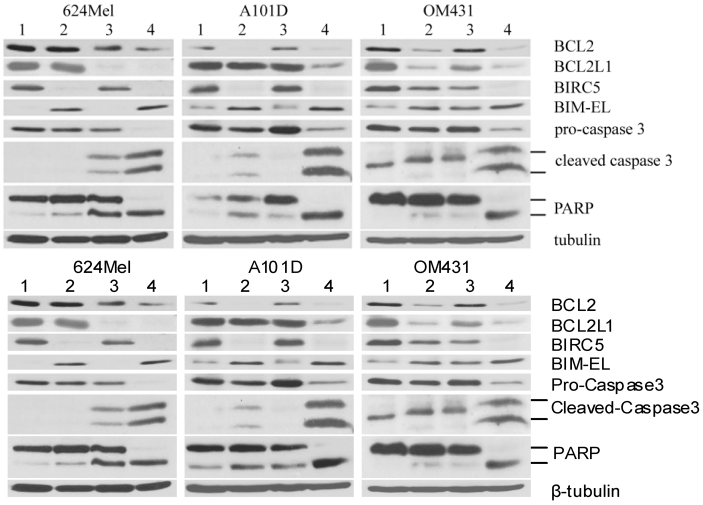

Figure 8. Changes in the expression of pro-survival and pro-apoptotic proteins. Cells were treated with solvent vehicle control (1), PD98059 (2), 219476 (3), and PD98059 plus 219476 (4) for 48 h in medium containing 5% FBS. Western blotting of 20 μg total cell extracts from 624Mel, A101D and OM431 cells using BCL2, BCL2L1, BIRC5, BIM, caspase-3, and PARP antibodies; tubulin was used as loading control, as described previously [1, 2].

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In this study, we simultaneously inhibited MEK and CDK4 kinases using pharmacological inhibitors PD98059 and 219476 and observed significantly increased apoptosis compared to control and single agent treatment. This is consistent with our previous report that simultaneous knockdown of BRAF using small interfering RNA (siRNA) and expression of *INK4A* cDNA in melanoma cells leads to a significant increase in apoptosis [1, 3]. These results demonstrate that an increase in apoptosis can be achieved through combinatorial targeting of ERK and RB pathways. It has been well established that constitutive activation of the ERK signaling induces the expression of cyclin D [1, 2, 61], which binds to and activates CDK4 leading to the phosphorylation of RB protein facilitating cell cycle entry [1, 2, 61]. Consistent with an epistatic regulation between ERK pathway and cyclin D:CDK4, amplification of cyclin D1, and CDK4 genes have been identified mainly in melanomas that harbor wild-type *NRAS* and *BRAF* [58, 60]. Additionally, cyclin D:CDK4 mediates resistance to inhibitors of the ERK signaling pathway [58]. Therefore, the enhanced apoptosis and decreased proliferation by simultaneously inhibiting ERK and RB pathways could result from the double hitting of ERK-cyclin D:CDK4-RB that regulate cell cycle progression and cell survival. Alternatively, in support of our previous results that *BRAF* and *INK4A* have a nonlinear functional interaction [1, 61], additional cellular processes could be affected when cells are exposed to both PD98059 and 219476. ERK pathway has pleiopotent activities that regulate cell proliferation, survival, and differentiation through both cyclin D:CDK4 dependent and independent routes [5, 61]. Likewise, cyclin D:CDK4 can be regulated and converges multiple cellular signals. For example, while PI3K signaling can activate CDK4 through downregulation of *INK4A* and upregulation of cyclin D [73], WNT signaling can turn on CDK4 through suppression of *INK4A* transcription [72], It is conceivable that inhibition of MEK and CDK4 not only affects ERK and RB pathways, but also PI3K, WNT, and other ERK signaling activities not mediated through the RB pathway. Therefore, simultaneous targeting of both ERK and RB pathways can generate enhanced effects by targeting both linear and non-overlapping activities.

Apoptosis resistance is a critical factor for therapy failure in melanoma patients. Encouragingly, combined treatment with PD98059 and 219476 leads to significant apoptosis in all the three melanoma cell lines studied (Fig. 7). The apoptotic rate caused by the combined treatment is higher than the combined apoptosis by monotreatment, suggesting that MEK and CDK4 kinases mediate each other's pro-survival effect. The apoptotic effect is associated with changes of apoptosis-related proteins (Fig. 8). PD98059 and 219476 combined treatment leads to significant down-regulation of the pro-survival proteins BCL2, BCL2L1, and BIRC5, and up-regulation of the pro-apoptotic protein BIM. We showed previously that BCL2 and BIM were regulated by *BRAF* and *INK4A* [1, 61]. BCL2L1 and BIRC5 are highly expressed in melanoma cells, and increased expression correlates with tumor progression [74, 75]. A straightforward explanation for the observed apoptosis is that the changes in the pro-apoptotic and anti-apoptotic factors offset the balance and lead to apoptosis [1]. Sequencing analysis of TP53 cDNA [1, 3] showed that 624Mel and OM431cells respectively harbor a T1076G (Cys275Trp) and a G1048A (Gly266Glu) mutations in the DNA binding domain that is likely to compromise the transcription and apoptosis function of p53 [76]. No TP53 mutation has been detected in A101D cells. Although apoptosis is enhanced in all the three cell lines, it is

more pronounced in A101D than 624Mel and OM431 cells (Fig. 7), suggesting that TP53 status may influence the magnitude of apoptosis. Combinatorial-treated cells have further inhibited phosphorylation of ERK and RB, reduced total RB, and increased expression of p27KIP1 (Fig. 5). We observed similar effects on ERK and p27KIP1 in a previous report of simultaneous expression of *BRAF* siRNA, and *INK4A* cDNA in melanoma cells [1, 3]. Yu et al. demonstrated that loss of Rb causes apoptosis without effect on cell proliferation [77], and Wang et al. found that overexpression of p27KIP1 leads to apoptosis in melanoma cells [78]. The mechanisms of these changes in relationship to each other and to the observed cooperative effects need to be further investigated. To our knowledge, this study is the first to demonstrate that combined inhibition of MEK and CDK4 using pharmacological inhibitors can cooperate to trigger significant apoptosis in melanoma cells. Deregulation of the RAS-RAF-MEK-ERK and p16-cycylin D:CDK4-RB pathways are common in human malignancies and appears to be important for melanoma development. There has been significant effort to target components of these pathways in cancer treatment. Pharmacologic agents targeting components of the ERK and RB pathways have been developed. However, clinical studies as monotherapy showed that the clinical responses have failed expectations and maximum tolerated doses are often reached before reaching clinical efficacy. Our current study further reinforces the notion that combination targeting of ERK and RB pathways is a promising strategy for melanoma treatment and should encourage further in-depth investigations.

Development of biomarkers to predict treatment response to BRAF, MEK, and CDK4 inhibitors. Apart from *BRAF* mutation, there is no other validated molecular assay to direct BRAFi and MEKi treatment. Comprehensive and standardized *INK4A* molecular assays have not been established in the context of BRAFi and MEKi treatment. Technical and clinical validation of *INK4A* molecular assays may lead to the clinical use of new molecular companion biomarkers to accurately predict clinical response to BRAF and MEK inhibitors, and may also direct future combination treatment that includes CDK4 inhibitors for metastatic melanoma. Because CDK4 is important in both normal and cancerous cells, CDK4 inhibitors are expected to decrease the ability of the bone marrow to make white blood cells, platelets, and red blood cells. Although these effects are expected to be reversible, they can increase the risk of infection, bleeding and fatigue. Like BRAF inhibitors, these drugs are also expected to be expensive. Therefore, development of predictive molecular markers, as in the case of *BRAF* mutation assay for BRAFi, should help selecting patients that are likely to response to the treatment, therefore to maximize efficacy and avoid unnecessary side-effect and treatment cost [79, 80].

Genetic and epigenetic changes of *INK4A* have been identified in 30-70% of melanomas irrespective of *BRAF* mutation [59, 70, 81]. Bi-allelic deletion of *INK4A* (p16 null) occurs in 10-27% of melanomas [60, 82]. Other changes include mono-allelic deletion, point mutation, or promoter hypermethylation, resulting in various levels of p16 expression/activity (Table 1) [57, 60, 81-83]. It is believed that the acquisition of p16 lesions allows melanoma cells to bypass senescence/growth arrest during melanoma development [84]. Although preliminary results with combination therapy of BRAFi and MEKi are encouraging with better clinical response over single agent BRAFi treatment [9], levels of treatment responses vary under

the combination treatment [9]. We hypothesize that clinical response to combination therapy of BRAFi and MEKi correlates with status of *INK4A*/p16 (Table 2). The development of clinically useful *INK4A* assays requires an understanding of the underlying biology and access to technology that allows high quality assay performance. Recent advances in molecular technology enable accurate, rapid, and cost-effective *INK4A* molecular testing that can be performed routinely on tumor specimens. However, validation of the technical performance characteristics of *INK4A* assays and understanding of assay limitations are necessary for the accurate interpretation of test results.

INK4A status	**p16 protein sequence and expression**
Wild-type	Normal sequence
Various mutations	Heterogeneous sequence changes
Bi-allelic deletion	Protein null
Promoter hypermethylation	Lower levels of p16

Table 1. Heterogeneity of *INK4A* and p16 in melanoma specimens

As examples, Table 2 is a list of molecular assays to comprehensively examine *INK4A*/p16 lesions in melanoma specimens. Technical and clinical validation studies are necessary before the routine use of these assays in the clinic.

Test	Method	Reference
INK4A deletion	fluorescent *in situ* hybridization (FISH) (p16 SpectrumOrange/ chromosome 9 centromeric probe (CEP9) SpectrumGreen Probe, Abbott Molecular, Des Plaines, IL)	[85, 86]
INK4A mutation	Sanger sequencing	[86, 87]
INK4A promoter methylation	Pyrosequencing (PyroMark Q24 CpG p16 Kit, Qiagen, Valencia, CA)	[82, 88, 89]
p16 expression	Immunohistochemical staining (IHC)	[90, 91]

Table 2. Summary of molecular assays

These assays need to be validated both technically and clinically with defined cut-off values. There should be correlation of results among assay methods; for example, cells with bi-allelic *INK4A* deletion show negative p16 IHC staining and cells with mono-allelic *INK4A* deletion show mutations with loss of heterozygosity (LOH), and p16 expression inversely correlates with levels of *INK4A* promoter methylation. The major obstacles in testing tumor specimens are the presence of non-tumor cells in the samples, the cellular heterogeneity within tumor specimens, and degradation/damage of nucleic acid and protein during sample processing. To ensure accurate testing results, SOPs need to be established with clearly

defined instructions on the selection and handling of tumor specimens. For example, FISH assay requires fixation time between 6-48 hrs [92]. Alterations in *INK4A* may also affect the overlapping *ARF* gene (Fig. 2). Although the proposed study focuses on *INK4A*, changes in *INK4A* may also affect *ARF*, which may also be analyzed. Assay clinical sensitivity, clinical specificity, positive predictive value, and negative predictive value of *INK4A* biomarkers for a given treatment response can be calculated as described in Table 4.

INK4A result	**Treatment resistant case**	**Treatment sensitive case**	
Lesion +ve	A	B	Positive predictive value = A / (A + B)
Lesion -ve	C	D	Negative predictive value = D / (C + D)
	Sensitivity = A / (A + C)	Specificity = D / (B + D)	

Table 3. Calculation of clinical sensitivity, clinical specificity and predictive values

3. Conclusion

Patients with metastatic melanoma have a median survival of 6-8 months [93]. Recently, ipilimumab (Yervoy, Bristol-Myers Squibb), an inhibitor of cytotoxic T-lymphocyte antigen 4 (CTLA-4) and vemurafenib (PLX4032, Zelboraf, Plexxikon/Roche), an inhibitor of mutant BRAF, gained FDA approval to treat patients with metastatic melanoma. Although both drugs offer new approaches to the treatment of advanced melanoma, their therapeutic efficacy is limited. Both drugs typically lengthen life by only several months in patients that initially responded to the treatment [94, 95]. There is mounting evidence that acquired resistance to BRAFi frequently correlates with reactivation of the RAS-RAF-MEK-ERK signaling pathway [52, 53, 64]. This finding led to clinical trials combining BRAFi and MEKi in patients with *BRAF*-mutant metastatic melanoma who progressed on a prior BRAFi treatment regimen [94]. Dabrafenib (GSK2118436, GlaxoSmithKline) is a potent and selective inhibitor of mutant BRAF and is comparable in safety and efficacy to vemurafenib. In phase I testing, it achieved a 67% response rate in metastatic melanoma patients with BRAF V600 mutations [96]. Trametinib (GSK1120212, GlaxoSmithKline) is a potent and selective inhibitor of MEK1/2, achieved a clinical response of 40% in patients with an activating *BRAF* mutation in phase I study [97]. A multicenter phase I/II trial of combined treatment with dabrafenib and trametinib demonstrated a disease control rate of 67% (12/18) in patients who failed prior single-agent treatment with a BRAFi [9]. We hypothesize that although reactivation of MEK-ERK-cyclin D-CDK4 in tumors previously treatment with BRAFi may be suppressed by the combination of dabrafenib and trametinib, cyclin D-CDK4 can also be reactivated by alternative resistance mechanisms that cannot be suppressed by the addition of MEKi (e.g.; activation of growth factor receptor and PI3K-AKT pathway) [51-53, 55, 56, 65, 66], if unopposed by p16, can lead to resistance to the BRAFi and MEKi combination therapy (Fig. 1). It has been shown that melanoma cells that harbor abnormal *INK4A* are more sensitive than *INK4A* wild-type cells to the growth inhibitory effect of a p16-mimicking pep-

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tide [98] or of flavopiridol, a pan-CDK inhibitor [99], and combination of BRAFi or MEKi with the expression of wild-type *INK4A* or a CDK4 inhibitor significantly suppresses growth and enhances apoptosis in melanoma cells [2, 3]. Therefore, melanoma combination treatments that include CDK4 inhibitors may overcome treatment resistance and enhance efficacy. There is a critical need to identify predictive markers for therapies not only to improve treatment outcomes, but to help avoid ineffective toxic therapies, also because of the likely high cost of combination regimens. Like *BRAF* mutation assay, testing of *INK4A*-p16 may predict which patients will response to BRAF, MEK, and CDK4 inhibitors. Therefore, *INK4A* biomarkers may also have great potential to guide future melanoma combination treatments that include CDK4 inhibitors.

Nomenclature

ASK1: apoptosis signal-regulating kinase-1

ARF: alternative open reading frame

BCL2: B-cell chronic lymphocytic leukemia/lymphoma 2

BCL2L1: BCL2-like 1

BIM: BCL2 interacting mediator

BIRC5: baculoviral IAP repeat-containing 5, also known as survivin

BRAF: v-raf murine sarcoma viral oncogene homolog B1

BRAFi: BRAF inhibitor

Caspase: cysteine-aspartic acid protease

CDK2: cyclin-dependent kinase 2

CDK4: cyclin-dependent kinase 4

CDK4i: CDK4 inhibitor

CEP9: chromosome 9 centromeric probe

CLL: chronic lymphocytic leukemia

DAPI: 4'-6-diamidino-2-phenylindole

DMEM: Dulbecco's modified Eagle medium

DMSO: dimethyl sulfoxide

DTIC: dacarbazine

ERK: extracellular-signal-regulated kinase

FBS: fetal bovine serum

FDA: Food and Drug Administration

FGF: fibroblast growth factor

FISH: fluorescent *in situ* hybridization

FITC: fluorescein isothiocyanate

HGF: hepatocyte growth factor

IAP: inhibitor of apoptosis family

IHC: immunohistochemical staining

INK4A: inhibitor of cyclin-dependent kinase 4A; part of cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*), also known as multiple tumor suppressor 1 (*MTS1*)

KIP1: kinase interacting protein 1

LOH: loss of heterozygosity

MEK: mitogen-activated protein kinase/ERK kinase

MEKi: MEK inhibitor

MST2: sterile 20- like-kinase 2

PAGE: polyacrylamide gel electrophoresis

PARP: poly (ADP-ribose) polymerase

PBS: phosphate buffered saline

PI3K: phosphatidylinositol 3-kinase

p-ERK: phopho-ERK

RAF: v-raf murine sarcoma viral oncogene homolog. Human has three RAF: CRAF, BRAF, and ARAF

RAS: rat sarcoma viral oncogene homolog. Human has three RAS: HRAS, NRAS, and KRAS (KRAS4A and KRAS4B proteins arise from alternative splicing)

RB: retinoblastoma proteins including pRB, p107, and p103

SDS: sodium dodecyl sulfate

siRNA: small interfering RNA

TdT: terminal deoxynucleotidyl transferase

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

UV: ultra violate

WNT: wingless

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