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CONTRACTING ORGANIZATION: University of Cincinnati

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14. ABSTRACT Reactive oxygen species (ROS) levels are elevated after acquisition of resistance to v-raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitors including dabrafenib and MEK inhibitors such as trametinib in BRAF-mutant melanoma. To circumvent toxicity to PI-103 (a pan PI3K inhibitor), we utilized a novel ROS-induced drug release (RIDR)-PI-103, with a self-cyclizing moiety linked to PI-103. Under high ROS conditions, RIDR-PI-103 releases PI-103, which inhibits conversion of phosphatidylinositol 4,5-bisphosphate (PIP ₂) to phosphatidylinositol 3,4,5-triphosphate (PIP ₃). Previous findings demonstrate that trametinib and dabrafenib resistant (TDR) cells maintain p-Akt levels compared to parental counterparts and have significantly higher ROS. This is a rationale to explore the efficacy RIDR-PI-103 in TDR cells. We tested the effect of RIDR-PI-103 on melanocytes and TDR cells. RIDR-PI-103 exhibited less toxicity compared to PI-103 at 5 µM in melanocytes. RIDR-PI-103 significantly inhibited TDR cell proliferation at 5 and 10 µM. 24 hour treatment with RIDR-PI-103 inhibited p-Akt, p-S6 (Ser240/244) and p-S6 (Ser235/236). We assessed the mechanism of activation of RIDR-PI-103, using glutathione (GSH) or t-butyl hydrogen peroxide (TBHP) on the TDR cells in the presence or absence of RIDR-PI-103. Addition of the ROS scavenger GSH to RIDR-PI-103 significantly rescued the cell proliferation in TDR cell lines while addition of the ROS inducer TBHP and RIDR-PI-103 inhibited cell proliferation in WM115 and WM983B TDR cell lines. Examining the efficacy of RIDR-PI-103 on BRAF and MEK inhibitor resistant cells will expand possible treatment options and open avenues for the development of new ROS based treatment therapies for BRAF-mutant melanoma patients.		

15. SUBJECT TERMS BRAF; melanoma; dabrafenib; trametinib; Reactive oxygen species (ROS)					
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INTRODUCTION: Malignant melanoma is one of the most aggressive human cancers. The incidence of cutaneous melanoma is rising and the disease is incurable for many due to its metastatic nature and notorious therapeutic resistance. Immunotherapy increases the cure rate for some advanced cases. Nevertheless, 50-60% of patients do not respond and some cannot tolerate therapy. Constitutive activation of the BRAF serine-threonine kinase (BRAF-V600E) is the most common genetic change in melanoma. BRAF mutant melanomas are treated with the combination of BRAF inhibition (vemurafenib, dabrafenib) and MEK inhibition (trametinib, cobimetinib). However, the vast majority still succumb to disease due to acquired resistance. For example, disease progression for BRAF-mutant metastatic melanoma patients treated with the combination of dabrafenib and trametinib occurs within 9-10 months. Tumors thwart the effects of BRAK and MEK inhibition via reactivation of ERK due to mutations in genes encoding MEK1, MEK2, COT, NRAS or KRAS, overexpression of receptor tyrosine kinases (RTKs), or expression of BRAF-V600E splice variants; overexpression of the master melanocyte transcription factor, MITF; or PI3K/AKT activation by RTKs or as a result of PTEN or AKT mutations. Furthermore, these resistant tumors have increased reactive oxygen species (ROS). Consequently, new drug combinations are urgently needed to increase the 5-year survival rate of patients with advanced disease. This project is focused on understanding and harnessing drug resistant tumors that have high ROS for treatment strategies.

KEYWORDS: BRAF; melanoma; dabrafenib; trametinib; Reactive oxygen species (ROS)

ACCOMPLISHMENTS:

What were the major goals of the project?

<u>Specific Aim 1: Determine the role of ROS in BRAF and MEK inhibitor-resistant melanoma.</u>	Timeline	Site 1
Determine if short-term treatment with BRAF and MEK inhibitors or cells/tumors rendered resistant to BRAF and MEK inhibitors have elevated ROS	Months	
Task 1. Generate dabrafenib/trametinib resistant (DTR) cells from 451Lu, A375, SK-MEL-24, WM-115, and WM983B cells by growing cells in increasing concentrations of dabrafenib and trametinib.	1-2	Garrett
Task 2. Measure ROS <i>in vitro</i> by flow cytometry using the following fluorescent oxidation probes: Mito-SoxRed (mitochondrial superoxide), DHE (cytosolic superoxide), luminol (cytosolic superoxide), CDCFH2- DA (hydrogen peroxide) in parental cells ± dabrafenib/trametinib treatment and DTR cell lines.	2-6	Garrett
Task 3. Measure 8-oxo-dG levels in parental cells ± dabrafenib/trametinib treatment and DTR cell lines.	6-8	Garrett
Task 4. Lentiviral transduction	6-8	Garrett
Task 5. ACURO Approval	6-8	Garrett

Task 6. Assess <i>in vivo</i> levels of hydrogen peroxide present in A375-Fluc-Puro, WM115-Fluc-Puro and WM983B-Fluc-Puro cells tumor xenografts treated $\pm$ dabrafenib/trametinib using a chemoselective bioluminescent reporter, Peroxy Caged Luciferin-1 (PCL-1). SCID mice 2 treatment groups (vehicle or dabrafenib/trametinib) x 6 mice/group x 3 cell lines= 36 mice needed	8-12	Garrett
Milestone 1: Establish ROS levels are increased upon short-term inhibition of BRAF and MEK and upon establishment of resistance to BRAF and MEK inhibitors.	1-12	Garrett
Milestone 2: Prepare manuscript for publication. Published Yuan et al. Cancers 2020	1-12	Garrett
Determine epigenetic and signaling changes upon establishment of resistance to BRAF and MEK inhibitors.		
Task 1. Perform methyl-seq on WM115, WM115-DTR, A375 and A375-DTR.	13-16	Garrett
Task 2. Perform RTK and Phospho-Kinase Antibody arrays on cell lysates from parental and DT resistant cells. Identified kinases with differential levels of activation in parental versus resistant cells followed up using standard immunoblotting techniques.	17-18	Garrett
Task 3. Follow up with proteins identified from the methyl-seq screen, RTK array and test PRDX1 and SOD2 proteins (identified from proteomics analysis, figure 3) to determine how shRNA knockdown of identified protein(s) modulates proliferation of cells and ROS levels.	18-24	Garrett
Milestone 1: Identify candidate genes that are up- or down-regulated upon BRAF/MEK inhibition/resistance and modulate ROS levels.	13-24	Garrett
Milestone 2: Prepare manuscript for publication. Published Patel et al. Cancers 2021	13-24	Garrett
Specific Aim 2: Evaluate the efficacy of combination ROS-activatable prodrug and dabrafenib and trametinib therapy for BRAF/MEK inhibitor-resistant melanoma	Timeline	Site 1
Identify the most efficacious ROS-prodrug compound <i>in vitro</i> and <i>in vivo</i>		
Task 1. Establish WM4237 PDXs that continue to grow in the presence of dabrafenib and trametinib, termed WM4237-DTR. NOD-SCID Gamma (NSG) mice. 15 tumors needed for continuous dabrafenib and trametinib treatment to develop resistance. 15 total mice needed for this task.	18-20	Garrett

Task 2. Examine cell growth of WM115 cells using an MTT assay testing a library of 75 compounds <i>in vitro</i> .	18-24	Merino/Garrett
Task 3. Test the top 3 ROS-prodrug compounds <i>in vivo</i> in a small pilot study with WM4237-DTR to determine the most efficacious compound. NSG mice, n=5 per group x 4 groups (3 ROS prodrugs and 1 vehicle control)= 20 total mice needed for this task	25-28	Garrett
Milestone 1: Identify the top-candidate ROS-prodrug for further <i>in vivo</i> studies.	18-28	Garrett
Evaluate the ROS pro-drug in combination with dabrafenib and trametinib therapy in WM4237 PDX		
Task 1. Establish WM4237-DTR tumors in 40 mice for large-scale study. NSG mice, n=10 per group x 4 groups = 40 total mice needed for this task	29-31	Garrett
Task 2. Treat mice with 1) 0.5% methylcellulose, 0.1% Tween 80 vehicle via orogastric gavage and 300 µL PBS vehicle x3/week i.p.), 2) dabrafenib (30 mg/kg/day via orogastric gavage) and trametinib (0.6 mg/kg/day via orogastric gavage), 3) ROS-prodrug (30 mg/kg x3/week i.p.), 4) dabrafenib, trametinib + ROS pro-drug for up to 6 weeks. Measure tumor growth.	32-36	Garrett
Task 3. Harvest tumors, fix in formalin, or snap-freeze in liquid N ₂ . Subject tumors to IHC to detect Ki-67, caspase 3, p27, P-Mek, P-Erk, P-ATM, P-ATR and γ-H2AX. Perform immunoblots of tumor lysates with the same antibodies.	32-36	Garrett
Milestone 1: Establish the efficacy of the ROS pro-drug	29-36	Garrett
Milestone 2: Prepare manuscript for publication Published Patel et al. AntiCancer Drugs 2023	29-36	Garrett

What was accomplished under these goals?

Please see appendices with peer-reviewed publications.

What opportunities for training and professional development has the project provided?

Attended and presented research findings at:

PanAmerican Society for Pigment Cell Research 2021 Conference and other conferences.

In academic year 2021-2022, I took part in UC Women Lead. UC Women Lead is an experiential leadership development program, designed to develop core leadership skills for women faculty and staff who wish to advance to senior academic and administrative positions at the University of Cincinnati. Leadership development is a critical strategy for transforming institutions of higher education. In this program I did the following:

- Develop a better understanding of their personal leadership skills and developmental needs

- Cultivate relationships across campus and within various disciplines
- Identify strategies for successfully navigating challenges in negotiation, conflict management, leadership identity, communication, and career-life balance
- Outline a leadership development plan tailored to their goals
- Receive both peer and executive coaching support to advance their leadership goals
- Engage in an institutional development project that is designed to draw on core competencies and have an impact on the University

How were the results disseminated to communities of interest?

We regularly attend and present our research at national scientific meetings like American Association for Cancer Research with the exception of the COVID-19 pandemic when national meetings were canceled.

On an annual basis our laboratory meets with cancer advocates. Members of the lab including myself give brief presentations on our research findings. We hear from patient advocates, getting their perspectives on what it's like to receive treatment and their experiences. The most recent lunch in June 2023 we heard from a patient advocate about the frustrating experience of being misdiagnosed.



June 28, 2021 meeting with patient advocates



June 29, 2023 meeting with patient advocates

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our lab has shown that a reactive oxygen species (ROS) activated PI3K inhibitor (termed RIDR-PI-103) may be efficacious in BRAF mutant melanomas that no longer respond to BRAK and MEK inhibition. Examining the efficacy of RIDR-PI-103 on BRAF and MEK inhibitor resistant cells will expand possible treatment options and open avenues for the development of new ROS based treatment therapies for BRAF-mutant melanoma patients.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

CHANGES/PROBLEMS:

Nothing to report.

PRODUCTS:

Publications, conference papers, and presentations

Yuan L, Mishra R, Patel H, Alanazi S, Wei X, Ma Z, Garrett JT. BRAF Mutant Melanoma Adjusts to BRAF/MEK Inhibitors via Dependence on Increased Antioxidant SOD2 and Increased Reactive Oxygen Species Levels. *Cancers (Basel)*. 2020 Jun 23;12(6); published; yes

Patel H, Yacoub N, Mishra R, White A, Long Y, Alanazi S, Garrett JT. Current Advances in the Treatment of BRAF-Mutant Melanoma. *Cancers* (Basel). 2020 Feb 19;12(2); published; yes

Patel, H., Mishra, R., Yacoub, N., Alanazi, S., Kilroy, M.K., Garrett, J.T. IGF1R/IR mediates resistance to BRAF and MEK inhibitors in BRAF-mutant melanoma. *Cancers* (Basel). 13(22):5863, 2021; published; yes

Patel, H., Mishra, R., Wier, A., Mokhtarpour, N., Merino, E.J., Garrett, J.T. RIDR-PI-103, ROS-activated PI3K inhibitor prodrug inhibits cell growth and impairs the PI3K/Akt pathway in BRAF and MEK inhibitor resistant BRAF-mutant melanoma cells. *Anticancer Drugs* 34: 519. 2023; published; yes

Presentations:

American Association for Cancer Research Virtual Annual Meeting (June 2020). Title: *Role of IGF1R in mediating resistance to MAPK pathway inhibitors in BRAF-mutant melanoma*.

PanAmerican Society for Pigment Cell Research 2021 Conference (October 2021). Title: *IGF1R and IR mediate resistance to MAPK pathway inhibitors in BRAF-mutant melanoma*

Cedars-Sinai Grand Rounds (November 2021) Los Angeles, CA. Title: *Mechanisms of Resistance to BRAF/MEK inhibitors in BRAF mutant melanoma*.

University of Nottingham Centre for Cancer Sciences (December 2022) Nottingham, U.K. Title: *Mechanisms of Resistance to BRAF/MEK inhibitors in BRAF mutant melanoma*.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Joan Garrett

Project Role: PI

Nearest person month worked: 3.6

Contribution to the project: Dr. Garrett is the Principal Investigator on this project. She is the primary person responsible for the conception, design and coordination of the experiments in this research proposal. Dr. Garrett directly supervises the *in vitro* and *in vivo* experiments. She is responsible for the analyses and interpretation of data, preparation of scientific reports, manuscripts, as well as presentations at national and international scientific meetings.

Name: Zalfa Abdel-Malek

Project Role: Co-I

Nearest person month worked: 0.1

Contribution to the project: Dr. Abdel-Malek participates in scheduled meetings for updates on the progress of the study.

Name: Long Yuan

Project Role: graduate student

Nearest person month worked: 3

Contribution to the project: Long co-lead many of studies in the first year resulting in a research publication in *Cancers*.

Funding Support: Graduate Teaching Assistant Stipend provided by University of Cincinnati

Name: Rosalin Mishra

Project Role: Research Associate

Nearest person month worked: 6

Contribution to the project: Dr. Mishra leads *in vitro* and *in vivo* studies and assists graduate students.

Name: Hima Patel
Project Role: graduate student
Nearest person month worked: 3
Contribution to the project: Hima has assisted Dr. Mishra on studies with RIDR-PI-103.
Funding Support: Graduate Teaching Assistant Stipend provided by University of Cincinnati

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

N/A

Article

BRAF Mutant Melanoma Adjusts to BRAF/MEK Inhibitors via Dependence on Increased Antioxidant SOD2 and Increased Reactive Oxygen Species Levels

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Abstract: B-Rapidly Accelerated Fibrosarcoma (BRAF) mutations are found in about 50% of melanoma patients. Treatment with Food and Drug Administration (FDA)-approved BRAF and MAP/ERK kinase (MEK) inhibitors has improved progression free and overall survival of patients with BRAF mutant melanoma. However, all responders develop resistance typically within 1 year of treatment with these inhibitors. Evidence indicates that reactive oxygen species (ROS) levels are elevated after BRAF pathway inhibition treatment. We aim to decipher the role of mitochondrial antioxidant proteins relative to ROS levels and BRAF pathway inhibitor resistance. We observed BRAF mutant melanoma cells treated with the combination of a MEK inhibitor (trametinib) and a BRAF inhibitor (dabrafenib), exhibited elevated ROS levels, both in in vitro and in vivo melanoma models. We next generated trametinib- and dabrafenib-resistant (TDR) cells and found increased ROS levels after acquisition of resistance. An immunofluorescence experiment showed an increase of DNA damage in TDR cell lines. Furthermore, we observed that TDR cells increased superoxide dismutase 2 (SOD2), an antioxidant, at both mRNA and protein levels, with the upregulation of the transcription factor Nuclear Factor (NF)- κ B. Knockdown of SOD2 significantly reduced the growth of BRAF pathway inhibitor-resistant cells. In addition, the results indicate that TDR cells can be re-sensitized to BRAF pathway inhibitors by the ROS scavenger, N-Acetyl Cysteine (NAC). Overall, these data indicate that BRAF pathway inhibitor-resistant cells can compensate for elevated ROS via increased expression of the antioxidant SOD2.

Keywords: BRAF; melanoma; dabrafenib; trametinib; reactive oxygen species (ROS); superoxide dismutase 2 (SOD2)

1. Introduction

Melanoma, which accounts for 4% of all skin cancers, is the deadliest form of skin cancer, which contributes to 80% of all skin cancer related deaths. About 100,350 new melanomas will be diagnosed and about 6850 people are expected to die of melanoma in the year 2020, according to the American Cancer Society statistics. The identification of the B-Rapidly Accelerated Fibrosarcoma (BRAF)-V600E mutation, which accounts for 50% of patients, piloted a new era in the treatment of advanced melanomas [1,2]. The advent of combination therapy using a BRAF inhibitor (dabrafenib, encorafenib, vemurafenib) with a MEK inhibitor (binimetinib, cobimetinib, trametinib) resulted in a longer median

survival rate than other treatments. However, most patients still have relatively short-lived responses with resistance, eventually causing their deaths [3].

Overcoming drug resistance has become an essential issue in metastatic melanoma [4]. Evidence shows that BRAF inhibitors induce reactive oxygen species (ROS) levels in melanoma cells through Peroxisome proliferator-activated receptor gamma coactivator 1 (PGC1 α)-induced mitochondria biogenesis [5]. ROS, which consists of superoxide, H₂O₂, hydroxyl free radicals etc., is generated due to increased oxidative stress related to enhanced metabolism and the transformation of cancer cells [6]. Recent data from our group indicated that increased ROS activity in dabrafenib-resistant (DR) melanoma cells sensitized these cells to A100, a ROS-activated prodrug that causes DNA double strand breaks. Furthermore, these dabrafenib-resistant melanoma cells had upregulation of the mitochondrial enzyme, manganese superoxide dismutase 2 (SOD2), which functions to reduce ROS levels in the mitochondria [7].

SOD2, a member of the Mn superoxide dismutase (MnSOD) family, is an important mitochondria antioxidant enzyme that protects cells from oxidative stress by converting free superoxide ions (O²⁻) into hydrogen peroxide (H₂O₂). Elevated MnSOD2 levels have been shown to reduce tumor growth, in part by suppressing cell proliferation [8]. It is believed that loss of SOD2 expression propagates early progression of metastatic diseases, while evidence shows that SOD2 levels increase in many tumors as progression goes from the early to late stage of the disease progression [9]. Herein, we found that SOD2 levels are upregulated in trametinib- and dabrafenib-resistant (TDR) melanoma cell lines.

Transcription factor NF- κ B proteins are of central importance in inflammation and immunity [10,11]. The SOD2 gene promoter is under control of NF- κ B, and thus the activation of the MnSOD gene is in part mediated by NF- κ B [12,13]. In addition, evidence has shown that SOD2 is the target of NF- κ B, with antioxidant activity [14–16]. As a key regulator of SOD2, we observed a significant upregulation in NF- κ B in TDR cell lines versus parental drug sensitive melanoma cells. Our data indicate that both NF- κ B and SOD2 are upregulated in response to BRAF pathway inhibitor resistance in melanoma cells, which correlated to high oxidative stress.

2. Results

2.1. ROS Levels Are Increased in BRAF Mutant Melanoma in Response to Treatment with BRAF and MEK Inhibitors

The role of BRAF inhibitors in upregulating ROS levels in melanoma cell lines through PGC1 α -induced mitochondria biogenesis has already been reported [5]. We extended our investigations to determine ROS levels after treatment with BRAF and MEK inhibitors. We observed a significant increase in ROS levels after treating BRAF mutant melanoma cell lines (WM-115 and WM-983) with BRAF and MEK inhibitors dabrafenib and trametinib (D+T) for 24 h, as measured using the MitoSOX assay (Figure 1A). ROS level alterations after treating with BRAF and MEK inhibitors in A375 and SK-MEL-24 BRAF mutant melanoma cell lines, are shown in Figure S1 with similar results. Next, we assessed hydrogen peroxide (H₂O₂) production using established reporter gene cell lines (WM-115-Fluc-Puro and WM-983-Fluc-Puro), which stably express the firefly luciferase gene. We used Peroxy Caged Luciferin-2 (PCL2), a H₂O₂-responsive boronic acid probe that releases 6-hydroxy-2-cyanobenzothiazole (HCBT) upon reacting with reactive oxygen species (ROS), as well as D-cysteine, which reacts with HCBT and forms firefly luciferin in situ [17]. Photons and bioluminescence can be released and detected upon reaction with firefly luciferase enzymes in our newly established reporter gene cell lines. We observed an increase of the total bioluminescence signal after WM-115-Fluc-Puro and WM-983-Fluc-Puro cell lines were treated with 2.4 μ M dabrafenib and 500 nM trametinib for 24 h, suggesting an increase of ROS levels after treatment of BRAF and MEK inhibitors (Figure 1B). In addition, the WM-115-Fluc-Puro reporter gene cell line was injected in nude mice and tumor xenografts were established. Mice were treated with vehicle or dabrafenib (30 mg/kg/day via orogastric gavage) and trametinib (0.6 mg/kg/day via orogastric gavage) for 3 days. The mixture of PCL2 and D-cysteine (30 μ mol each) was injected via intra peritoneal (i.p.) as a probe to

measure the bioluminescence signal using the IVIS Spectrum in vivo imaging system [12]. We observed an increase in ROS levels after a short-term 3 day treatment of BRAF and MEK inhibitors, indicating that ROS levels were upregulated in an in vivo mouse melanoma model subjected to acute treatment of dabrafenib and trametinib (Figure 1C).

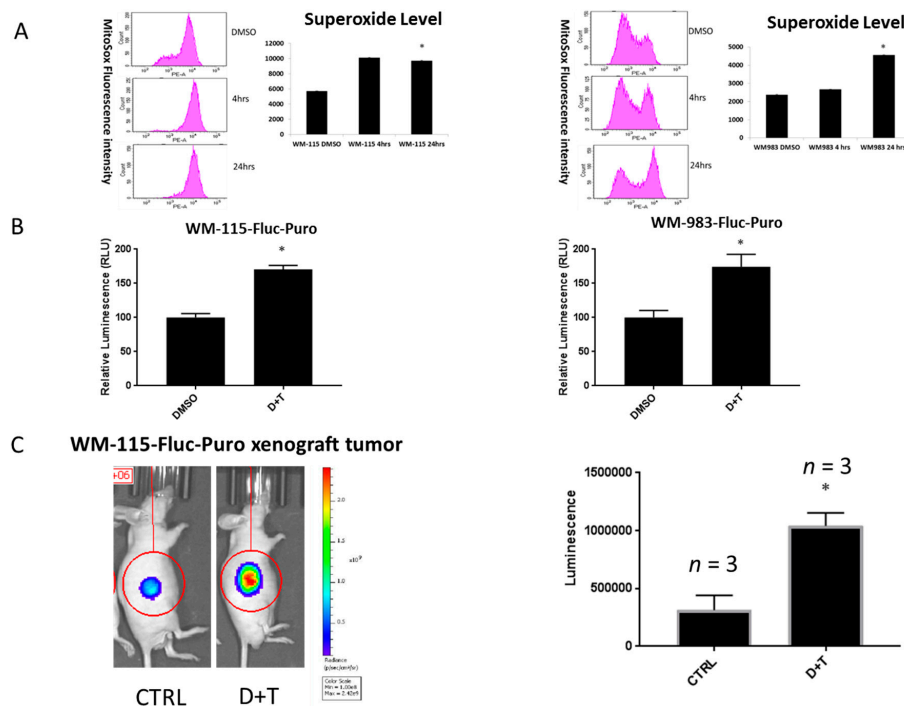


Figure 1. Reactive oxygen species (ROS) level is upregulated upon BRAF pathway inhibitor treatment. (A) WM-115 and WM-983 cell lines were treated with 2.4 μ M dabrafenib and 500 nM trametinib. Superoxide levels were measured by MitoSOX assay at 0, 4, and 24 h. (B) WM-115-Fluc-Puro and WM-983-Fluc-Puro cell lines were treated with 2.4 μ M dabrafenib and 500 nM trametinib for 24 h. The total bioluminescent signal, integrated over 5 min, added with a mixture of Peroxy Caged Luciferin-2 (PCL2) (25 mM) and D-cysteine (25 mM) were measured. Signals were normalized to the Dimethyl Sulfoxide (DMSO) control group. (C) Left panel: Representative image (45 min post-injection) for mice injected with a mixture of PCL2 and D-cysteine (i.p., 30 μ mol each, in 50 μ L of 1:1 DMSO: phosphate buffered saline (PBS)) after daily oral gavage of vehicle (CTRL) or dabrafenib and trametinib (D+T) (30 mg/kg/day dabrafenib + 0.6 mg/kg/day trametinib oral gavage for 3 days). Right panel: Total bioluminescent signal quantification ($n = 3$ per group). * $p < 0.05$ versus control group. Statistical analysis by one way ANOVA.

2.2. ROS Level Is Upregulated upon Drug Resistance in BRAF Mutant Melanoma Cell Lines

Our previous data indicated that ROS levels are upregulated in response to acute dabrafenib and trametinib treatment in BRAF mutant melanoma. Our lab has shown that dabrafenib-resistant (DR) cell lines have more ROS levels compared to parental cell lines [7]. We were interested by the alterations in ROS levels in response to dual BRAF and MEK inhibitor-resistant melanoma cells. We generated trametinib- and dabrafenib-resistant (TDR) WM115 and WM983 cell lines via a gradual dose escalation of each drug [18]. We checked superoxide and hydrogen peroxide (H_2O_2) levels in TDR melanoma cell lines. Superoxide levels were significantly increased in WM-115 TDR and WM-983 TDR cell lines compared to WM-115 DR, WM-983 DR [7] and parental cell lines, as measured using the MitoSOX assay (Figure 2A). Levels of H_2O_2 were measured using the DCFDA/H2DCFDA assay kit to measure ROS, which indicated that WM-115 TDR and WM-983 TDR cells had higher H_2O_2 compared to respective DR or parental cell lines (Figure 2B). Overall, the data indicated that ROS levels were

significantly augmented in response to chronic treatment with BRAF and MEK inhibitors in TDR melanoma cell lines.

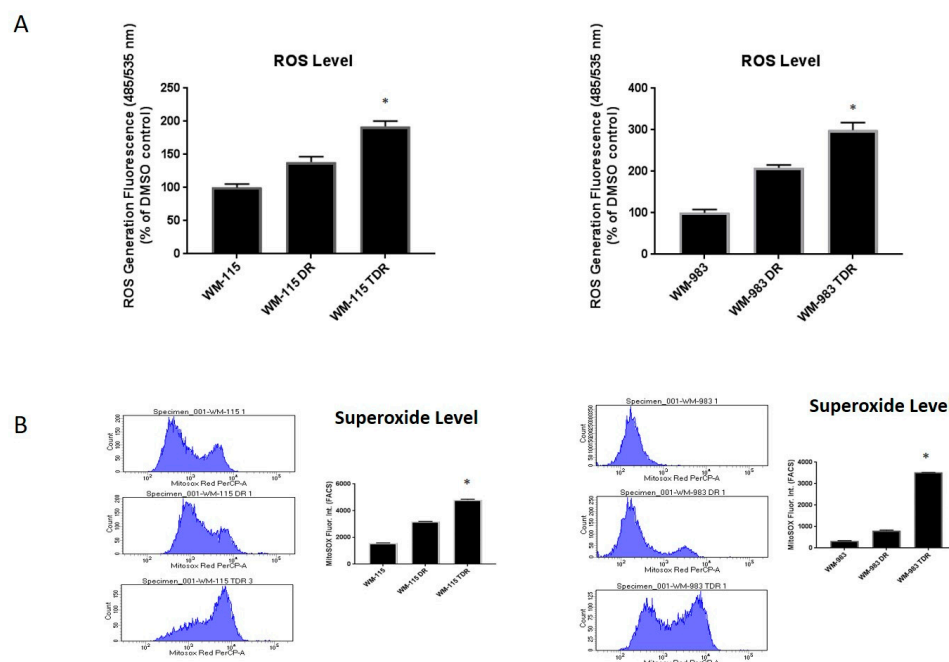


Figure 2. ROS level is upregulated upon BRAF and MEK inhibitor resistance. **(A)** ROS levels in WM-115 (parental, dabrafenib-resistant (DR), and trametinib- and dabrafenib-resistant (TDR) and WM-983 (parental, DR, and TDR) were measured by the DCFDA assay. **(B)** Basal superoxide levels in WM-115 (parental, DR, and TDR) and WM-983 (parental, DR, and TDR) cells were measured by the MitoSOX assay. * $p < 0.05$ versus parental cell lines. Statistical analysis by one way ANOVA.

2.3. BRAF Mutant Melanoma Cells Resistant to BRAF and MEK Inhibition Show Increases in DNA Damage

Given the fact that acute and chronic BRAF and MEK inhibitor treatment results in upregulation of ROS levels, we assessed the alteration in expression of the DNA damage marker, 8-oxo-dG, in TDR cells versus the DR and parental cell lines via immunofluorescence assay [19]. In nuclear and mitochondrial DNA 8-oxo-dG [20] is one of the predominant forms of free radical-induced oxidative lesions and has therefore been widely used as a biomarker for oxidative stress. Our data indicated that WM-115 TDR and WM-983 TDR have higher levels of 8-oxo-dG than WM-115 DR and WM-983 DR and parental cell lines (Figure 3A,B). These data suggest the possible involvement of DNA damage signaling induced in response to high ROS generated via chronic dabrafenib and trametinib treatment in BRAF mutant melanoma cells.

2.4. SOD2 and NF- κ B Levels Are Increased in BRAF Pathway Inhibitor-Resistant Melanoma Cells

Oxidative stress and intracellular redox state balance are essential. Evidence also shows that high intracellular ROS levels could cause an increase in antioxidant enzymes for compensation and homeostasis balance [7,21–23]. Based on our previous findings that antioxidants, such as SOD2 and peroxiredoxin-1 (PRDX1), are elevated in dabrafenib resistant (DR) melanoma cells identified from a proteomics screen [7], we aimed to assess SOD2 and PRDX1 levels in BRAF and MEK inhibitor-resistant cells. Two-step real time-qPCR was performed to assess the mRNA level of the antioxidants, SOD2 and PRDX1. Our results show that both SOD2 and PRDX1 antioxidant levels were upregulated at the mRNA and protein levels for WM-115 TDR cell lines versus the dabrafenib resistant (DR) and parental cell lines (Figure 4A). We observed increased mRNA and protein levels of SOD2 in WM-983 TDR versus WM-983 DR and WM-983 parental cell lines. However, we observed a reduction in PRDX1 mRNA and protein levels in both WM-983 DR and TDR cells, as compared to WM-983 parental cells (Figure 4B).

NF- κ B plays an important role in modulating SOD2 levels as a transcription factor [15,24]. Our results show that NF- κ B increased in WM-115 and WM-983 TDR cells, indicating the possible involvement of NF- κ B in ROS-mediated adaptive response in the TDR cell lines. Previous data indicated the role of p53 in modulating SOD2 [25]. Since TDR cells are shown to have high SOD2 compared to parental cells, we checked the p53 levels in TDR cell lines. There was no consistent pattern in p53 levels amongst the TDR cells versus the parental melanoma cell lines (Figure 4C). Original whole blot images can be found in Figures S2–S6. Densitometry analysis of western blotting can be found in Figures S7–S15.

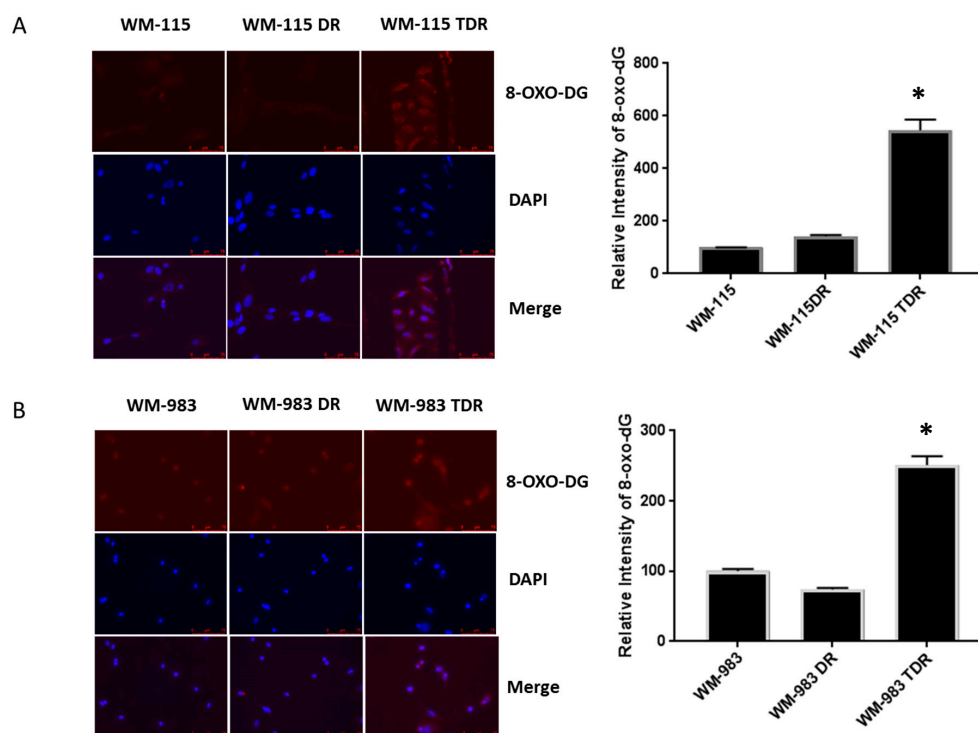


Figure 3. 8-oxodG, a DNA damage marker, is increased in BRAF pathway inhibitor-resistant melanoma cells. (A) Representative images showing anti-8-oxodG expression for WM-115 (parental, DR, and TDR) cell lines, as detected using immunofluorescent assay. 4',6-diamidino-2-phenylindole (DAPI) stain is used to identify nuclei. Quantifications are shown on the right panel. (B) Representative images showing anti-8-oxodG staining for WM-983 (parental, DR, and TDR) cell lines, as detected by immunofluorescent staining. DAPI stain is used to identify nuclei. Quantifications are shown on the right panel. * $p < 0.05$ versus parental cell lines. Statistical analysis by one way ANOVA.

2.5. Knockdown of SOD2 Suppresses the Growth of BRAF Pathway Inhibitor Resistant Melanoma Cells

Previous reports have shown that SOD2 levels appear to surge during metastatic progression in highly aggressive tumor cells [26,27]. Several studies have shown that elevated antioxidant enzyme expression is necessary for tumor progression and survival [28,29]. We investigated whether knockdown of SOD2 has a role in cell growth and proliferation. WM-115 TDR and WM-983 TDR cells were transfected with siRNA, specifically targeting SOD2 (SOD2 siRNA). The specificity of the siRNA was analyzed by western blotting (Figure 5A). The effect of SOD2 knockdown on WM-115 TDR and WM-983 TDR cell proliferation and growth was assessed using 3-4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and crystal violet assays. We observed a decrease in cell growth for both TDR cell lines versus the control after SOD2 knockdown, suggesting that SOD2 could be a factor in modulating tumor proliferation and survival in trametinib- and dabrafenib-resistant (TDR) melanoma cell lines (Figure 5B,C). The original whole blot can be found in Figure S7.

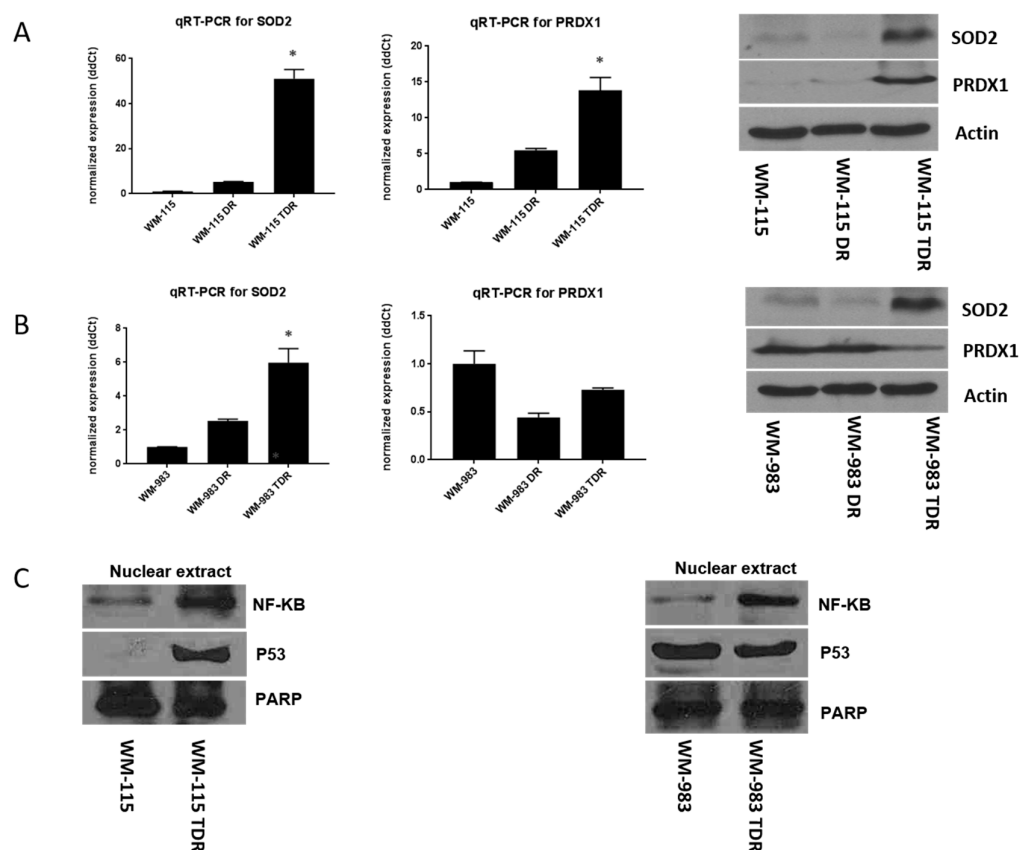


Figure 4. Superoxide dismutase 2 (SOD2) and NF- κ B levels are increased in BRAF pathway inhibitor-resistant cells. (A) An equal number of WM-115 (parental, DR, and TDR) cells were plated. Total RNA was extracted from these cell lines using the RNA extraction mini kit, according to the manufacturer's instructions. Two-step real time-qPCR was performed to assess the mRNA level of SOD2 and PRDX1. First strand cDNA was synthesized using the iScriptTM cDNA synthesis kit qPCR, which was set up using the CFX96 real-time system. Actin was used as an internal control. Relative SOD2 and PRDX1 expression was presented by the $2^{-\Delta\Delta C_T}$ method (left panel). In separate experiments, whole cell lysates were prepared and separated in a 12.5% sodium dodecyl sulfate (SDS) gel, followed by immunoblot analysis with indicated antibodies. Western blot data is shown on the right panel. (B) An equal number of WM-983, WM-983 DR, and WM-983 TDR cells were plated. Two-step real time-qPCR and immunoblot analysis with indicated antibodies were performed under the above conditions (left and right panels). (C) Nuclear extracts were collected and prepared from WM-115 and WM-983 (parental and TDR). * $p < 0.05$ versus parental cell lines. Statistical analysis by one way ANOVA.

2.6. NAC, a ROS Scavenger, Can Re-Sensitize the BRAF Inhibitor in Melanoma Cells

We next aimed to examine if we could re-sensitize TDR cells to BRAF and MEK inhibitors via treatment with a ROS scavenger. Hence, we assessed the sensitivity of these cells in the presence of the ROS scavenger, N-Acetyl Cysteine (NAC), using crystal violet and MTT growth assays. We found that TDR cells maintain resistance to dabrafenib and trametinib after drugs have been withdrawn for 21 days. We observed a decrease in cell proliferation and growth of WM-115 TDR and WM-983 TDR cell lines when subjected to the combination of dabrafenib (D) and trametinib (T) in the presence of NAC, compared to the D+T group or DMSO controls (Figure 6A,B). Our data indicated the possible involvement of a ROS-mediated signaling pathway in regulating TDR cell proliferation and growth.

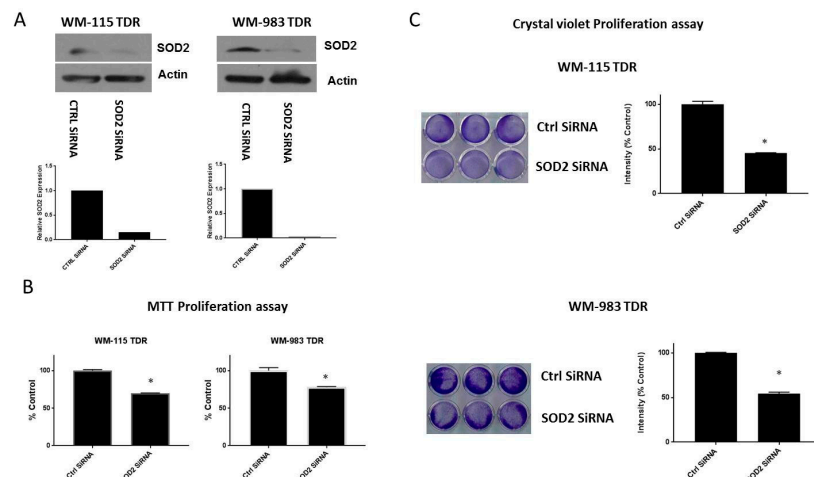


Figure 5. Knockdown of SOD2 reduces growth in BRAF pathway inhibitor-resistant cells. (A) WM-115 TDR and WM-983 TDR cells were transfected with a SOD2 specific siRNA or control (siCon) for 48 h and analyzed using specific antibodies. Actin served as the loading control. Densitometric analysis of SOD2 expression was normalized to Actin. (B) Both WM-115 TDR and WM-983 TDR cells transfected with Con siRNA and SOD2 siRNA cells were plated in triplicate in 96 well plates for 72 h. (C) Both WM-115 and WM-983 TDR cells transfected with Con siRNA and SOD2 siRNA (30,000 cells/well) were plated in triplicate, as indicated. * $p < 0.05$ vs. CTRLsiRNA. Statistical analysis by one way ANOVA.

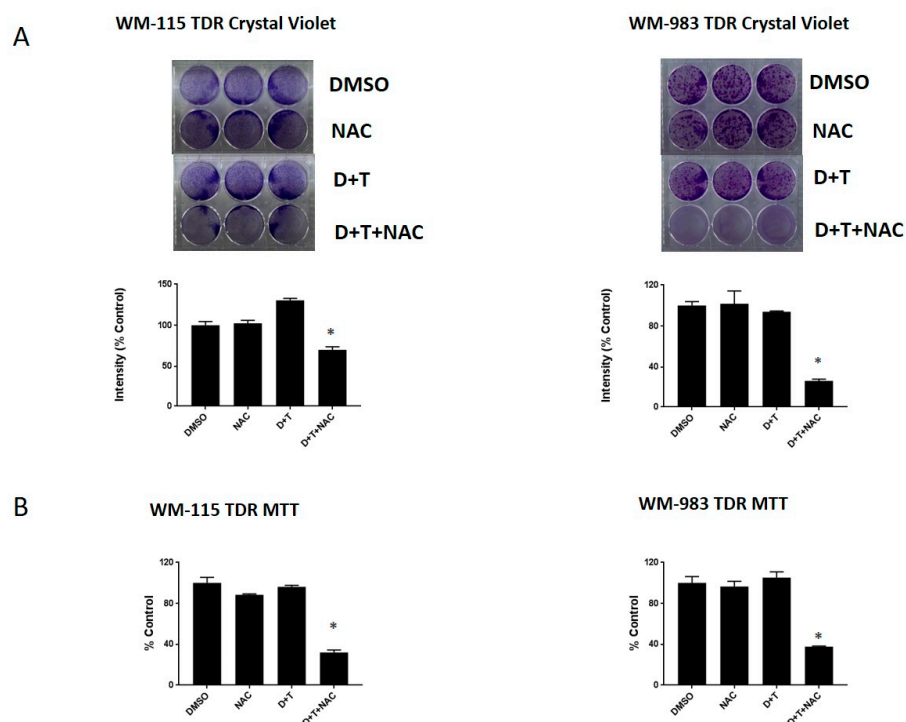


Figure 6. N-Acetyl Cysteine (NAC), a ROS scavenger, can re-sensitize BRAF pathway inhibitor-resistant melanoma cells. (A) WM-115 TDR and WM-983 TDR cells (30,000 cells/well) were plated in triplicate and treated with DMSO, 5 mM N-Acetyl Cysteine (NAC), and/or 2.4 μ M dabrafenib and 500 nM trametinib, as indicated. (B) WM-115 TDR and WM-983 TDR cells were plated in triplicate in 96 well plates and treated for 72 h with DMSO, 5 mM NAC, and/or 2.4 μ M dabrafenib and 500 nM trametinib, as indicated. * $p < 0.05$ versus DMSO control. Statistical analysis by one way ANOVA.

3. Discussion

Current targeted therapy for the treatment of the metastatic melanoma with BRAF-V600E mutation focuses on the use of combination of BRAF and MEK inhibitors as a treatment strategy. Several studies and clinical trials have demonstrated improved patient outcomes with this combination therapy. However, there are several challenges met with the current standard of care [4]. Hence, the current research is focused on understanding the resistant mechanisms in response to BRAF and MEK inhibitors. Our study is focused on the role of oxidative stress-induced ROS in trametinib- and dabrafenib-resistant (TDR) melanoma cells with BRAF mutation. Our data indicated that melanoma cells in response to acute and chronic exposure of BRAF pathway inhibitors induce ROS in both in vitro and in vivo melanoma models (Figures 1 and 2) [30,31].

We observed that dual resistant (TDR) melanoma cell lines have higher antioxidant levels (SOD2 and PRDX1), accompanied with higher ROS levels compared to counterpart parental and dabrafenib resistant (DR) cells (Figure 4). The involvement of SOD2 in mediating an effect on proliferation in TDR cells was confirmed by siRNA mediated silencing, where knockdown of SOD2 significantly suppressed melanoma cell proliferation and growth (Figure 5). There is evidence that downregulation or knockout of SOD2 would be lethal in mice models [32,33]. SOD2 is a target for the transcription factor NF- κ B. The levels of NF- κ B are also influenced by the level of ROS, thus affecting the antioxidant SOD2 levels [15,34,35]. NF- κ B binding sites are known to be located in the promoter region and within the intronic enhancer element of the SOD2 gene [36–38]. In addition, NF- κ B is known to participate actively in the regulation of SOD2 expression in tumor cells [39–41]. In our study, we observed an upregulation of NF- κ B in the nuclear fraction of BRAF pathway inhibitor-resistant cells relative to parental cells. The increased SOD2 could be caused by NF- κ B transcriptional regulation in BRAF pathway inhibitor-resistant cells (Figure 4). Further experiments are needed to delineate if SOD2 levels are directly regulated by NF- κ B. p53 levels were upregulated in correlation with enhanced NF- κ B levels in the WM-115 TDR cell line. However, there were no alterations in p53 levels in the WM-983 TDR cell line compared to the control. While both WM-983 TDR and WM-115 TDR have increased SOD2, notably, WM-115 TDR cells have much higher levels of SOD2 mRNA compared to parental cells, relative to SOD2 mRNA in WM-983 TDR cells (~50 fold increase in WM-115 TDR cells versus ~6 fold increase in WM-983 TDR cells (Figure 4A,B). Thus, it is possible that WM115 TDR cells have a larger increase in SOD2 levels, as enhanced expression of both the transcription factors (NF- κ B and p53) in WM-115 TDR cells may have resulted in increased transcriptional activity of SOD2 [24,42,43].

Furthermore, we observed treatment of BRAF pathway inhibitor-resistant cells with the ROS quenching NAC restored sensitivity to BRAF and MEK, whereas NAC alone had no effect on cell growth (Figure 6). Treatment of cancer with antioxidants, such as Vitamin C, has been studied extensively with inconsistent results. Supplementing diets with the antioxidants NAC and vitamin E in mouse models of BRAF and KRAS lung cancers increased tumor progression and reduced survival [44] by reducing ROS, DNA damage, and p53 expression in mouse and human lung tumor cells. We note the differences in our in vitro administration of NAC in the context of acquired drug resistance. There has been a renewed interest using high dose vitamin C potentially administered intravenous, as there is evidence that large doses of vitamin C can kill cultured colon cancer cells with BRAF or KRAS mutations by raising free radical levels [45].

Our study has several limitations. The bulk of results are obtained in only two cell lines, WM115 and WM983. WM983 is derived from a metastatic melanoma, whereas WM115 is a primary melanoma with metastatic potential [46,47]. Furthermore, the majority of data presented are from in vitro studies and are not derived directly from human patients. We acknowledge the limitations of our data. Nevertheless, crucial information about the interplay between antioxidants, ROS and BRAF pathway inhibitor resistance is obtained from this work, which can be bolstered by future work examining the effects of high dose antioxidants in BRAF pathway inhibitor-resistant tumors in vivo.

4. Material and Methods

4.1. Cell Culture and Inhibitors

Human melanoma cells (WM-115 and WM-983) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Dabrafenib resistant melanoma cell lines were generated in a previous protocol in our lab [7]. Dabrafenib and trametinib were obtained from LC Laboratories. WM-115 cells were maintained in Minimum Essential Medium Eagle (MEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in 5% CO₂ in a humidified incubator. WM983 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in 5% CO₂.

4.2. Generation of Trametinib- and Dabrafenib-Resistant (TDR) Cell Lines

Briefly, melanoma cell lines (WM-115, WM-983) were initially treated with 0.01 µM dabrafenib and trametinib subjected to gradual dose escalation of dabrafenib (0–2.4 µM) and trametinib (0–500 nM) over a span of 3–4 months and finally maintained in 2.4 µM dabrafenib and 500 nM of trametinib. Media and dabrafenib were replenished every alternate day. The cell lines were authenticated using short tandem repeat (STR) profiling by ATCC.

4.3. Immunoblotting

TDR melanoma cells were maintained in 2.4 µM dabrafenib and 500 nM trametinib and lysed in RIPA buffer containing protease and phosphatase inhibitors. Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagents Kit (Thermo Fisher, Waltham, MA, USA), as per the manufacturer's guidelines. Immunoblotting was performed using specific primary antibodies for NF-κB, poly ADP ribose polymerase (PARP), P53, PRDX1, SOD2 (Cell Signaling Technology, Danvers, MA, USA), and Actin (Santa Cruz biotechnology, Dallas, TX, USA). Anti-rabbit IgG-horse radish peroxidase (HRP) was used as the secondary antibody. Actin and PARP served as loading controls.

4.4. Transient Transfection

WM-115 TDR and WM-983 TDR cells were transfected with siRNAs, specifically targeting SOD2 (SOD2 siRNA; Dharmacon, Lafayette, CO, USA) and control siRNA (control siRNA; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

4.5. MTT Cell Proliferation Assay

Both WM-115 and WM-983 TDR cell lines were transfected with Con siRNA and SOD2 siRNA (WM-115 TDR Con siRNA, WM-115 TDR SOD2 SiRNA, WM-983 TDR Con SiRNA, and WM-983 TDR SOD2 SiRNA melanoma cells). Cells were plated into a 96-well plate at a density of 3.0×10^4 cells per well in triplicate and allowed to grow for 72 h. After 72 h, the media containing the drug was replaced with 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dissolved in cell line specific media, and incubated for 4 h. After 4 h, the media was aspirated and crystals were dissolved with isopropanol (Molecular grade, Fisher BioReagents, Waltham, MA, USA). The absorbance was read at 570 nm using a microplate reader (SPECTR Amax PLUS Microplate Spectrophotometer Plate Reader, Molecular Devices Corporation, San Jose, CA, USA). Data is represented as the mean of at least two independent experiments ± SEM.

4.6. Crystal Violet Cell Proliferation Assay

Both WM-115 and WM-983 TDR (30,000 cells/well) were transfected using Con siRNA and SOD2 siRNA for 48 h and were plated in triplicate in a 6-well plate. Medium and inhibitors were replenished every 2 days. After 4–6 days, cells were stained with crystal violet in 5% methanol after visible difference was noticed. The intensities were measured using the Odyssey infrared System. The values

were expressed as the mean of intensities obtained from three independent experiments and bar graphs were generated using graph pad prism 7 (GraphPad Software, Inc., La Jolla, CA, USA).

4.7. Measurement of Intracellular ROS

The production of intracellular ROS was measured using the oxidation sensitive MitoSOX assay using flow cytometry. Cellular O_2^- generation was measured using the mitochondria-targeting probe, MitoSOX Red. WM-115 and WM-983 parental cells (4×10^5) were treated in DMSO, 2.4 μ M of dabrafenib, and 500 nM trametinib for 24 h. WM-115 DR and WM-983 DR cells were maintained in 2.4 μ M of dabrafenib, while WM-115 TDR and WM-983 TDR cells were maintained in 2.4 μ M of dabrafenib and 500 nM trametinib, respectively. The cells were resuspended in Hank's Balanced Salt Solution (HBSS) and incubated with 5 μ M of MitoSOX Red for 30 min at 37 °C. The cells were washed, resuspended in HBSS on ice, and centrifuged at 4 °C. The red fluorescence (MitoSOX, Waltham, MA, USA) were measured using the FL-2 channel of the FACS Diva and analyzed using the CellQuest software.

The production of H_2O_2 was measured using the oxidation-sensitive Dichloro-dihydrofluorescein diacetate (DCFH-DA). WM-115 and WM-983 (parental, DR, and TDR) cells (2×10^4) were plated and maintained with DMSO, 2.4 μ M of dabrafenib, 2.4 μ M dabrafenib, and 500 nM trametinib, respectively. The cells were resuspended in DCFH-DA for 30 min and analyzed using Cell Quest software. The ROS levels were measured and represented using a bar graph.

4.8. qPCR Analysis of Antioxidant mRNA

Total RNA was extracted from both WM-115 and WM-983 (parental, DR, and TDR) cell lines using the RNA extraction mini kit (Qiagen, 74104, Germantown, MD, USA), according to the manufacturer's instructions. Two-step real time-qPCR was performed to assess the mRNA level of SOD2 and PRDX1. First strand cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Biorad, 1708890, Hercules, CA, USA). qPCR was set up using the CFX96 Real-Time System (Biorad, USA). Actin was used as an internal control. Relative SOD2 and PRDX1 mRNA expression was presented by the $2^{-\Delta\Delta CT}$ method. Paired primer sequences were used for SOD2: 5'-GCTCCGGTTTTGGGGTATCTG-3' (forward) and 5'-GCGTTGATGTGAGGTTCCAG-3' (reverse); for PRDX1: 5'-CCACGGAGATCATTGCTTTCA-3 (forward) and 5'-AGGTGTATTGACCCATGCTAGAT-3' (reverse); and for Actin: 5'-AAGGAGCCCCACGAGAAAAAT-3' (forward); 5'-ACCGAACTTGCATTGATTCCAG-3'.

4.9. Immunofluorescence

An equal number of WM-983 and WM-115 (parental, DR, and TDR) were plated on coverslips at a density of 0.15×10^5 cells in each chamber for 24 h. The cells were fixed in 4% formalin and then 0.5% triton for 10 min, respectively. Cells were incubated with 20% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h at room temperature and analyzed for 8-oxo-dG expression using immunofluorescence assay with antigen retrieval, as described below. Cells (parental, DR, and TDR) were incubated with an antibody mix of 8-oxo-dG mouse monoclonal antibody (Trevigen, 1:1000 dilution) for 2 h at room temperature in a humidified chamber. A total of 5 drops of Red Vectafluor (Vector Lab, Burlingame, CA, USA) fluorescent secondary antibodies were applied to slides for 30 min. Cells were counterstained with a 10 ng/mL DAPI (SigmaAldrich, St. Louis, MO, USA) for 5 min. Immunofluorescence images were captured using a Zeiss fluorescence microscope. Quantification was performed using ImageJ, with three images per treatment group.

4.10. Generation of Stable Reporter Cell Lines

Lentiviral vector LV-Luc2-P2A-Puro was purchased from Imanis Life Sciences (Rochester, MN, USA). WM-115 and WM-983 parental cell lines were seeded in complete medium to achieve 60–70% confluency and cells were transduced at a multiplicity of infection (MOI) of 10 in the presence of

6 µg/mL polybrene, as per the manufacturer's guidelines. Cells transduced with lentiviral vector containing a puromycin (Puro) resistance gene were selected using 625 nM puromycin to generate WM-115-Fluc-Puro and WM-983-Fluc-Puro stable cell lines.

4.11. *In Vitro Luciferase Assays*

A Xenogen IVIS Spectrum instrument (Vontz Core Imaging Laboratory, Cincinnati, OH, USA) was used for bioluminescent imaging in cellular experiments. WM-115-Fluc-Puro and WM-983-Fluc-Puro cells were plated at a density (10^4 , 10^5 , 10^6 cells/well) of black 96-well plates with clear bottoms and were allowed to grow overnight (Becton Dickinson and Co., Franklin Lakes, NJ, USA). A mixture of PCL2 and D-cysteine (25 mM each) was added and bioluminescence was measured by a Xenogen IVIS Spectrum instrument.

In separate experiments, cells (WM-115-Fluc-Puro and WM-983-Fluc-Puro) were plated in 96-well plates with clear bottoms at a density of 2.0×10^5 cells per well in triplicate and allowed to grow overnight. After 24 h, cells were treated with 2.4 µM of dabrafenib and 500 nM trametinib with DMSO as the control. After 24 h, a mixture of PCL2 and D-cysteine (25 mM each) was added and bioluminescence was measured by a microplate reader (SPECTRAMax PLUS Microplate Spectrophotometer Plate Reader, Molecular Devices Corporation).

4.12. *In Vivo Xenograft Experiments*

Four-week-old, male homozygous Foxn1nu mice were purchased from Jackson Labs and maintained at the University of Cincinnati Laboratory Animal Medical Services (LAMS) facility, with free access to food and water. All studies were approved and performed according to the guidelines of the Animal Care and Use Committee of the University of Cincinnati. Mice were injected subcutaneously with 5×10^6 WM-115-Fluc-Puro cells via the right flank with matrigel (Corning, NY, USA) in a ratio of 1:1. Once tumors had reached a volume ≥ 200 mm³, mice were randomized to the following groups: vehicle control group or 30 mg/kg/day dabrafenib + 0.6 mg/kg/day trametinib oral gavage group ($n = 3$ per group). Control mice were dosed with the vehicle by oral gavage daily for 3 days. Dabrafenib and trametinib were treated daily for 3 days to mimic the standard of care treatment in patients. Dabrafenib and trametinib were suspended in 0.5% methyl cellulose (Sigma, St. Louis, MO, USA) and 1% Tween 80 and administered by orogastric gavage. Tumor diameters were measured serially with calipers and volume in mm³.

4.13. *In Vivo Luciferase Experiments*

A Xenogen IVIS Spectrum instrument (Vontz Core Imaging Laboratory) was used for bioluminescent imaging in animal experiments. Mice were anesthetized prior to injection and during imaging via inhalation of isoflurane. Phosphate buffered saline (PBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), Isoflurane was purchased from Phoenix Pharmaceuticals, Inc. (St. Joseph, MO, USA), and pharmaceutical-grade DMSO was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mice were imaged via i.p. injection with a mixture of PCL2 and D-cysteine (i.p., 30 µmol each, in 50 µL of 1:1 DMSO:PBS), both for the DMSO and D + T treated groups after a daily oral gavage of D + T. Imaging data were quantified by averaging the region of interest from the maximal photon emitting exposure.

4.14. *Statistical Analysis*

Data are shown as the mean $\pm$ standard error of mean (SEM) and are representative of at least three independent experiments, unless indicated otherwise. Statistical analysis was performed by two sample *t*-tests, using ANOVA (Graph Pad Prism 7). The data was considered statistically significant if $p < 0.05$.

5. Conclusions

Our study suggests that SOD2 is induced in BRAF mutant melanomas rendered resistant to dabrafenib and trametinib treatment. In addition, SOD2 plays a substantial role in WM-115 TDR and WM-983 TDR cell growth and proliferation. BRAF pathway inhibitor-resistant cells (both WM-115 TDR and WM-983 TDR) had increased DNA damage and increased amounts of the transcription factor NF- κ B. Furthermore, BRAF pathway inhibitor-resistant cells can be rendered sensitive to BRAF and MEK inhibitors with the addition of the antioxidant NAC, which reduces ROS levels. This could be a potential strategy to treat BRAF-mutant melanoma patients whose tumors no longer respond to BRAF pathway inhibition.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/12/6/1661/s1>, Figure S1: Whole blot image of PRDX1 in WM-115, WM-115 DR, WM-115 TDR from Figure 4, Figure S2: Whole blot image of SOD2 in WM-983, WM-983 DR, WM-983 TDR, WM-115, WM-115 DR, WM-115 TDR from Figure 4, Figure S3: Whole blot image of PRDX1 in WM-983, WM-983 DR, WM-983 TDR from Figure 4, Figure S4: Whole blot image of Actin in WM-983, WM-983 DR, WM-983 TDR, WM-115, WM-115 DR, WM-115 TDR from Figure 4, Figure S5: Whole blot image of p53 in WM-115, WM-115 TDR, WM-983, WM-983 TDR from Figure 4, Figure S6: Whole blot image of Actin, CTRL SiRNA, SOD2 SiRNA in WM-983, WM-983 TDR, WM-115, WM-115 TDR from Figure 5, Figure S7: Densitometry reading of SOD2 expression in Figure 4A, Figure S8: Densitometry reading of PRDX1 expression in Figure 4A, Figure S9: Densitometry reading of SOD2 expression in Figure 4B, Figure S10: Densitometry reading of PRDX1 expression in Figure 4B, Figure S11: Densitometry reading of NF-KB expression in Figure 4C, Figure S12: Densitometry reading of NF-KB expression in Figure 4C, Figure S13: Densitometry reading of p53 expression in Figure 4C, Figure S14: Densitometry reading of p53 expression in Figure 4C.

Author Contributions: Data curation, L.Y., R.M., H.P. and S.A.; Methodology, L.Y., X.W. and Z.M.; Project administration, J.T.G.; Resources, Z.M.; Writing—original draft, L.Y.; R.M., J.T.G., Writing—review & editing, L.Y., R.M., H.P., S.A. and J.T.G. All authors have read and agreed to the published version of the manuscript.

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Review

Current Advances in the Treatment of BRAF-Mutant Melanoma

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Abstract: Melanoma is the most lethal form of skin cancer. Melanoma is usually curable with surgery if detected early, however, treatment options for patients with metastatic melanoma are limited and the five-year survival rate for metastatic melanoma had been 15–20% before the advent of immunotherapy. Treatment with immune checkpoint inhibitors has increased long-term survival outcomes in patients with advanced melanoma to as high as 50% although individual response can vary greatly. A mutation within the MAPK pathway leads to uncontrollable growth and ultimately develops into cancer. The most common driver mutation that leads to this characteristic overactivation in the MAPK pathway is the B-RAF mutation. Current combinations of BRAF and MEK inhibitors that have demonstrated improved patient outcomes include dabrafenib with trametinib, vemurafenib with cobimetinib or encorafenib with binimetinib. Treatment with BRAF and MEK inhibitors has met challenges as patient responses began to drop due to the development of resistance to these inhibitors which paved the way for development of immunotherapies and other small molecule inhibitor approaches to address this. Resistance to these inhibitors continues to push the need to expand our understanding of novel mechanisms of resistance associated with treatment therapies. This review focuses on the current landscape of how resistance occurs with the chronic use of BRAF and MEK inhibitors in BRAF-mutant melanoma and progress made in the fields of immunotherapies and other small molecules when used alone or in combination with BRAF and MEK inhibitors to delay or circumvent the onset of resistance for patients with stage III/IV BRAF mutant melanoma.

Keywords: melanoma; metastatic; resistance; BRAF; immunotherapy

1. Introduction

Melanoma is the uncontrollable division of melanocytes located within the deep layer of the epidermis [1]. Although invasive melanoma is the third most common type of skin cancer, it is the most serious compared to its other two counterparts- basal cell carcinoma and squamous cell carcinoma. The American Cancer Society estimates that in 2019 there will be 96,480 new cases of melanoma diagnosed accompanied by 7,230 expected deaths. The five-year survival rate for metastatic melanoma has been 15–20% [2], although these statistics are rapidly improving with the success of immune checkpoint inhibitors. Treatment with immune checkpoint inhibitors demonstrated substantial clinical efficacy along with long-term survival outcomes in patients with advanced melanoma [3–5]. There are several clinical trials such as KEYNOTE-002, CheckMate067 and CheckMate064, which validate these findings, as detailed in Table 1.

An important risk factor for melanoma is exposure to direct and chronic ultraviolet (UV) radiation which, increases the risk for DNA damage and leads to genetic changes. Familial history is also one of the risk factors for developing melanoma, with cyclin-dependent kinase inhibitor 2A (CDKN2A) and cyclin-dependent kinase 4 (CDK4) being the most common heritable mutations [1]. Germ-line polymorphisms of the melanocortin-1 receptor (MC1R) also confers susceptibility due to its ability to control the level of skin pigmentation in response to UV radiation [2,6].

The molecular changes occurring in the progression of melanoma serve as points for therapeutic interference. Typically, the preliminary change of a melanocyte into a benign nevus remains controlled and is non-cancerous. However, some molecular changes can lead to overactivation of growth regulatory pathways, such as the mitogen-activated protein kinase (MAPK) signaling pathway [2]. The MAPK pathway is crucial in relaying extracellular signals in order to keep a balance of growth/proliferation and apoptosis within the cell. A mutation within MAPK pathway leads to uncontrollable growth and ultimately develops into cancer [7]. The most common driver mutation that leads to this characteristic overactivation in the MAPK pathway is the B-RAF mutation [2]. Raf is a family of oncogenic serine-threonine protein kinases within the MAPK pathway with three isoforms: A-RAF, B-RAF, and C-RAF [7,8]. B-RAF-mutant melanoma accounts for nearly 50% of metastatic melanoma cases. Substitution of valine (V) for glutamic acid (E) at amino acid position 600 (V600E) represents 84.6% of the B-RAF mutations. A second common substitution of valine (V) for lysine (K) at amino acid position 600 (V600K), representing 7.7% of the B-RAF mutations [9]. B-RAF mutant melanoma is typically found in younger patients and is characterized by a superficial spreading tumor or nodular tumor that can be found in areas without chronic sun exposure. It has a higher chance of metastasizing into brain along with a shorter survival time as compared to the non-BRAF mutant melanoma. A B-RAF mutation alone may not contribute to the development of melanoma; but accompanying driver mutations in tumor suppressor genes are commonly indispensable leading to the development of malignant melanoma [9].

Several studies have addressed the need for molecular testing with respect to B-RAF mutations in order to tailor the best course of treatment available for each patient. [9,10]. Treatment options include surgery, immunotherapy, targeted therapy, chemotherapy, inclusion in a clinical trial and radiation [11]. Stage I and II melanoma can typically be surgically excised in concert with a sentinel lymph node biopsy if there is concern for metastasis. Stage III and IV melanoma require more systemic interventions due to the aggressiveness of the tumor and increased tumor burden [8]. The current standard of care for a patient diagnosed with B-RAF mutant metastatic melanoma is to first consider eligibility for a metastasectomy. Regardless of qualifications for the metastasectomy, the next step is to design a treatment regimen with either checkpoint inhibition immunotherapy or a molecularly targeted therapy [9,10].

Current targeted therapies include a combination of B-RAF and MEK inhibitors. Vemurafenib was the first FDA-approved B-RAF inhibitor in 2011, followed by approval of dabrafenib in 2013 [8]. The most recent FDA-approved B-RAF inhibitor is encorafenib, approved in 2018 [12]. In parallel with the discovery and use of B-RAF inhibitors opened up the avenue for development of MEK inhibitors, targeting a molecule downstream of the B-RAF protein. The first MEK-inhibitor, trametinib, was approved by FDA in 2013, followed by approval of cobimetinib in 2015 [8]. Another recently FDA-approved MEK inhibitor was binimetinib [12]. Current combinations of BRAF and MEK inhibitors that have demonstrated improved patient outcomes include dabrafenib with trametinib, vemurafenib with cobimetinib or encorafenib with binimetinib [13]. Treatment with BRAF and MEK inhibitors has met with some challenges as patient responses began to drop due to the development of resistance to these inhibitors which paved the way for development of immunotherapies and other small molecule inhibitor approaches to address this.

Current immunotherapies include the anti-cytotoxic T-lymphocyte antigen 4 antibody (anti-CTLA-4) and two anti-programmed death protein 1 antibodies (anti-PD1) [8]. Current preclinical and clinical trials are underway to determine the efficacy and benefits of combining immunotherapy

treatment regimen alone or in combination with BRAF and MEK inhibitors for treatment of patients with BRAF mutant melanoma [13–16]. New avenues exploring the possible combination therapies of BRAF/MEK inhibitors with immunotherapy drugs are being tested. Combination therapies are not only limited to MAPK pathway targeted therapies plus immunotherapy but have expanded to include other molecules such as AXL and ROS that have a role in the development of drug resistance. These have emerged as alternative treatment options for treating metastatic melanoma patients. Preclinical and clinical trials evaluating the efficacy of various PI3K and CDK4/6 inhibitors in combination with BRAF and MEK inhibitors are also initiated [17–22].

This review focuses on the current landscape of resistance with the chronic use of BRAF and MEK inhibitors in BRAF -mutant melanoma and progress made in the fields of immunotherapies and other small molecules when used alone or in combination with BRAF and MEK inhibitors to delay or circumvent the onset of resistance for patients with stage III/IV melanoma.

2. Mechanisms of Resistance

The development and use of the BRAF targeted inhibitors, Vemurafenib and dabrafenib, has improved the treatment arena for patients with metastatic melanoma. However, over half of patients treated with these single agent inhibitors begin to show signs of tumor recurrence within 6 to 7 months of treatment [23,24]. Several mechanisms of drug resistance have been proposed. There are two general types of resistance—primary resistance/intrinsic resistance and secondary or acquired resistance. Intrinsic resistance refers to those patients who do not respond to any type of BRAF inhibitor therapy and accounts for approximately 15% of patients [25]. Acquired resistance, refers to those patients who show tumor regression after an initial positive response to therapy and this is commonly observed in most melanoma patients [26].

2.1. Intrinsic Resistance

PTEN is a negative regulator of phosphoinositide 3-kinase (PI3K) and loss of PTEN can lead to an upregulation of the PI3K/Akt pathway whose activation can explain tumor resistance [27,28]. Loss of PTEN alone does not confer a resistance state; it is typically accompanied with Akt phosphorylation and activation [26,29]. Cyclin D1 amplification (CCND1) is observed in about 15–20% of all BRAF-mutant melanoma [30,31]. CCND1 alone can accelerate the resistance in BRAF-mutant melanoma and is intensified when there is both cyclin D1 overexpression along with a cyclin dependent kinase-4 (CDK4) mutation [32].

Neurofibromin-1 (NF1) is a tumor suppressor and a negative regulator of RAS. Loss of NF1 is typically seen in 14% of melanoma cases and leads to activation of RAS and other downstream pathways including the MAPK and PI3K-Akt [26,33]. Loss of NF1 can also mediate resistance to RAF and MEK inhibitors [34].

RAC1 is part of the Rho family of small GTP binding proteins. Mutations in RAC1 are found in 4–9% of patients and is the third “hotspot” mutation in melanoma, following BRAF and NRAS [35–37]. RAC1 mutation status is being considered a biomarker for resistance to RAF inhibitor therapy [35]. It has been shown that pre-existing mutations in MEK1 can confer resistance to RAF inhibitor therapy [38].

2.2. Acquired Resistance

Starting from the cell surface, several receptor tyrosine kinases (RTK) converge onto parallel pathways such as the MAPK and the PI3K-Akt pathway [39]. Upregulation of RTKs has been shown to directly activate the MAPK pathway via RAS activation [30]. Additionally, upregulation of specific RTKs such as the insulin like growth factor 1 receptor (IGFR1), platelet derived growth factor receptor β (PDGFR β) can activate the PI3K-Akt pathway in a non-ERK dependent manner [40,41]. Epigenetic changes affecting epidermal growth factor receptor (EGFR) have been shown to also induce the PI3K-Akt pathway in melanoma resistant cells [42]. Dual activation of these pathways strongly contributes to drug resistance, as these pathways promote cell survival and proliferation.

NRAS activating mutations are present in approximately 15–20% of melanomas [43]. Q61 mutations in NRAS keeps it constitutively active in the ‘RAS-GTP’ state [44]. The activated mutant NRAS can activate the MAPK pathway via induction of dimerization of CRAF and BRAF [45].

Treatment with BRAF inhibitors will only inhibit the mutant monomer in BRAF mutant melanoma cells [46]. This plays an important role in the ability for these cells to maintain RAF dimerization which in turn keeps MAPK signaling active. A phenomenon known as the ‘BRAF-inhibitor Paradox’ describes the event in which the BRAF inhibitor blocks MAPK signaling in mutant cells but activates the MAPK pathway in non-mutant cells by allowing the drug-free RAF protein to be transactivated and dimerize [47]. RAF dimerization can be fulfilled through a variety of mechanisms including alternative BRAF splicing, amplification of BRAF, and expression of different RAF isoforms such as CRAF overexpression [48–51]. Resistance to RAF inhibition include activation of HGF and its receptor MET which lead to the reactivation of the MAPK and PI3K-AKT pathways [52]. Screening for the presence of RAF inhibitor resistance genes, found in a high percentage of patients can help improve treatment outcomes especially when used in conjunction with appropriate therapeutic combinations [53].

Secondary mutations in both MEK1 and MEK2 have also been linked to acquired resistance in melanoma cell lines [54]. The resistance to MEK inhibitors is attributed to mutations in MEK1/2 which lead to constitutive activation of MEK1/2 or a mutation in the drug binding site [55]. MAPK reactivation occurs via secondary mutations in MEK1 (Q56P or E203K) which help reactive the MAPK pathway downstream [56]. Additionally, BRAF amplification along with KRAS mutations can be contributing factors to MEK1/2 inhibitor resistance [57].

The RTK AXL has also been identified as a player in both intrinsic and acquired resistance. Patients relapsed of BRAF and MEK inhibitors overexpress AXL as an adaptive response [58]. Non-genomic mechanism of acquired resistance include high expression of transcriptomic alterations and intra-tumoral immunity which involves cytolytic T-cell inflammation [59].

Combination therapy using BRAF and MEK inhibitors has also shown signs of resistance. Proposed mechanisms of resistance include BRAF gene amplification, BRAF splice-variants and mutations in MEK2 [60]. Resistance to BRAF and MEK inhibitors exists by combining or augmenting the mechanisms related to single agent BRAF inhibitor resistance. The overexpressed BRAFV600E and MEK mutants interact via the regulatory interface of BRAFV600E, R662 [61]. Acquired resistance to combination targeted therapy has other factors contributing to it, including whether the patient had received inhibitor monotherapy prior to the combination therapy or is ‘monotherapy naïve’ [62].

Many recent advancements in the treatment of metastatic melanoma have been in the field of immunotherapy. There are proposed mechanisms of resistance to BRAF and MEK inhibitor therapy involving immune system molecules. Cancer cells, in general, aim to avoid immune recognition by downregulating surface receptors that participate in co-activation of T cells as well as upregulating negative feedback pathways, such as the immune checkpoint inhibitor receptors programmed cell death protein (PD-1) and T-lymphocyte associated protein-4 (CTLA-4). PD-1 and CTLA-4 are localized on the T-cells [63]. It is documented that after 2 weeks of treatment with BRAF and MEK inhibitors, melanoma cells have been able to downregulate melanoma differentiation antigens (MDA) surface expression, decrease T cell activity, and surface display of increase immune checkpoint inhibitory receptors [64,65]. This manipulation of key immune system regulators gives melanoma cells yet another way to bypass drug resistance. The rationale to combine an immune checkpoint inhibitor therapy with targeted therapy is that the treatment with a BRAF and MEK inhibitor renders the tumor microenvironment more immunoresponsive [66].

The sequence of treatment for a patient with targeted therapy and immune therapy is not well established. Starting a patient on a BRAF inhibitor or anti-PD1 inhibitor is effective regardless of the treatment order, but more randomized controlled trials are required to address and establish the superiority and sequencing of one therapy over the other [67,68]. Studies have also shown that the efficacy of immunotherapy is improved in previously untreated patients compared to patients who have single agent immunotherapy failure or failure to targeted therapy [69]. Despite showing

exceptional clinical efficacy, treatment with immune checkpoint inhibitors has met some difficulties with respect to development of innate and acquired resistance [70,71]. Various clinical trials evaluating immune therapies such as Toll-like receptor 9 (TRL9) agonists, neoantigen vaccines and oncolytic viruses alone or in combination with immune checkpoint inhibitors are underway. Combination of these therapies may help combat resistance to immune checkpoint inhibitors [72–77].

3. Therapies

Figure 1 summarizes the therapies in pre-clinical and clinical phases, described in this review to treat patients with metastatic melanoma.

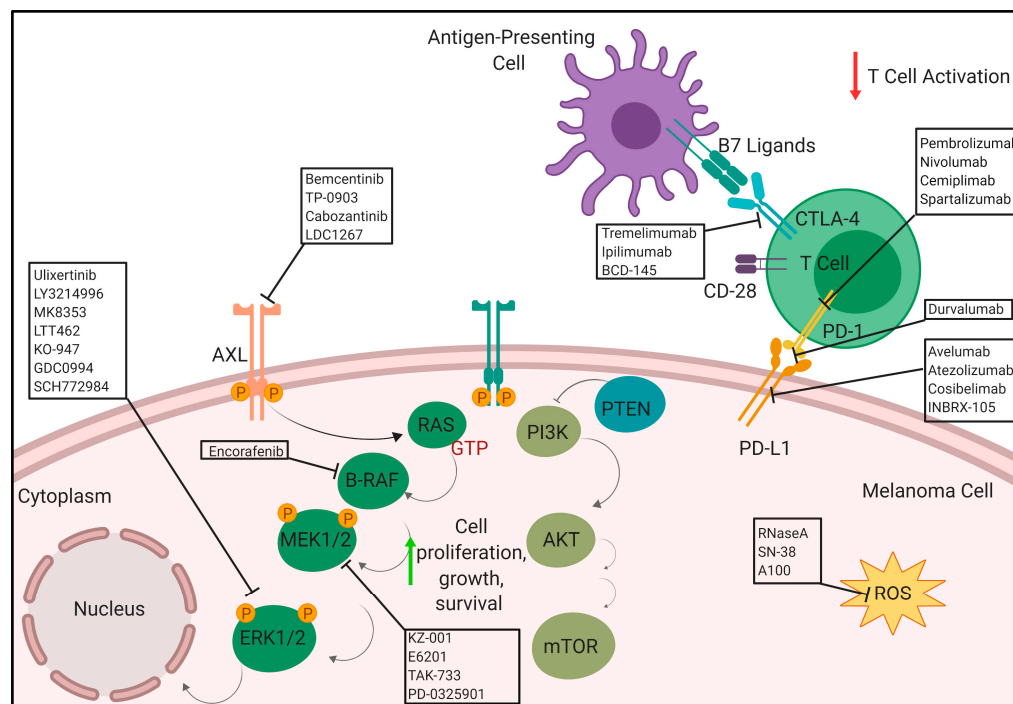


Figure 1. Novel therapies in pre-clinical and clinical phases (Anti PD-1/anti-PD-L1, anti CTLA-4, AXL inhibitors. BRAF inhibitors. ERK inhibitors and ROS activated prodrugs) to treat patients with metastatic melanoma (created with BioRender; www.biorender.com).

4. Anti-PD-1/PD-L1

The immunogenic nature of melanoma was utilized to develop several immunotherapeutic treatment strategies especially with regards to the programmed cell death (PD-1) receptor and its ligand, PD-L1. Antibodies targeting the PD-1 axis has shown significant promise in the clinic for treatment of metastatic melanoma either as a monotherapy or in combination with Ipilimumab. There are several ongoing clinical trials using anti-PD1 and anti-PD-L1 antibodies. Programmed cell death protein 1 (PD-1) is an inhibitory receptor expressed on the surface of the cancer cells that inhibits the immune system via suppressing the T-cell activity. Anti-PD-1 monoclonal antibodies block the PD-1 receptor which maintains T-cells in activated state to suppress the tumor growth [78]. There are several anti-PD-1/PD-L1 monoclonal antibodies including pembrolizumab (Keytruda®), nivolumab (Opdivo®), avelumab (Bavencio®), durvalumab (Imfinzi®), cemiplimab (Libtayo®), atezolizumab (Tecentriq®), cosibelimab and INBRX-105 in several stages of clinical trial in melanoma. Pembrolizumab, nivolumab and nivolumab in combination with iIpilimumab (anti-CTLA-4 inhibitor) have been approved by FDA for treatment of melanoma.

4.1. Pembrolizumab/Lambrolizumab/MK-3475/SCH 900475/Keytruda

This is a humanized monoclonal antibody targeting the PD1 receptor in the lymphocytes. It was developed by Merck and approved for treatment of metastatic melanoma in 2017 [79].

4.2. Nivolumab/ONO-4538/BMS-936558/MDX1106/Opdivo

This is a human IgG4 anti-PD-1 monoclonal antibody. Nivolumab works as a checkpoint inhibitor that inhibits T-cell activation. [80]. It was developed by Medarex and Ono Pharmaceutical, and is marketed by Bristol-Myers Squibb (BMS) and Ono. Nivolumab was approved by the FDA for melanoma in 2014 [81,82].

4.3. Avelumab/MSB0010718C/Bavencio

This is a humanized monoclonal antibody developed by Merck and Pfizer that targets the PD-L1. It has been approved by FDA for treatment Merkel-cell carcinoma, an aggressive type of skin cancer [83]. It blocks the PD-1/PD-L1 receptor/ligand complex formation leading to suppression of CD8+ T cells action [84]. There is a current clinical trial (NCT01772004) investigating the safety, tolerability, pharmacokinetics and clinical activity of avelumab in melanoma [85].

4.4. Durvalumab/MEDI4736/Imfinzi

This is monoclonal antibody that blocks the interaction of PD-L1 with the PD-1 and CD80 (B7.1) molecules developed by Medimmune/AstraZeneca [86,87]. A phase I clinical trial (NCT02586987) is ongoing evaluating the safety and efficacy of selumetinib (AZD6244 Hyd-sulfate) in combination with durvalumab (MEDI4736) along with tremelimumab in patients with advanced solid tumours, including melanoma [88].

4.5. Atezolizumab/MPDL3280A/Tecentriq

This is a fully humanized engineered monoclonal antibody of IgG1 isotype against PD-L1 developed by Genentech [89,90]. There is an active ongoing phase II trial (NCT02303951) which includes the combination of vemurafenib, cobimetinib and atezolizumab in stage III/IV advanced melanoma patients [91]. Another phase III study (NCT02908672) compares the efficacy of atezolizumab in combination with cobimetinib and vemurafenib versus placebo control plus cobimetinib and vemurafenib in unresectable and advanced melanoma patients with BRAFV600 mutation [92].

4.6. Spartalizumab/PDR001

Spartalizumab (PDR001) is a humanized monoclonal antibody against the negative immuno-regulatory human cell surface receptor programmed death-1 (PD-1, PCD-1) was developed by Novartis. This suppresses T-cell activation as it binds to PD-1 on activated T-cells and inhibits the interaction with its ligands, (PD-L1, PD-1L1) and (PD-L2, PD-1L2) [93]. A phase I/Ib (NCT03891953) study evaluating the efficacy of spartalizumab in combination with DKY709 (immunomodulatory agent) in patients with advances solid tumors including melanoma is ongoing [94]. A phase II PLATforM (NCT03484923) study evaluating the efficacy and safety of spartalizumab in combinations with LAG525 (monoclonal antibody targeting LAG-3), capmatinib (MET inhibitor), canakinumab (monoclonal antibody targeting IL-1 β) and ribociclib (CDK4/6 inhibitor) is ongoing in previously treated unresectable or metastatic melanoma [95]. A phase III COMBI-i study (NCT02967692) comparing the combination of spartalizumab, dabrafenib and trametinib versus dabrafenib and trametinib in previously untreated patients with unresectable or metastatic BRAFV600 mutant melanoma is initiated [96].

5. Anti-CTLA-4

In addition to PD-1, another immune checkpoint inhibitor, cytotoxic T-lymphocyte antigen 4 (CTLA-4), is important in melanoma. It is found on the surface of regulatory T cells (Treg) and activated T cells [97]. CTLA-4 competes with CD28, another receptor expressed on the surface of T cells, to interact with its two ligands CD80 and CD86, collectively known as the B7 ligands. When CTLA-4 binds with the B7 ligands, commonly found on antigen presenting cells (APC), it results in an immunosuppressive response, which is the inhibition of T cell activation via transendocytosis of CD80 and CD86 from their surfaces [98,99]. Typically, T cell activation requires co-stimulation from the CD28-B7 ligand interaction and the TCR-MHC interaction [100]. However, CTLA-4 has a stronger affinity for the B7 ligands, making it a good immune checkpoint inhibitor that keeps the immune response from turning into an autoimmune one [97]. CTLA-4 is expressed on tumor cells, infiltrating Tregs, and exhausted, activated T cells [101]. Tumor cells, therefore, take advantage of this natural immunosuppressive system in order to prevent an immune response against them. This provides a therapeutic approach which involves anti-CTLA-4 therapy. There are currently three main anti-CTLA-4 antibodies under preclinical and clinical trials for the treatment of melanoma: Tremelimumab, Ipilimumab (Yervoy), and BCD-145.

5.1. Tremelimumab/Ticilimumab/CP 675.206

This human monoclonal antibody against CTLA-4 was developed by AstraZeneca [102]. A phase I active, clinical trial (NCT02141542) is -evaluating tremelimumab in combination with MEDI3617 (human anti-angiopoietin 2 monoclonal antibody) for unresectable Stage III/IV melanoma patients [103]. Another phase I active, clinical trial (NCT01103635) is examining tremelimumab in combination with CP-870,893 CD40 agonist monoclonal antibody) for metastatic melanoma [104].

5.2. Ipilimumab/MDX010/BMS-734016

This human monoclonal antibody against CTLA-4 was developed by YERVOY Medarex/BMS. It was approved by the FDA in 2011 for the treatment of unresectable or metastatic melanoma [105]. There are current, active clinical trials devoted to assess the efficacy of ipilimumab in combination with other immunotherapies or targeted therapies for metastatic melanoma. A phase I clinical trial (NCT02115243) that assessed ipilimumab as a neoadjuvant followed by melphalan (chemotherapeutic) via isolated limb perfusion in patients with unresectable in-transit extremity melanoma is completed [106]. A phase Ib clinical trial (NCT02117362) evaluating ipilimumab in combination with GR-MD-02 (galnectin inhibitor) in metastatic melanoma patients has been completed [107]. A phase II clinical trial (NCT03153085) examining ipilimumab in combination with TBI-1401(HF10) in Japanese patients with Stage IIIb, IIIc, IV unresectable or metastatic malignant melanoma has been completed [108]. A phase II clinical trial (NCT01970527) looking at SBRT followed by Ipilimumab in patients with stage IV and recurrent melanoma has been completed [109].

5.3. BCD-145

This human monoclonal antibody against CTLA-4 is developed by BIOCAD [110]. A completed phase I clinical trial (NCT03472027) studied the efficacy of BCD-145 in unresectable/metastatic melanoma [111]. The combination of anti-PD-1/PD-L1 and anti-CTLA-4 are also being tested in the clinic for stage III/IV melanoma patients. A phase I clinical trial (NCT02935790) evaluating ipilimumab and nivolumab in combination with ACY-241 (selective HDAC inhibitor) is completed [112]. Current clinical trials, outcomes and adverse events investigating the efficacy of anti-CTLA-4, anti-PD-1/PD-L1 therapies and their combinations used to treat metastatic melanoma patients are listed in Table 1 [113–160].

Table 1. Summary of clinical trials, outcomes and adverse events associated with anti-PD-1/PD-L1, anti-CTLA-4 and their combination in metastatic melanoma patients.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Anti-PD-1/PD-L1					
Pembrolizumab in Japanese patients with advanced melanoma (KEYNOTE-041) [113]	Completed	Merck Sharp & Dohme Corp	Ib, NCT02180061	Pembrolizumab has promising anti-tumor activity and an acceptable safety profile in patients with cutaneous melanoma ($n = 29$). As per central review, the median overall survival (OS) and median duration of response was not reached. The 1 year OS was 82.7%. The median progression-free survival (PFS) and 6 months PFS was 4.2 months (95% CI: 2.8–7 months) and 41.4% respectively. The overall response, complete response (CR) and partial response (PR) were 24.1% (95% CI: 10.3–43.55), 6.9% (95% CI: 0.8–22.8%) and 17.2% (95% CI: 5.8–35.8%) respectively.	Pruritus, anemia, maculopapular rash, malaise, and hypothyroidism
Study of pembrolizumab (MK-3475) versus chemotherapy in patients with advanced melanoma KEYNOTE-002) [114]	Completed	Merck Sharp & Dohme Corp.	II, NCT01704287	The progression-free survival was improved in patients assigned to pembrolizumab 2 mg/kg (HR 0.57, 95% CI 0.45–0.73; $p < 0.0001$) and those assigned to pembrolizumab 10 mg/kg (0.50, 0.39–0.64; $p < 0.0001$) compared with those assigned to chemotherapy. 6-month progression-free survival was 34% (95% CI 27–41) in the pembrolizumab 2 mg/kg group, 38% (31–45) in the 10 mg/kg group, and 16% (10–22) in the chemotherapy group.	Fatigue, generalised oedema, myalgia, hypopituitarism, colitis, diarrhoea, anemia decreased appetite, hyponatremia, pneumonitis, neutropenia and leucopenia.
Pembrolizumab versus ipilimumab in advanced melanoma (KEYNOTE-006) [115]	Completed	Merck Sharp & Dohme Corp.	III, NCT01866319	Pembrolizumab showed superiority over ipilimumab at 5 year follow up time. 834 patients were stratified into three groups: (i) Pembrolizumab (10 mg/kg i.v. every 2 weeks), (ii) Pembrolizumab (10 mg/kg i.v. every 3 weeks) and Ipilimumab (3 mg/kg i.v. every 3 weeks). The median follow up was 57.5 months (IQR: 56.7–59.2 months). Combined Pembrolizumab groups: The median OS and PFS were 32.7 months (95% CI: 24.5–41.6 months) and 8.4 months (95% CI: 6.6–11.3 months) respectively. The ORR was 42% (95% CI: 38.1–46.5%). Ipilimumab groups: The median OS and PFS were 15.9 months (95% CI: 13.3–22 months; HR 0.73; 95% CI for HR- 0.61–0.88; $p = 0.00049$) and 3.4 months (95% CI: 2.9–4.2 months; HR 0.57; 95% CI for HR: 0.48–0.67; $p < 0.0001$) respectively. The ORR was 17% (95% CI: 12.4–21.4%).	Fatigue, colitis, diarrhea, asthenia, arthralgia, rash, pruritus, vitiligo.
Durvalumab in combination with Dabrafenib and Trametinib in patients with advanced melanoma [116]	Completed	MedImmune LLC	I. NCT02027961	Durvalumab in combination with dabrafenib and trametinib had manageable safety profile. No maximum tolerated dose was identified ($n = 50$) and durvalumab 10 mg/kg was selected for further studies.	Pyrexia, fatigue, diarrhea, rash, vomiting and other drug related toxicities

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Nivolumab in metastatic melanoma patients [117]	Active, not recruiting	BMS in collaboration with Ono Pharmaceutical Co. Ltd.	I, NCT00730639	Treatment with nivolumab is associated with long-term survival in patients with melanoma ($n = 72$). The median duration to response and objective response rate (ORR) was 22.9 months (95% CI: 19.7–31.8 months) and 31.8% respectively. The median, estimated 3 years and 5 year overall survival rates were 20.3 months (95% CI: 12.5–37.9 months), 42.3% (95% CI: 32.7–51.6%) and 34.2% (95% CI: 25.2–43.4%) respectively. Patients who had an ORR had significantly higher mean baseline absolute lymphocytes count (1480 cells/uL) as compared to patients without response (1300 cells/uL; $p = 0.4$).	Anemia, hypothyroidism, gastrointestinal disorder, general disorder, muscular disorder, nasopharyngitis, decreased appetite, nervous and respiratory problems, vascular and skin disorder
Atezolizumab in combination with vemurafenib alone or in combination with cobimetinib [118]	Active, not recruiting	Genentech, Inc.	Ib, NCT01656642	The triple combination was safe, tolerable and had a promising anti-tumor activity. Atezolizumab + Vemurafenib ($n = 17$): The best objective response rate and complete response rate was 76.5% (95% CI: 50.1–93.2%) and 17.6% respectively. All the patients demonstrated a reduction in the sum of the longest diameter of the target lesion. The median duration of response, PFS and OS was 10.6 months (95% CI: 9.1–37.6 months), 10.9 months (95% CI: 5.7–22 months) and 46.2 months (95% CI: 24.1-not reached) respectively. Estimated OS rates for 1 year were 82%. Atezolizumab + Vemurafenib + Cobimetinib ($n = 39$): The best objective response rate and complete response rate was 71.8% (95% CI: 55.1–85%) and 20.5% respectively. All the patients demonstrated a reduction in the sum of the longest diameter of the target lesion. The median duration of response and PFS was 17.4 months (95% CI: 10.6–25.3 months), 12.9 months (95% CI: 8.7–21.4 months) respectively. The median OS was not reached. Estimated OS rates for 1 year were 83%. Treatment with vemurafenib alone or in combination with cobimetinib exhibited an increase in the proliferating CD4+ T-helper cells and addition of atezolizumab led to further escalation in these cells. CD8+ cytotoxic T cells were augmented on addition of atezolizumab.	Increase in AST, ALT and blood bilirubin, hyponatremia, blood alkaline phosphatase, rash, diarrhea, vomiting.
Anti-CTLA-4					

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Tremelimumab in patients with advanced refractory and/or relapsed melanoma [119]	Completed	AstraZeneca	II, NCT00254579	Tremelimumab showed a durable response in these patients ($n = 241$). The ORR was 6.6% and the duration of response was 8.9–29.8 months. The median OS and clinical benefit rate was 10.1 months (95% CI: 7.9–11.7 months) and 21% respectively. The survival rate at 1 and 2 years was 40% (95% CI: 34–46%) and 22% (95% CI: 17–27%) respectively. Median PFS and 6 months PFS was 2.8 months (95% CI: 2.7–2.8 months) and 15% respectively. As per Response Evaluation Criteria in Solid Tumors (RECIST) criteria, 3.3% of the patients achieved response at the target lesion.	Diarrhea, pruritus, rash, nausea, fatigue, vomiting and colitis.
Ipilimumab alone or in combination with dacarbazine, paclitaxel and carboplatin [120]	Completed	BMS in collaboration with Medarex	I, NCT00796991	Ipilimumab could be combined safely with these chemotherapies with no major pharmacokinetic/pharmacodynamic interactions being observed in these patients. The combinations exhibited a good anti-tumor activity. Ipilimumab alone ($n = 20$): Estimated geometric mean for Area Under the Curve (AUC) (0-infinity) and maximum serum concentration (C_{max}) for Ipilimumab metabolite (AIC) in presence of ipilimumab was changed by 0.970 (90% CI: 0.891–1.056) and 1.058 (90% CI: 0.974–1.150) fold respectively. Based on World Health Organization (WHO) and immune-related criteria ORR were 29.4% and 33.3% respectively and disease control rates were 59.2% and 73.3% respectively. Ipilimumab + Decarbazine ($n = 19$): Estimated geometric mean for AUC (0-infinity) and C_{max} for dacarbazine in presence of ipilimumab was changed by 0.912 (90% CI: 0.757–1.099) and 1.027 (90% CI: 0.848–1.243) folds respectively. Based on WHO and immune-related criteria ORR were 27.8% and 33.3% respectively, and disease control rates were 55.6% and 61.1% respectively. Ipilimumab + paclitaxel + carboplatin ($n = 20$): Estimated geometric mean for AUC (0-infinity) and C_{max} for carboplatin/paclitaxel in presence of ipilimumab was changed by 0.970 (90% CI: 0.891–1.056) and 1.058 (90% CI: 0.974–1.150) folds respectively. ORR based on WHO and immune related criteria were 11.1% and 27.8% respectively. Disease control rate based on WHO and immune related criteria were 44.4% and 55.6% respectively. There was a significant increase in the mean relative frequency and counts of HLA-DR+ CD4+ and CD8+ T cells after treatment initiation in all the three groups.	Rash, fatigue, diarrhea, pruritus, nausea, increase in ALT and AST, decreased neutrophil count.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Ipilimumab in combination with imatinib mesylate in patients with advanced malignancies [121]	Completed	M.D. Anderson Cancer Center in collaboration with NCI	I, NCT01738139	The combination was well tolerated and safe and the MTD and recommended phase 2 dose for intravenous ipilimumab was 3 mg/kg every 3 weeks and imatinib mesylate at 400 mg orally twice daily. Twenty six patients were enrolled in dose escalation cohort Expression of ICOS and OX40 was increased on the CD4 + T cells upon ipilimumab treatment.	Fatigue, nausea, vomiting, edema, anemia, diarrhea, rash, fever.
Ipilimumab and high dose IFN- α 2B as a neoadjuvant combination for locally/regionally advanced/recurrent melanoma [122]	Completed	Diwakar Davar, University of Pittsburgh	I, NCT01608594	The combination was well tolerated and exhibited promising durable clinical response rates. 30 patients were enrolled. The median follow-up was 32 months and the pathologic complete response rate was 32% (95% CI: 18–51%). The radiologic response rate was 36% (95% CI: 21–54%). The median PFS was not reached and the probability of PFS at 12 and 6 months was 0.79 (95%CI: 0.65–0.95) and 0.86 (95% CI: 0.74–1) respectively. The probability of OS at 2 years and 1 year was 0.89 (95% CI: 0.79–1) and 0.93 (95% CI: 0.84–1) respectively. The peripheral blood mononuclear cell was significantly lower at 12 weeks ($p = 0.025$). The tumor-infiltrating lymphocyte (TIL) was significantly higher in primary melanoma tumors for patients with pathologic complete response ($p = 0.033$). There was an increase in the number of tumor associated clones following the neoadjuvant treatment and it showed a strong correlation with TIL fraction ($p = 0.7299$; $p = 0.0003$) and TIL clone diversity ($p = 0.882$; $p = 2.7–7$). The increase in the tumor T-cell clonality in the primary tumor and a further increase in the clonality after neoadjuvant therapy was statistically significant with relapse-free survival. ($p = 0.048$ for tumor clonality and $p = 0.018$ for post treatment metastatic clonality).	Fever, fatigue, creatinine increase, skin, GI, hepatic, endocrine and hematologic disorders.
Ipilimumab in combination with PEG-Interferon (IFN) α 2B on for Stage IIb/c/IV unresected melanoma [123]	Completed	H. Lee Moffitt Cancer Center and Research Institute in collaboration with Merck Sharp & Dohme Corp.	Ib, NCT01496807	The maximum tolerated dose established was 3 mg/kg of Ipilimumab and 2 ug/kg/week of peginterferon alfa-2 b was efficacious and well tolerated. 30 patients were enrolled. Immune related response criteria: 3.33% and 36.67% of the subjects achieved a CR and PR respectively. The overall response rate was 40%. The median follow up time was 35.8 months (Range: 19.7–50.2 months). The median PFS was 5.9 months and median OS was not reached. At 40.3 months, 16.7% of the patients had a prolonged PFS without any need for further therapy. 85.6% of the subjects had an objective response. Here was a significant correlation between autoimmune vitiligo and objective response ($p = 0.009$).	Anemia, dry eye, GI disorders, chills, fatigue, fever, increase in blood enzymes, anorexia, musculoskeletal and connective tissue disorders, nervous system disorders. Cough, dyspnea, depression, skin and subcutaneous tissue disorders

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Intratumoral injection of Ipilimumab and IL-2 for unresectable stage III/IV melanoma [124,125]	Completed	University of Utah	I, NCT01672450	The combination was well tolerated and generated an enhanced systemic immune response at injected and non-injected lesions in these patients. No dose limiting toxicities were observed in the 12 enrolled patients. Immune-related response criteria: Clinical benefit rate was 50% (95% CI: 19–81%). The PR and overall ORR was 30% and 40% (95% CI: 10–70%) respectively. 67% of the subjects (95% CI: 40–94%) had local response on the injected lesion which was assessed by pathology and/or measurement. 88.9% of the patients (95% CI: 68–100%) had an abscopal effect observed at distant and locoregional sites. Based on imaging and/or pathology, 40% of the patients (95% CI: 10–70%) demonstrated an objective response. 60% of the subjects had an increase in the frequency of CD8+ T cells expressing Tbet (fold increase: 1.75; 95% CI: 1.14–2.36) and IFNgamma (fold increase: 2.02; 95% CI: 1.31–2.73). 40% (fold increase: 1.83; 95% CI: 1.61–2.05) and 50% (fold increase: 1.50; 95% CI: 1.32–1.68) of the patients had an increase in the CD8+ T cells expressing granzyme-B and perforins.	Chills, fatigue, flu like symptoms, pain and ulceration at site of injection, rash, soft tissue infection. No grade 4/5 toxicities were observed.
Ipilimumab in combination with Stereotactic Radiosurgery (SRS) or Whole Brain Radiation Therapy (WBRT) [126]	Completed	Sidney Kimmel Cancer Center at Thomas Jefferson University in collaboration with BMS	I, NCT01703507	The combination of ipilimumab (3 mg/kg; $n = 7$ or 10 mg/kg; $n = 9$) and SRS was safe without any dose limiting toxicities. Arm A (WBTR; $n = 5$): The median follow-up time was 8 months (Range: 3.5–24.1 months). Median time to intracranial progression, PFS and OS were 2.53 months (Range 0.3–18 months), 2.5 months and 8 months respectively. Arm B (SRS; $n = 11$): the median follow-up time was 10.5 months (Range: 1.8–36.8 months). Median time to intracranial progression and PFS was 2.45 months (Range: 1–37 months) 2.1 months respectively. The median OS was not reached. Immune-related PR was 7%.	Neurotoxic effects, headache, GI toxicity, vomiting, subclinical intracranial hemorrhage, increase in ALT, dizziness, tinnitus. No grade 4/5 and radionecrosis were observed.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Ipilimumab in subjects with previously treated unresectable stage III/IV melanoma [127]	Completed	BMS in collaboration with Medarex	II NCT00289640	The three fixed doses used in this study were ipilimumab 10 mg/kg, 3 mg/kg or 0.3 mg/kg administered every 3 weeks for four cycles (induction phase) followed by maintenance therapy administered every 3 months. Ipilimumab had a dose-dependent efficacy and safety in these subjects and 10 mg/kg dose was well tolerated with manageable safety. 10 mg/kg ($n = 73$): The median follow-up was 10.7 months (Interquartile range [IQR]: 3.6–20.4 months). Best overall response rate was 11.1% (95% CI: 4.9–20.7). This dose had greater increase in absolute lymphocyte count and serum ipilimumab concentrations compared to the other doses at 4 weeks. 3 mg/kg ($n = 72$): The median follow-up was 8.3 months (IQR: 4–20.4 months). Best overall response was 4.2% (95% CI: 0.9–11.7). 0.3 mg/kg ($n = 32$): The median follow-up was 8.3 months (IQR: 3.5–15.3 months). Best overall response was 0% (95% CI: 0–4.9). The best overall response was significant for 10 mg/kg group ($p = 0.0015$). Median OS for 10 mg/kg vs 3 mg/kg had an HR of 0.875 (95% CI: 0.593–1.291). Median OS for 10 mg/kg vs 0.3 mg/kg had a hazard ratio (HR) of 0.77 (95% CI: 0.525–1.130).	Immune-related grade 3–4 events were GI related, skin related, nausea, vomiting, pruritus, rash, endocrine. Some immune related grade 5 adverse events were observed.
Ipilimumab in combination with Fotemustine for unresectable locally advanced or metastatic malignant melanoma with or without brain metastasis (NIMIT-M1) [128]	Completed	Italian Network for Tumor Biotherapy in collaboration with BMS	II, NCT01654692	The combination was efficacious and fotemustine did not impair the activity of Ipilimumab. The median follow up time was 39.9 months. The median duration of response, 3 and 2 year duration of response rates were 30.3 months (95% CI: 15.5–46.5 months), 49.2% (95% CI: 27.4–71%) and 55.4% (95% CI: 34.7–76.1%). Whole Study Population ($n = 86$): The median OS was 12.9 months (95% CI: 7.1–18.7 months). The 3 and 2 year survival rates were 28.5% and 33.4% respectively. The PFS and median brain PFS were 4.5 months (95% CI: 3.1–5.9 months) and 8.3 months (95% CI: 4.7–11.8 months) respectively. For patients with brain metastasis: The median OS, 3 and 2 years survival rates were 12.7 months (95% CI: 2.7–22.7 months), 27.8% and 38.9% respectively. The PFS and median brain PFS were 3.4 months (95% CI: 2.3–4.5 months) and 3 months (95% CI: 2.9–3.1 months). The median OS was not significant for patients with BRAF WT and V600E mutation. Subjects with improved OS had increased levels of circulating CD3⁺ CD4⁺ ICOS⁺ CD45RO⁺ T cells as opposed to CD3⁺ CD4⁺ ICOS⁺ CD45RA⁺ T cells. The expansion of memory T cells over naïve T-cells shows that the combination favors an increase in T-cell antigen-primed populations.	Rash, pruritus

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Ipilimumab in advanced melanoma patients with preexisting humoral response to NY-ESO-1 [129]	Completed	National Center for Tumor Diseases, Heidelberg in collaboration with University Hospital Heidelberg	II, NCT01216696	Ipilimumab demonstrated a higher clinical efficacy in patients with NY-ESO-1, maybe used as a surrogate for preexisting immune response to tumor antigens. 25 patients were enrolled in the study. The median duration of treatment was 64 days (Range: 1–352 days). Immune-related response criteria: the disease control rate was 52% (90% CI: 34.1–69.5%). 36% of the subjects had a PR. The PFS was 7.8 months (95%CI: 2.6-nr months). No significant association was observed between best response and the amount of NY-ESO-1-specific T-cells. RECIST criteria: 24% of the subjects had a PR. The PFS was 2.9 months (95%CI: 2.5–8.1 months). The median OS was 22.7 months (95%CI: 9.5-nr months). The 1 year survival rate was 66.8% (95%CI: 0.44–0.82). The best overall response (BOR) had a statistically significant association with the OS ($p = 0.0031$). For a small subset of patients there was a statistically significant association between PFS and NY-ESO-1-specific CD3+ T-cells (HR: 1.039; $p = 0.0478$).	Endocrine, GR, hepatobiliary, musculoskeletal and connective tissue disorders, headache, skin and subcutaneous tissue disorders
Vemurafenib followed by ipilimumab in V600 BRAF mutant, untreated metastatic melanoma patients [130]	Completed	BMS	II, NCT01673854	The study was divided into two parts: one where patients received vemurafenib followed by ipilimumab and the other where subjects who progressed after ipilimumab received vemurafenib. The sequential treatment was efficacious and had a manageable safety profile. The use of targeted therapy followed by immune modulation therapy has helped to understand the optimum regimen of these therapies. VEM1-IPI: 46 patients were treated with vemurafenib followed by 46 patients on ipilimumab induction and eight patients on ipilimumab maintenance. The median duration of response and follow-up was 23.1 months (95%CI: 5.03-not reached). The BOR was 32.6%. The median PFS and OS was 4.5 months (95%CI: 4.17–6.57 months) and 18.5 months (95%CI: 11.96-not evaluated). VEM2: 19 patients progressed on ipilimumab were treated with vemurafenib. The median follow-up and overall survival was 15.3 months and 18.5 months (95% CI: 11.96-not evaluated) respectively. The median PFS was 4.5 months (95% CI: 4.17–6.57 months). The BOR rate was 36.8%. 4.3% of patients had a CR and 28.3% had a PR.	Rash, erythema, pruritus, GI toxicities, hepatobiliary toxicities, nausea, vomiting.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Ipilimumab in combination with HF-10 (replication-competent HSV-1 oncolytic virus) in stage IIIb/c/IV unresectable or metastatic malignant melanoma [131]	Completed	Takara Bio Inc. in collaboration with Theradex	II, NCT02272855	The combination was well tolerated, beneficial and elicited anti-tumor activity. The combination induced an immune-cell infiltration in the TME. 46 patients were enrolled. The best overall response rate was at 24 weeks. Immune-related response criteria: 18% and 23% of the patients had a CR and PR respectively. The median PFS and OS were 19 months and 26 months respectively. There was increase on the total tumor infiltrating CD8+ T-cells and lymphocytes along with a decrease in the CD4+ T-cells.	Treatment related grade 3/4 events. Majority of the AEs were due to Ipilimumab which are immune related events.
Ipilimumab in combination with standard melphalan and dactinomycin to isolate limb infusion (ILI) for advanced unresectable melanoma of the extremity [132]	Completed	Memorial Sloan Kettering Cancer Center in collaboration with BMS	II, NCT01323517	The combination was safe and efficacious. 18 patients were enrolled. The median follow-up time was 18 months. At 3 months timepoint, 89% of the patients had a limb response from which 65% and 24% had a CR and PR, respectively. At 18 months, the median OS was 78%. The PFS at one year was 57%. The levels of eosinophils and ALC were elevated in all the subjects.	Limb toxicity, colitis, hypophysitis, rash.
Ipilimumab in Japanese patients with unresectable or metastatic melanoma [133]	Completed	BMS	II, NCT01990859	Ipilimumab was well tolerated and demonstrated an anti-tumor response in these patients ($n = 20$). The median OS and PFS was 8.71 months (95% CI: 3.71–nr months) and 2.74 months (95% CI: 1.25–2.83 months) respectively. The disease control rate and best overall response rates were 20% (95% CI: 5.7–43.7) and 10% (95% CI: 1.2–31.7) respectively. 10% of the subjects had a CR.	Rash, pruritus, pyrexia, GI disorder, increase in AST and ALT, skin, liver and endocrine related immune events. No grade 4 drug related adverse events were observed.
Ipilimumab in combination with temozolamide in metastatic melanoma patients [134,135]	Completed	M.D. Anderson Cancer Center in collaboration with BMS	II, NCT01119508	The combination exhibited an enhanced antitumor activity. 64 patients were enrolled. The median duration of response and median follow-up was 35 months (Range: 2–57 months) and 20 months (Range: 2–60 months). 15.6% of the subjects had a CR and PR. The median PFS and OS was 5 months and 24.5 months respectively. 6 months PFS was 45%. The PFS for patients with bone metastasis was significantly decreased ($p = 0.014$) but not for subjects with liver metastasis. 21% and 7% of the subjects with liver metastasis had a CR and PR respectively while no ORR was observed in subjects with bone metastasis	Pruritus, skin rash, nausea, constipation, diarrhea, colitis, increase in ALT and AST, hematologic toxicities. No drug related grade 5 toxicities were observed.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Ipilimumab as monotherapy for previously treated unresectable stage III/IV melanoma [136,137]	Completed	BMS in collaboration with Medarex	II, NCT00289627	Ipilimumab demonstrated good clinical activity in these patients which consists of subjects who did not respond to prior therapy. 155 patients were enrolled in the study. The median follow-up and OS was 10 months (Range 0.32–33.1 months) and 10.2 months (95% CI: 7.6–16.3%). The best overall response rate and disease control rate was 5.8% (95% CI: 2.7–10.7%) and 27% (95% CI: 20–35%) respectively. 5.8% of the subjects had a PR and no CR was observed. The 1 year, 18 months and 2 year survival rates were 47.2% (95% CI: 39.5–55.1%), 39.4% (95% CI: 31.7–47.2%) and 32.8% (95% CI: 25.4–40.5%), respectively.	Immune related grade 3 and 4 events - skin and GI tract, liver and endocrine. No grade 5 immune related adverse events were observed.
Ipilimumab in combination with autologous TriMix-DC therapeutic vaccine for previously treated unresectable stage III/IV melanoma [138,139]	Completed	Bart Neyns, Universitair Ziekenhuis Brussel in collaboration with Vrije Universiteit Brussel	II, NCT01302496	The combination was well tolerated and demonstrated a durable anti-tumor response. 39 patients were enrolled in the trial. The median follow-up time, estimated median PFS and OS were 36 months (Range: 22–43 months), 27 weeks (95% CI: 9–44 weeks) and 59 weeks (Range: 72–185 weeks) respectively. PFS and OS rates at year one- 33% (95% CI: 18–48%), 59% (95% CI: 43–74%); year two- 22% (95% CI: 9–36%), 38% (95% CI: 23–53%) and year three- 18% (95% CI: 5–31%), 34% (95% CI: 19–50%). Immune-related response criteria: 6 months disease control rate was 51% (95% CI: 36–67%). The ORR was 38%. 20% and 18% of the subjects had a CR and PR respectively. The outcome was poor in patients with brain metastasis. There was a significant increase in the eosinophils, peripheral blood lymphocytes and monocytes after from the baseline treatment with the combination. A significant increase in the CD8+ ($p < 0.001$), CD4+ ($p < 0.001$), HLA-DR+ activation marker on CD4+ cells ($p < 0.001$), CD3+ ($p < 0.001$), ratio of CD4+ to CD8+ ($p = 0.003$) and Tregs ($p = 0.016$) was observed after the combination treatment.	Dermal injection site reactions, post-infusion chills, flu-like symptoms. Immune related adverse events- dermatitis, colitis, diarrhea, hypophysitis, pneumonitis, lymphadenopathy. Grade 3/4 immune related adverse events were observed with no grade 5 events.
Ipilimumab in combination with WBRT for melanoma patients and brain metastases (GEM STUDY, GRAY-B) [140,141]	Completed	Grupo Español Multidisciplinar de Melanoma in collaboration with BMS	II, NCT02115139	The combination was well tolerated and safe. Fifty eight patients were enrolled in the study. The overall survival at 1 year was 31.8% (95% CI: 18.8–44.8%). The median OS and PFS was 5.8 months (95% CI: 3.6–5.9 months) and 4.8 months (95% CI: 2.2–3.4 months).	WBRT related- vomiting and headache. Immune therapy related- diarrhea, intestinal perforation, increase in AST and ALT, headache.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Ipilimumab in combination with SRS for subjects with brain metastasis [142]	Completed	University Hospital, Lille in collaboration with BMS	II, NCT02662725	High dose of ipilimumab and SRS was effective with a manageable safety profile. 57 patients were enrolled in the study. Median survival time for reference population vs study population was 5.6 months vs 13.2 months (HR: 0.29, 95% CI: 0.19–0.39; $p < 0.0001$) with 49% disease control rate.	Colitis, hepatitis, hypophiitis, headache. One subject showed radionecrosis.
Ipilimumab alone or in combination with decarbazine in previously untreated metastatic melanoma patients [143]	Completed	BMS	II, NCT00050102	The combination was well tolerated and a durable, clinically meaningful responses were observed. Decarbazine did not affect the PK of ipilimumab. Ipilimumab alone ($n = 37$): The median follow up time and median OS were 16.4 months and 11.4 months (95% CI: 6.1–15.6 months) respectively. The objective response rate and disease control rate were 5.4% (95% CI: 0.7–18.2%) and 21.6% (95% CI: 9.8–38.2%), respectively. 5.4% of the subjects had a PR. The survival rates for 1 year, 2 year and 36 months were 45%, 21% and 9%, respectively. Combination ($n = 35$): The median follow up time and the OS were 20.9 months and 14.3 months (95% CI: 10.2–18.8 months) respectively. The objective response rate and disease control rate was 14.3% (95% CI: 4.8–30.3%) and 37.1% (95% CI: 21.5–55.1%) respectively. 5.7% and 8.6% of the patients had a CR and PR for more than 24 weeks respectively. The survival rates for 1 year, 2 year and 36 months were 62%, 24% and 20% respectively. CD4+ and CD8+ expressing HLA-DR T cells were increased in both the groups.	Colitis, muscle weakness, anemia, tachycardia, abdominal pain. Fatigue, dehydration, increased ALT/AST, rash, pruritus, vasculitis.
Ipilimumab in combination with HF10 for unresectable Stage IIb/c/IV or metastatic malignant melanoma [144]	Completed	Takara Bio Inc. in collaboration with Theradex	II, NCT02272855	The combination was well tolerated with positive antitumor activity and there were no dose limiting toxicities. 46 patients were enrolled. The median PFS and OS was 19 months and 21.8 months respectively. Immune-related response criteria: The best overall response rate and disease stability rate was 41% and 68%, respectively. 16% and 25% of the patients had CR and PR, respectively.	Embolism, lymphedema, diarrhea, hypoglycemia, groin pain, immune related events.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Decarbazine alone or in combination with ipilimumab for unresectable stage III/IV melanoma [145]	Completed	BMS in collaboration with Medarex	II, NCT00324155	The combination of ipilimumab and decarbazine was well tolerated and had a long-term durable overall survival. Ipilimumab and decarbazine group ($n = 250$): The median survival follow-up time and OS was 11 months (Range: 0.4–71.9 months) and 11.2 months (95% CI: 9.5–13.8 months) respectively. At 5 years, 18.2% of the patients were alive which was significantly higher than that in the other group ($p = 0.002$). 7.5% and 42.5% of the patients had a CR and PR respectively. No median OS was reached for the responders while that for the non-responders was 14.3 months (95% CI: 11.4–16.9 months; HR: 0.28, 95% CI: 0.16–0.47). Decarbazine and placebo group ($n = 252$): The median survival follow-up time and OS was 8.9 months (Range: 0.1–73.2 months) and 9.1 months (95% CI: 7.8–10.5 months; HR: 0.69; 95% CI: 0.57–0.84) respectively. At 5 years, 8.8% of the patients were alive. 35% of the patients had a PR and no CR was achieved. The median OS for non responders and responders was 12.3 months (95% CI: 10.9–15.4 months) and 20.2 months (95% CI: 14.6–45.3 months; HR: 0.51, 95% CI: 0.32–0.84), respectively.	Rash, pruritus, vitiligo, GI, liver and endocrine related events. Grade 3 to 4 immune related adverse events were observed in skin with no grade 5 events.
Ipilimumab antibody (MDX-010) alone or in combination with melanoma peptide vaccine (MDX-1379; gp100) for previously untreated unresectable stage III/IV melanoma [146]	Completed	BMS	III, NCT00094653	Overall ipilimumab resulted in survival of 20% of the patients for more than 2 years. 45% of the patients who survived for more than 2 years survived for more than 3 years. Ipilimumab + placebo ($n = 137$): 25% of the patients survived for more than 2 years and 3 years. The disease control rate for on-study response and for patients surviving more than 2 years was 28.5% (1.5% CR and 9.5% PR) and 83.3% (8.3% CR and 41.7% PR) respectively. Gp100 vaccine alone ($n = 136$): 17% and 10% of the patients survived for more than 2 year and 3 years respectively. The disease control rate for on-study response and for patients surviving more than 2 years was 11% (1.5% PR) and 43.8%, respectively. Combination ($n = 403$): 19% and 15% of the patients survived for more than 2 years and 3 years respectively. The disease control rate for on-study response and for patients surviving more than 2 years was 20.1% (0.2% CR and 5.5% PR) and 66.7% (1.9% CR and 22.2% PR), respectively.	Immune related adverse events—colitis, vitiligo, diarrhea, hypogonadism, proctitis, dermatologic, GI, endocrine related events, increase ALT. No grade 4/5 immune related adverse effects were observed.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Ipilimumab doses- 3 mg/kg vs 10 mg/kg for previously treated or untreated unresectable or metastatic melanoma [147]	Completed	BMS	III, NCT01515189	Both doses were well tolerated, with 10 mg/kg dose having more treatment-related events. 10 mg/kg group ($n = 364$): The median follow-up time, OS and PFS were 14.5 months (IQR: 4.6–42.3 months), 15.7 months (95% CI: 11.6–17.8 months) and 2.8 months (95% CI: 2.8–3 months) respectively. 2% and 13% of the patients had a CR and PR respectively. The 1 year, 2 year and 3 year overall survival was 54.3% (95% CI: 49–59.3%), 38.5% (95% CI: 33.4–43.5%) and 31.2% (95% CI: 26.4–36%), respectively. 3 mg/kg group ($n = 362$): The median follow-up time, OS and PFS was 11.2 months (IQR: 4.9–29.4 months), 11.5 months (95% CI: 9.9–13.3 months) and 2.8 months (95% CI: 2.8–2.8 months), respectively. 2% and 10% of the patients had a CR and PR respectively. The 1 year, 2 year and 3 year overall survival was 47.6% (95% CI: 42.4–52.7%), 31% (95% CI: 26.2–35.8%) and 23.2% (95% CI: 18.9–27.7%), respectively. HR between both the groups for median overall survival was 0.84 (95% CI: 0.7–0.99; $p = 0.04$). EORTC QLQ-C30 global health status were significantly declined in both the groups from the baseline.	Headache, diarrhea, colitis, increase in ALT, hypophysitis. No grade 5 toxicities were observed.
Ipilimumab alone or in combination with talimogene laherparepvec (T-VEC) in patients with previously untreated unresected, Stage IIIb-IV melanoma [148]	Active, not recruiting	Amgen	Ib/II, NCT01740297	The combination was well tolerated and had a greater systemic antitumor response (in uninjected and visceral lesions) as compared to single agent. Ipilimumab + Laherparepvec ($n = 98$): The median duration of treatment with laherparepvec and ipilimumab was 21.1 and 9.1 weeks, respectively. The median followup time and time to response was 68 weeks (Range: 0–156 weeks) and 5.8 months (95% CI: 5.4–10.9 months) respectively. 39% of the patients had an ORR (13% CR and 26% PR) and visceral lesions decrease was observed in 52% of the patient population. The median PFS was 8.2 months (95% CI: 4.2–21.5 months). Ipilimumab alone ($n = 100$): The median duration of treatment of ipilimumab was 9.1 weeks. The median followup time was 58 weeks (Range: 0–152 weeks). The median time to response was not estimated (HR = 1.41; 95% CI: 0.8–2.5). 18% of the patients had an ORR (7% CR and 11% PR; OR: 2.9, 95% CI: 1.5–5.5, $p = 0.002$) and visceral lesion decrease was observed in 23% of the patient population. The median PFS was 6.4 months (95% CI: 4.2–21.5 months).	Fatigue, chills, GI disorders, pruritus, rash and nausea.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Ipilimumab in Stage IV melanoma patients receiving palliative radiation therapy [149]	Active, not recruiting	Stanford University	II, NCT01449279	The combination was safe and efficacious. 22 patients were enrolled and treated in the study. 50% of the patients had benefited from the therapy including CR and PR at 55 week follow-up. 27.3% of the patients had an ongoing systemic complete response to the combination (95% CI- 9.7–56.9%) with no evidence of disease at 55 week. 27.3% (95% CI- 9.7–56.96%) of the patients has an initial PR without progression for median of 40 weeks (Range: 29–53). The median PFS was 26 weeks (Range: 2–65; 95% CI: 16.3–35.7) and the median overall survival was 55 weeks (Range: 8–141; 95% CI: 39.2–70.8) with the patients receiving the combination. The median time for response for patients who had CR or PR was 19 weeks (Range: 12–52). The strong antitumor response in patients with CR or PR can be attributed to increased levels of IL-2 producing CD8+ T cells and central memory CD8+ T cells in comparison to patients with melanoma could be used as biomarkers further.	Colitis, hypophysitis, rash, anemia, nausea and radiation dermatitis.
Ipilimumab alone or in combination with sargramostim (GM-CSF) in Stage III/IV melanoma that cannot be removed surgically [150]	Active, not recruiting	NCI	II, NCT01134614	The combination was advantageous and had a lower toxicity profile. The median follow-up was 13.3 months (Range: 0.03–19.9 months). <u>Ipilimumab + Sargarmostim ($n = 132$):</u> The median OS was 17.5 months (95% CI: 14.9-not reached). The one year survival rate was 68.9% (95% CI: 60.6–85.5%). <u>Ipilimumab alone ($n = 122$):</u> The median OS was 12.7 months (95% CI: 10-not reached). The one year survival rate was 52.9% (95% CI: 43.6–62.2%). There was no difference in PFS across both the groups.	GI toxicity, pulmonary toxicities,
Ipilimumab as adjuvant therapy after complete resection of high risk stage III melanoma [151]	Active, not recruiting	BMS	III, NCT00636168	Addition of Ipilimumab as adjuvant therapy benefited patients with microscopic involvement only (sentinel node-positive) and for patient with macroscopic or palpable nodes. The median follow up was 5.3 years. <u>Ipilimumab group ($n = 475$):</u> The 5 years recurrence-free survival and OS was 40.8% and 65.4% respectively. The rate of distant metastasis free survival at 5 years was 48.3%. <u>Placebo group ($n = 476$):</u> The 5 years recurrence free survival and OS was 30.3% (HR: 0.76; 95% CI: 0.64–0.89; $p < 0.001$) and 54.4% (HR: 0.72; 95% CI: 0.58–0.88; $p = 0.001$) respectively. The rate of distant metastasis free survival at 5 years was 38.9% (HR: 0.76; 95% CI: 0.64–0.92; $p = 0.002$).	Immune related adverse events-GI, hepatic, endocrine, skin and neurologic.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Combination of Anti-PD-1/PD-L1 and anti-CTLA-4					
Ipilimumab in low dose as an adjuvant in combination with nivolumab after resection of melanoma macrometastases [152]	Completed	Universitair Ziekenhuis Brussel	Ib, NCT02941744	Ipilimumab at low doses in combination with nivolumab had an acceptable safety profile. Ipilimumab (50 mg) + Nivolumab (10 mg) ($n = 34$): the median follow up was 86 weeks. One year relapse-free survival, overall survival and distant metastasis-free survival was 55% (95% CI: 39–72%); 97% (95% CI: 94–100%) and 79% (95% CI: 65–92%) respectively. Median relapse free survival was 84 weeks (95% CI: 28–139 weeks). Nivolumab (10 mg) ($n = 22$): The median follow up was 36 weeks. One year relapse-free survival and overall survival was 78% (95% CI: 73–82%) and 100% respectively. Distant metastasis was not observed. The median relapse free survival was not reached.	4–8% grade 3 immune related adverse events were across both the cohorts.
LTX-315 alone or in combination with Ipilimumab or Pembrolizumab in patients with transdermally accessible tumors [153,154]	Completed	Lytix Biopharma AS in collaboration with Theradex and ICON plc	I, NCT01986326	Combination of immune checkpoint inhibitors with LTX-315 was safe and tolerable and demonstrated a potent anti-tumor activity. Of 6 melanoma patients received LTX-315 in combination with Ipilimumab, stable disease was observed in 33% of the patients. LTX-315 when administered to patients with solid tumors resulted in increase in number of CD8+ T cells at the site of treated lesions along with tumor infiltrating lymphocyte population. Clonal expansion of T-cells in blood was observed after treatment with LTX-315 as revealed by T-cell receptor sequencing.	LTX-315-related grade 3 and 4 adverse events (allergic/anaphylaxis) were observed along with tingling post injection, rash, fatigue, diarrhea, hypo and hyper tension, weakness.
Nivolumab and Ipilimumab alone or in combination in patients with previously untreated unresectable or metastatic melanoma (CheckMate067) [155]	Active, not recruiting	BMS	III, NCT01844505	Combination of ipilimumab and nivolumab or nivolumab alone was superior over monotherapy with ipilimumab. No new toxic effects associated with chronic use of these therapies were observed. Nivolumab plus Ipilimumab group ($n = 314$): Median overall survival was more than 60 months. Hazard ratio for death versus Ipilimumab group was 0.52. 5 year overall survival rate was 52%. Nivolumab group ($n = 316$): Median overall survival was 36.9 months. Hazard ratio for death versus Ipilimumab group was 0.63. 5 year overall survival rate was 44%. Ipilimumab group ($n = 315$): Median overall survival was 19.9 months. 5 year overall survival rate was 26%.	

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Ipilimumab in combination with Nivolumab in patients with unresectable Stage III/IV malignant melanoma [156]	Active, not recruiting	BMS in collaboration with Medarex and Ono Pharma USA Inc	Ib, NCT01024231	The combination of Ipilimumab and nivolumab had durable clinical activity in patients with advanced melanoma. 94 patients were enrolled in the study. At the target lesions, the mean reduction in tumor burden was around 64.7%. The median follow-up was 30.3 to 55 months while the median OS was not reached at 3 years. The median PFS was 6.2 months (95% CI: 3.2–11 months). The median duration of response was 22.3 months (95% CI: 13.8–25.8 months). The best overall response rate and objective response rate by modified WHO criteria were 19.1% and 41.5% (95% CI: 31.4–52.1%). The CR and PR rates were 22.3% and 16% respectively. The OS and median PFS rates at 1 year were 81% (95% CI: 71–87%) and 37% (95%CI: 27–47%) respectively. The OS and median PFS rates at 2 years were 72% (95% CI: 62–80%) and 28% (95% CI: 19–38%) respectively. The OS and median PFS at year 3 were 63% (95% CI: 52–72%) and 17% (95% CI: 8–29%) respectively.	Grade 3 and 4 immune related toxicities such as rash, diarrhea, increase in lipase, AST, ALT and amylase, rthralgia, colitis, were observed.
Nivolumab alone or in combination with ipilimumab in melanoma patients with brain metastases [157]	Active, not recruiting	Melanoma Institute Australia in collaboration with Melanoma and Skin Cancer Trials Limited and BMS	II, NCT02374242	The combination of Nivolumab and ipilimumab was active in melanoma brain metastases with a durable intracranial and extra cranial response. Cohort A ($n = 36$): Nivolumab (3 mg/kg) + Ipilimumab (3 mg/kg) Cohort B ($n = 27$): Nivolumab (3 mg/kg) Cohort C ($n = 16$): patients with brain metastases for whom local therapy failed/showing neurological symptoms/leptomeningeal disease were administered nivolumab (3 mg/kg). As per RECIST criteria, the median follow up was 17 months (IQR: 8–25 months). The overall survival at 6 months in cohorts A,B and c were 78% (95% CI: 65–94%), 68% (95% CI: 52–89%) and 44% (95% CI: 25–76%) respectively. Intracranial response: The overall response in cohorts A, B and C was 46% (95% CI: 29–63), 20% (95% CI: 7–41%) and 6% (95% CI: 0–30%) respectively. The PFS at 6 months in cohorts A, B and C was 53% (95% CI: 38–73%), 25% (95% CI: 9–44%) and 13% (95% CI: 5–65%) respectively. Extra cranial response: the overall response in cohorts A, B and C was 57% (95% CI: 37–75%), 29% (95% CI: 11–52%) and 25% respectively. The PFS at 6 months in cohorts A, B and C were 51% (35–76%), 35% (95% CI: 19–64%) and 19% (95% CI: 5–65%) respectively.	Grade 1 and 2 treatment related adverse events were commonly observed such as skin, GI, endocrine, musculoskeletal, respiratory related and fatigue. Grade 3/4 related adverse events were infrequent.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Pembrolizumab in combination with reduced dose Ipilimumab or Pegylated Interferon Alfa-2b in patients with advanced melanoma (KEYNOTE-29) [158]	Active, not recruiting	Merck Sharp & Dohme Corp.	I/II, NCT02089685	While the combination of Pembrolizumab and ipilimumab had good antitumor activity and manageable safety profile, the combination of pembrolizumab and PEF-INF did not. <u>Pembrolizumab and Ipilimumab ($n = 12$):</u> The median follow-up was 25.1 months (Range: 0.8–38.7 months). The median duration of response was not reached. The objective response rate as per independent central review was found to be 42% (95% CI: 15–72%). The CR and PR rates were 8.33% and 33.33% respectively. As per investigator review, the objective response rate and PR were 33% (95% CI: 10–655) and 33.33% respectively. <u>Pembrolizumab and PEG-IFN ($n = 17$):</u> The median follow up was 22.2 months (Range- 25–377 months). As per central and investigator review, the objective response rate was 20% and the partial response rate was 20%.	Pembrolizumab and Ipilimumab: Grade 1/2 treatment related adverse events- fatigue, diarrhea, rash, nausea, colitis, increased lipase, ALT and AST. Immune-related adverse events- colitis, hyper and hypo thyroidism. <u>Pembrolizumab and PEG-IFN:</u> Treatment related AEs- elevation is AST, ALT nerve disorder, fatigue, chills, pyrexia, diarrhea, rash, pruritus, nausea, anemia. Immune-related AEs- hyperthyroidism, pneumonitis, hepatitis.
Nivolumab combined with Ipilimumab or Ipilimumab alone in patients with untreated, unresectable or metastatic melanoma [159]	Active, not recruiting	BMS	II, NCT01927419	The median follow-up was 24.5 months (IQR: 9.1–25.7 months). <u>Ipilimumab alone ($n = 47$):</u> The 2 years overall survival was 53.6% (95% CI: 38.1–66.8%). The objective response rate and PR were 11% (95% CI: 3–23%) and 11% respectively. The median PFS was 3 months (95% CI: 27–5.1 months; HR: 0.36; 95% CI for HR: 0.22–0.56, $p < 0.0001$). The PFS and 1 and 2 year was 16% (95% CI: 6.6–28.9) and 152% (95% CI: 3.8–25.2%) respectively. <u>Nivolumab plus Ipilimumab ($n = 95$):</u> The 2 years overall survival was 63.8% (95% CI: 53.3–72.6%). The objective response rate, CR and PR were 59% (95% CI: 48–69%), 22% and 37% respectively. The median PFS was not reached. The PFS at 1 and 2 years was 52.5% (95% CI: 41.6–62.3%) and 51.3% (95% CI: 40.4–61.2%) respectively. The median overall survival was not reached in either group (HR: 0.74, 95% CI 0.43–1.26; $p = 0.26$).	Colitis, Diarrhea, Hypophysitis, Pneumonitis, Anaemia, Hypothyroidism, increased ALT.

Table 1. Cont.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
Nivolumab administered sequentially with Ipilimumab in subjects with advanced or metastatic melanoma (CheckMate064) [160]	Active, not recruiting	BMS	II, NCT01783938	Nivolumab followed by ipilimumab was more clinically beneficial. Nivolumab followed by Ipilimumab ($n = 70$): The median overall survival was not reached. The 1 year overall survival was 76% (95% CI: 64–85%). The overall response rate, CR and PR was 56% (95% CI: 43.3–67%), 12% and 44% respectively. Ipilimumab followed by Nivolumab ($n = 70$): The median overall survival was 16.9 months (95% CI: 9.2–26.5 months; HR: 0.48; 95% CI for HR: 0.29–0.8). The 1 year overall survival was 54% (95% CI: 42–65%). The overall response rate, CR and PR was 31% (95% CI: 20.9–43.6%), 6% and 26% respectively.	Pruritus, rash, fatigue, chills, pyrexia, vitiligo, diarrhoea, nausea, increased ALT, AST and lipase.

6. AXL Inhibitors

The TAM family of receptor tyrosine kinases (RTKs) is comprised of Tyro-3, Axl and Mer (TAM). These TAMs regulate cell proliferation, survival, adhesion, migration, invasion and metastasis of neoplasms [161]. The AXL gene is located on chromosome 19q13.2; encoded by 20 exons. The protein structure consists of an extracellular domain consisting of a combination of two IgG like domains and two fibronectin type III repeats; a conserved intracellular kinase domain and a transmembrane domain [162,163]. Even though all three TAMs have transforming potential, the aberrant overexpression of Axl is associated with cancer progression, drug resistance and supports tumor immune escape in several cancers including melanoma [164–172]. In primary and acquired resistance in melanoma, Axl levels inversely correlate with levels of melanocyte lineage factor- Microphthalmia-associated transcription factor (MITF). The high Axl, low MITF drug resistance phenotype is found frequently among BRAF mutant melanoma cell lines. This is associated with a phenotype switch of cells from proliferative to an invasive phenotype and promotes metastasis [165,173,174]. The Axl inhibitors can be classified into 2 types. Type I encompasses inhibitors that compete with ATP and bind to the active conformation of the receptor, DGF-in (constitutes of the aspartate-phenylalanine-glycine (DFG) motif oriented towards the active site). Type II inhibitors interact with the DFG residues of the activation loop which open up an allosteric region, adopt an extended conformation and prefer binding to the inactive DFG-out conformation. [175]

6.1. BGB-324/Bemcentinib (Type I)

This highly selective orally bioavailable inhibitor was developed by BerGenBio [176]. Upregulation of Axl leads to drug resistance of BRAF directed therapies in the context of melanoma and also reduces response to PD-1 blockade. A Phase Ib/II trial is ongoing (NCT02872259) evaluating BGB324 in combination with dabrafenib/trametinib or pembrolizumab in advanced non-resectable Stage IIIc/IV melanoma. The interim results for this study indicate that the combination was well tolerated at the recommended phase 2 dose of 200 mg daily of Bemcentinib. The common adverse events were diarrhea, fatigue, rash and pyrexia [177].

6.2. TP-0903 (Type I)

This oral Axl kinase inhibitor was developed by Tolero Pharmaceuticals, Inc. A first-in-human phase Ia/Ib trial (NCT02729298) evaluating TP-0903 in patients with advanced solid tumors encompassing BRAF mutated melanoma patients who haven't responded to BRAF/MEK inhibitor combination or immunotherapy is currently recruiting patients [178,179].

6.3. Cabozantinib/XL184/BMS-907351 (Type II)

This inhibitor, developed by Exelixis [180], is an orally bioavailable small molecule inhibitor against various tyrosine kinases which include Axl, MET and VEGF. A phase II trial (NCT00940225) evaluating cabozantinib in patients with metastatic melanoma was discontinued as the study was underpowered to detect statistical significance [181]. A phase I/II trial (NCT03957551) evaluating the combination of cabozantinib and pembrolizumab as a front-line therapy has been initiated for patients with advanced metastatic melanoma [182].

6.4. LDC1267 (Type II)

This inhibitor preferentially inhibits Axl, Mer and Tyro3. In an in vivo model, it was observed that treatment with LDC1267 unleashes natural killer (NK) cells to target and kill tumor cells. Treatment with LDC1267 reduced the metastatic spreading of melanoma in an in vivo B16F10 melanoma model [183]. Further pre-clinical and clinical trials need to be initiated to test the efficacy of this drug in melanoma.

6.5. AXL-1047-MMAE

This is an antibody-drug conjugate (ADC) in which the Axl targeting human antibody is conjugated to monomethyl auristatin E (MMAE), a microtubule disrupting agent by a valine citrulline linker which is protease-cleavable. This ADC induces cytotoxicity in vitro and in vivo in melanoma models. Treatment with the ADC prevents the emergence of BRAF-inhibitor resistant clones and potentiates the efficacy of BRAF and MEK inhibitors and co-operatively targets the growth of resistant cells. This ADC along with BRAF and MEK inhibitors has shown efficacy in treatment naïve and MAPK pathway inhibitor resistant melanoma. A phase I/II trial (NCT02988817) evaluating enapotamab vendotin (HuMax-AXL-ADC) has been initiated in patients with solid tumors, including melanoma [184,185].

7. BRAF Inhibitors

BRAF inhibitors are small molecule inhibitors that selectively target mutant BRAF isoforms, preferentially V600E but also other isoforms such as V600K or V600D [186]. BRAF inhibitors are typically used in combination with inhibitors of MEK, the downstream target of BRAF, in order to delay the development of resistance to BRAF inhibitor monotherapy as in the current standard of care for late-stage BRAF^{V600E} melanoma, dabrafenib and trametinib. Vemurafenib/PLX4032/RG7204, a serine/threonine kinase inhibitor, was the first selective BRAF inhibitor that was approved by the FDA. It binds to the ATP-binding domain of BRAF mutants such as V600E, V600R and V600D [187]. 960 mg twice daily was established as the recommended phase 2 dose in the phase 1 (NCT00405587) dose escalation clinical trial [186]. The FDA approval was granted based on the Phase 3 trial (BRIM-3) results (NCT01006980) which exhibited improved overall survival and progression-free survival rate in patients with BRAFV600E mutant melanoma [188]. Dabrafenib, a type I-kinase inhibitor was the second BRAF inhibitor that was approved by the FDA. This reversible ATP-competitive inhibitor, inhibits BRAFV600E, V600D and V600K proteins [189]. The Phase 2 trial (BREAK-2; NCT01153763) trial established a dose of 150 mg twice daily which can either be used as a single agent or in combination with trametinib [190,191]. It was granted FDA approval on the basis of the outcomes of Phase 3 trial (NCT01227889) in which it exhibited improved progression-free survival vs. decarbazine [24].

Encorafenib/LGX818

This molecule is an oral BRAF inhibitor selective for BRAF^{V600E} that was approved by the FDA in June 2018 for use in combination with the MEK inhibitor binimetinib (MEK162) in treating metastatic melanoma patients with the BRAF^{V600E} mutation [192]. A phase II trial (NCT02631447) to determine the optimal sequencing of BRAFi + MEKi (encorafenib + binimetinib) therapy and immunomodulatory antibody (ipilimumab + nivolumab) therapy in stage III-IV metastatic BRAF V600 melanoma is ongoing [193]. A phase II trial (NCT02159066) evaluating the use of third agent in encorafenib + binimetinib therapy in stage III-IV metastatic BRAF V600 melanoma [194].

8. MEK Inhibitors

MEK inhibitors are small molecule inhibitors targeting MEK1/2 proteins in the MAPK pathway. The addition of a MEK inhibitor in combination with a BRAF inhibitor delayed the development of resistance and decreased the toxicities associated with BRAF inhibitor monotherapy [195]. Trametinib/GSK1120212, a reversible, non-ATP-competitive inhibitor of MEK1/2 was the first MEK inhibitor approved by the FDA. The phase 1 study (NCT00687622) identified 2 mg daily dose of trametinib, which could be safely administered to the patients [196]. The phase 3 COMBI-D trial (NCT01584648) provided evidence of combining dabrafenib and trametinib in patients with metastatic BRAFV600E/K mutant melanoma as compared to monotherapy with dabrafenib [197]. Cobimetinib/GDC-0973 is used in combination with vemurafenib and is approved for patients with BRAFV600E/K mutant metastatic melanoma. The FDA approval was granted based on the efficacy results of combination of vemurafenib and cobimetinib in Phase-3 co-BRM trial (NCT01689519) [198].

Binimetinib/MEK162 is used in combination with encorafenib and is used in patients harbouring BRAFV600E/K mutation.

8.1. KZ-001

This selective MEK1/2 inhibitor is a benzoxazole compound with high potency and exhibits anti-tumor activity in BRAF- and NRAS-mutant tumor cell lines. It presented a synergistic effect in in vitro and in vivo xenograft models when used in combination with docetaxel (microtubule-stabilizing chemotherapeutic agent) and vemurafenib [199].

8.2. E6201

This MEK1 inhibitor was developed by Eisai Inc. This ATP-competitive MEK inhibitor is a synthetic analog of a natural product f152A1 occurring from the fungus *Curvularia verruculosa* [200]. NCT00794781 was a Phase I trial, evaluating the efficacy and safety of E6201 in patients with BRAF mutant advanced melanoma was terminated early due to futility based on response data [201].

8.3. TAK-733

This selective, oral, potent, non-ATP competitive allosteric site MEK inhibitor was developed by Millenium Pharmaceuticals, Inc. It demonstrated anti-tumor effects in vitro in melanoma cell lines and in vivo in patients-derived xenograft models [202]. NCT00948467 was a Phase 1 dose escalation trial evaluating TAK-733 in advanced solid tumors including patients with advanced metastatic melanoma had manageable toxicity profile but had limited antitumor activity and based on this result the further investigations are not planned [203].

8.4. PD-0325901/Mirdametinib

This selective, potent, oral, noncompetitive MEK inhibitor was developed by Pfizer. It inhibited ERK phosphorylation in in vitro model. It inhibited growth of melanoma cell lines in vitro and in xenograft models. This molecule also inhibited angiogenesis by inhibiting VEGF production and induced apoptosis in in vitro models [204]. NCT00147550, a phase I/II clinical trial evaluating the efficacy of PD-0325901 in advanced melanoma has been terminated due to ocular, neurological and musculoskeletal toxicities at higher doses (> 15 mg twice a day) [205].

9. ERK Inhibitors

ERK plays a unique role in the MAPK/ERK pathway; it has more than 100 substrates, some of which are involved in MAPK/ERK activating/de-activating feedback loops, yet it has only one upstream effector, MEK1/2 [206]. Due to this role, ERK inhibitors may show promise as a method of overcoming the development of resistance and re-activation of BRAF and MEK in BRAF^{V600E} melanoma. While presently far behind BRAF and MEK inhibitors in development, there has been a recent increase in the development and evaluation of ERK inhibitors for treating BRAF^{V600E} melanoma.

9.1. Ulixertinib/BVD-523

Ulixertinib is a novel, selective ERK1/2 inhibitor developed by BioMed Valley Discoveries, that inhibits ERK1/2 in a reversible and competitive manner. Importantly, ulixertinib presented equivalent efficacy in BRAF mutant cells and BRAF + MEK double mutant cells, while the efficacy of BRAF and MEK inhibitors decreased in the double mutant line [207]. Currently, it has been designated for fast track status by the FDA in the treatment of metastatic BRAFV600E-mutant melanoma [208].

9.2. LY3214996

A selective ERK1/2 inhibitor developed by Eli Lilly currently in phase I clinical trials [209,210]. Further details of this pre-clinical characterization have not been made publicly available, and Phase I trials are on-going.

9.3. MK8353

An orally dosed, selective inhibitor of activated ERK1/2, and non-activated ERK2 developed by Merck & Co. currently recruiting for phase I trials [211,212]. A phase I clinical trial was initiated following these results in healthy volunteers (NCT01358331); however, the study was terminated after phase Ia MTD determination (400 mg orally once daily) for strategic reasons [213].

9.4. LTT462

An oral ERK inhibitor developed by Novartis. LTT-462 has completed a phase I clinical trial for use in advanced cancers, including melanoma. A phase I clinical trial (NCT02711345) evaluating the use of LTT462 in advanced melanoma and other advanced cancer has concluded; however, the results are not yet publicly available [214].

9.5. KO-947

A highly potent and selective ERK1/2 inhibitor developed by Kura Oncology [215].

9.6. GDC0994

An orally-dosed, selective ERK1/2 inhibitor developed by Genentech [216]. A phase Ia trial (NCT01875705) was conducted on MAPK-dysregulated cancers not including melanoma. The phase Ia trial found a safety profile consistent with MAPK inhibition with tolerable adverse events [217]. A following phase Ib trial (NCT02457793) investigating the use of GDC0994 in combination with cobimetinib (MEKi) in advanced cancers including advanced melanoma has completed; however, the comprehensive results have not yet been released [218].

9.7. SCH772984

This is a selective, ATP-competitive ERK inhibitor developed by Merck. It exhibits antitumor activity against BRAFV600E mutant and NRAS mutant melanoma. It blocks proliferation of melanoma cell lines in BRAF and MEK inhibitor resistant cell lines in vitro [219]. The synergistic combination of SCH772984 with Vemurafenib delayed the onset of acquired resistance in in vitro models [220]. Intermittent dosing with RAF inhibitor, MEK inhibitor and ERK inhibitor (SCH772984) inhibited tumor growth in low-level BRAF amplification patient derived xenograft model of melanoma [221].

Table 2 summarizes the clinical trials, outcomes and adverse effects of novel BRAF and ERK inhibitors that are under investigation in the clinic to treat metastatic melanoma patients [211,222–224].

Table 2. Summary of clinical trials, outcomes and adverse events associated with novel BRAF inhibitors and ERK inhibitors in patients with metastatic melanoma.

Treatment	Status	Sponsor	Phase and NCT	Clinical Outcomes	Adverse Events
BRAF inhibitors					
LGX818 in patients with advanced or metastatic BRAF mutant melanoma [222]	Active, not recruiting	Array BioPharma	I, NCT01436656	25 BRAF-naïve and 29 BRAF inhibitor pretreated patients were enrolled in the study. The treatment was tolerable up to the MTD of 450 mg once daily, however the RP2D was declared as 300 mg once daily due to the increased risk of adverse events at 450 mg. BRAFi-naïve patients treated with 300–450 mg once daily saw an RR of 60% and PFS of 12.4 months (95% CI: 7.4–Not Reached), while for BRAFi-pretreated patients the RR was 22% and the PFS was 1.9 months (95% CI: 0.9–3.7 months).	Nausea, myalgia, PPED
LGX818 in combination with MEK162 in patients with advanced solid tumors [223]	Active, not recruiting	Array BioPharma	Ib/II, NCT01543698	The combination was safe with no substantial adverse events observed. Nine BRAF naïve and 14 BRAF inhibitor pretreated patients were enrolled. The MTD was unable to be determined and the RP2D was 450–600 mg LGX818 + 45 mg MEK162 orally once daily. CR and PR for BRAF-naïve patients were 11% and 78% respectively. The PR for BRAF inhibitor pretreated patient groups was 21%.	Nausea, abdominal pain, headache, diarrhea, fatigue, visual impairment.
Encorafenib in combination with Binimetinib or Vemurafenib in patients with BRAF-mutant melanoma (COLUMBUS) [224]	Active, not recruiting	Array BioPharma	III, NCT01909453	The combination was efficacious, safe and tolerable. The median follow up and followup for overall survival was 32.1 months (95% CI: 29.5–32.3 months) and 36.8 months (95% CI: 35.9–37.5 months) respectively. Encorafenib + Binimetinib ($n = 192$): The median overall survival was 33.6 months (95% CI: 24.4–39.2 months). One and two year OS was 75.5% (95% CI: 68.8–81%) and 57.6% (95% CI: 50.3–64.3%) respectively. The median PFS was 14.9 months (95% CI: 0.2–2 months). PFS was longer in this group as compared to vemurafenib only group (HR: 0.51, 95% CI: 0.39–0.67, $p < 0.0001$) and encorafenib only group (HR: 0.77, 95% CI: 0.59–1, $p = 0.05$). Overall response rates by masked independent central review was 64%. Vemurafenib only ($n = 191$): The median overall survival was 16.9 months (95% CI: 14–24.5 months). Hazard ratio compared to combination group was 0.61 (95% CI: 0.47–0.79, $p < 0.0001$). The overall survival did not differ significantly. One and two year OS was 63.1% (95% CI: 55.7–69.6%) and 43.2% (95% CI: 35.9–50.2%) respectively. The median PFS was 7.3 months (95% CI: 5.6–7.9 months). Overall response rates by masked independent central review was 41%. Encorafenib only ($n = 194$): Overall survival was longer in this group compared to Vemurafenib only group (HR: 0.81, 95% CI: 0.58–0.98, $p = 0.033$). One and two year OS was 74.6% (95% CI: 67.6–80.3%) and 49.1% (95% CI: 41.5–56.2%) respectively. The median PFS was 9.6 months (95% CI: 7.4–14.8 months). Overall response rates by masked independent central review was 52%.	Palmar-plantar erythrodysesthesia, nausea, diarrhea, vomiting, fatigue, myalgia, arthralgia, increased γ -glutamyltransferase, increased blood creatine phosphokinase, hypertension
ERK inhibitor					
Ulixertinib in patients with advanced solid tumors [211]	Completed	BioMed Valley Discoveries, Inc.	I, NCT01781429	135 patients were enrolled in the study. The treatment was well tolerated and had an acceptable safety profile at doses of 600 mg twice daily and this dose was established as MTD and RP2D. This dose demonstrated anti-tumor activity in patients with treatment naïve or those progressed on MAPK pathway inhibitors. The PR was 12%.	Diarrhea, fatigue, dehydration, nausea, rash, dermatitis acneiform, increased blood creatinine.

10. ROS Activated Prodrugs

Redox homeostasis is essential for cell transcription, proliferation and survival. Failure of regulating redox homeostasis can cause DNA damage and cell apoptosis [225]. Cancer cells are known to express increased reactive oxygen species (ROS) such as superoxide, H_2O_2 and the hydroxyl radicals [226–229]. Evidence has shown a significant increase in ROS levels after B-Raf inhibition in melanoma cells [230]. ROS-activated prodrugs can be potentially utilized in combination with B-Raf inhibitors to target metastatic melanoma cells.

10.1. Protein Ribonuclease A (RNase A)/SN-38

These ROS activated drugs usually contain two separate functional domains- a ROS-accepting moiety, “Trigger”, and an “Effector”. In the presence of H_2O_2 , the B-C bond within the aryl Byronic acid or esters will become oxidized, releasing the phenol group and activating the pro-drug. Protein ribonuclease A (RNase A) and SN-38 are being studied in the B16F10 murine melanoma cell line which mimics primary tumor growth [231,232]. SN-38 significantly decreases the proliferation of B16F10 murine melanoma cell line [231]. The enzymatic activity of RNase A will be reduced and cytotoxicity is improved when being activated via high ROS levels against skin melanoma cancer cells (B16F10) [232]. The success of these prodrugs in reducing tumor proliferation in murine melanoma cells gives the potential to study these drugs in human melanoma cell lines and potentially into clinical trials.

10.2. A100/RAC1

A100 is a quinone derivative. Quinones have substituents on the activated alkene, which are also called Michael acceptors. Cell damage and cytotoxicity occurs through the alkylation of DNA or cellular proteins. A100 sensitizes dabrafenib-resistant melanoma cells to BRAF protein kinase inhibitors [233]. A100, in the presence of high ROS levels, can self-cyclize into a bicyclic ring and cause DNA double strand breaks in cancer cells [234]. This compound and related ROS activated pro-drugs could be useful therapeutic agents where a BRAF inhibition has failed as the first line of treatment in melanoma patients harboring BRAF^{V600E} mutation.

11. Conclusions

Resistance to therapies continues to push the need to expand our understanding of melanoma treatment. This has led to exploring new treatments that utilizes combination therapies in order to achieve maximum anti-tumor efficacy over long durations of treatment avoiding resistance. Advances in the treatment of metastatic melanoma are on the rise with progress in targeted molecular therapy and immunotherapy. Targeted therapies are now expanding to include new BRAF and MEK inhibitors together and in combination with other therapies. Progress is being made in the field for targeting Axl and with ROS activated prodrugs. Immunotherapies are a new area of interest focusing on manipulation of checkpoint inhibition with durable clinical responses in patients. Melanoma has a strong molecular and genetic basis of pathogenicity, which allows for the development of personalized medicine. Selecting unique and individual treatments for melanoma patient makes it more likely to achieve high success rates in the clinic. Continual research and clinical trials are ongoing to further elucidate and expand knowledge on mechanisms of resistance and novel treatment strategies such as immunotherapies, new small molecule inhibitors and ROS-activated prodrugs to provide effective care to patients with metastatic melanoma.

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RIDR-PI-103, ROS-activated prodrug PI3K inhibitor inhibits cell growth and impairs the PI3K/Akt pathway in BRAF and MEK inhibitor-resistant BRAF-mutant melanoma cells

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Reactive oxygen species (ROS) levels are elevated after acquisition of resistance to v-raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitors including dabrafenib and MEK inhibitors such as trametinib in BRAF-mutant melanoma. To circumvent toxicity to PI-103 (a pan PI3K inhibitor), we utilized a novel ROS-induced drug release (RIDR)-PI-103, with a self-cyclizing moiety linked to PI-103. Under high ROS conditions, RIDR-PI-103 releases PI-103, which inhibits conversion of phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-triphosphate (PIP₃). Previous findings demonstrate that trametinib and dabrafenib-resistant (TDR) cells maintain p-Akt levels compared to parental counterparts and have significantly higher ROS. This is a rationale to explore the efficacy RIDR-PI-103 in TDR cells. We tested the effect of RIDR-PI-103 on melanocytes and TDR cells. RIDR-PI-103 exhibited less toxicity compared to PI-103 at 5 μ M in melanocytes. RIDR-PI-103 significantly inhibited TDR cell proliferation at 5 and 10 μ M. Twenty-four hour treatment with RIDR-PI-103 inhibited p-Akt, p-S6 (Ser240/244) and p-S6 (Ser235/236). We assessed the mechanism of activation of RIDR-PI-103, using glutathione or t-butyl hydrogen peroxide (TBHP) on the TDR cells in the presence or absence of RIDR-PI-103. Addition of the

ROS scavenger glutathione to RIDR-PI-103 significantly rescued the cell proliferation in TDR cell lines while addition of the ROS inducer TBHP and RIDR-PI-103 inhibited cell proliferation in WM115 and WM983B TDR cell lines. Examining the efficacy of RIDR-PI-103 on BRAF and MEK inhibitor-resistant cells will expand possible treatment options and open avenues for the development of new ROS-based treatment therapies for BRAF-mutant melanoma patients. *Anti-Cancer Drugs* 34: 519–531 Copyright © 2023 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Melanoma, a type of skin cancer originates in the pigment-producing melanocytes of the skin. Although it accounts for about 1% of skin cancers, it causes the majority of skin cancer-related deaths [https://www.cancer.org/cancer/melanoma-skin-cancer/about/key-statistics.html]. The 5-year survival rate for patients with metastatic melanoma is around 30% [1]. 50% of melanomas harbor activating mutations in v-raf murine sarcoma viral oncogene homolog B1 (BRAF) oncoprotein with over 90% of these mutations being V600E (valine at position 600 substituted by glutamic acid) [2]. The combination

of dabrafenib, a BRAF inhibitor and trametinib, a MEK inhibitor is Food and Drug Administration (FDA)-approved targeted therapy for patients with BRAF-mutant melanoma [3]. Chronic treatment with these inhibitors leads to the development of acquired resistance as the clinical response to these therapies drops [4]. Constitutive activation of mitogen-activated protein kinase pathway (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt pathway has been reported to contribute to resistance in melanoma [5–9]. Previous studies from our group have shown that BRAF-mutant melanoma cells resistant to dabrafenib and trametinib maintained p-Akt levels compared to their drug-sensitive counterparts [10].

Reactive oxygen species (ROS) are signaling molecules for normal biologic processes. An excess of ROS damages cellular organelles and DNA [11]. ROS are generated by partial reduction of oxygen comprised of radical and non-radical oxygen species such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical

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(HO•). ROS is mainly generated endogenously in cells during oxidative phosphorylation in the mitochondria or in response to exogenous stimuli such as xenobiotics, ionizing radiations, and nutrients [12,13]. An imbalance in ROS and antioxidants, which serve as a defense mechanism to ROS, has been implicated in cancer [11,14,15]. ROS has been implicated in the occurrence and development of skin carcinogenesis, most importantly melanoma [16,17]. BRAF inhibition induces and renders melanomas addictive to oxidative phosphorylation via peroxisome proliferator-activated receptor gamma coactivator 1- α and melanocyte-inducing transcription factor [18]. Previous studies from our group and other have demonstrated that elevated levels of ROS mediate resistance to BRAF and MEK inhibitors in melanoma. ROS levels are augmented on acute and chronic treatment with BRAF and MEK inhibitors [19–22]. The PI3K/Akt pathway is also subjected to redox regulation in presence of excess ROS [23].

The PI3K proteins are heterodimeric lipid kinases. These PI3Ks are divided into three classes based on their structure and specificity. The class I PI3Ks are further subdivided into two subclasses- PI3K α , PI3K β , and PI3K δ into class IA and PI3K γ into class IB. Class IA PI3Ks are composed of one p110 catalytic subunit encoded by PIK3CA (p110 α), PIK3CB (p110 β), or PIK3CD (p110 δ) and a regulatory p85 subunit. PI3K γ consists of a p110 γ subunit encoded by PIK3CG and one p101 regulatory subunit [24]. Upon ligand binding to cognate RTKs, PI3K is recruited to the cell surface and catalyzes the conversion of secondary messenger molecule phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-triphosphate (PIP₃). This conversion activates several downstream effectors such as PDK and AKT serine/threonine kinases which in turn activate several downstream signaling pathways. This pathway plays a major role in tumorigenesis in malignant melanoma [25].

PI3Ks are promising anticancer targets and many small molecule inhibitors have been developed as cancer therapy. The two classes of PI3K inhibitors are pan inhibitors (targeting the four isoforms of class I PI3Ks) and isoform-specific inhibitors [25]. Even though several PI3K inhibitors are being investigated in clinical trials, only a few have been FDA approved due to drug-related toxicities [26]. PI-103 is a pan PI3K inhibitor with strong activity against PI3K α isoform. PI-103 did not enter clinical trials due to limited solubility and extensive metabolism [27]. A boron-containing bioisostere of PI-103 offered improved bioavailability [28]. ROS-induced drug release (RIDR)-PI-103 is a prodrug of PI-103, which in presence of high ROS releases the biologically active component, PI-103 [29,30].

In this study, we have tested the efficacy of RIDR-PI-103 on trametinib and dabrafenib-resistant (TDR) melanoma cells. We observed that RIDR-PI-103 inhibited

cell proliferation in TDR cells as compared to control melanocytes. RIDR-PI-103 impaired PI3K/Akt signaling in a dose-dependent manner in TDR cells along with inhibition of downstream p-ribosomal protein 6 (S6). We demonstrate that RIDR-PI-103 in combination with glutathione, ROS scavenger rescues cell proliferation of TDR cells. RIDR-PI-103 in combination with t-butyl hydrogen peroxide (TBHP), ROS inducer inhibits cell proliferation. We demonstrate that RIDR-PI-103 in a dose-dependent manner inhibited the migration of TDR cells. These findings provide mechanistic insight and rationale for targeting drug-resistant melanomas with RIDR-PI-103.

Materials and methods

Reagents

RIDR-PI-103 was synthesized as described [29]. PI-103 (Cat# S1038) was obtained from Selleckchem (Houston, Texas, USA). All drugs were dissolved in dimethyl sulfoxide (DMSO) (Fisher Scientific, Waltham, Massachusetts, USA) at desired concentrations and stored at -20°C . Glutathione was obtained from Fisher Scientific (Cat# BP25211). N-acetyl cysteine (NAC) was obtained from Fisher Scientific (Cat# AAA1540936). TBHP was obtained from Fisher Scientific (Cat# AC180340050). Dabrafenib (Cat# D-5699) and trametinib (Cat# T-8123) were purchased from LC Laboratories (Woburn, Massachusetts, USA).

Cell lines and culture conditions

TDR cells were generated as previously described [10]. A375 cells were cultured in Dulbecco's Modified Eagle Medium (cat# MT15017CV; Corning, New York, USA); WM115 cells were cultured in MEM (cat# MT10009CV; Corning); WM983B cells were cultured in RPMI-1640 medium (cat# MT10040CM; Corning) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (cat# 89510-188; VWR) and 1% penicillin-streptomycin solution 100 $\times$ (cat# 30-002-CI; Corning). Cultured cells were incubated in a humidified incubator at 37°C with 5% (vol/vol) CO₂ and 95% (vol/vol) air. A375 TDR cell lines were maintained in 250 nM dabrafenib and 12.5 nM trametinib. WM983B TDR cells were maintained in 2.4 μM dabrafenib and 500 nM trametinib. WM115 TDR cells were maintained in 800 nM dabrafenib and 200 nM trametinib. 1788C and 1789B melanocytes were kindly provided by Dr. Zalfa Abdel-Malek from the University of Cincinnati, College of Medicine.

Transient transfection

5×10^4 cells were plated and transfected with control empty vector plasmid (pECE; Addgene: 26453) and constitutively active AKT plasmid (myrAkt delta4-129; Addgene: 10841). In separate experiments, the above cells were transfected with control empty vector plasmid (LZRS-Rfa; Addgene: 31601) and constitutively active

MEK plasmid (L1E-1; Addgene: 21193) using lipo-fectamine 2000 for 48 h. Cell lysates were collected and analyzed for AKT and MEK pathway activation using western blotting.

Cell proliferation and long-term growth assays MTT assay

Growth kinetics of WM115 TDR, WM983B TDR, A375 TDR, 1789C melanocytes, and 1788B melanocytes was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. 1×10^4 cells/wells were seeded in 96-well plates in quadruplicate. After 72 h of treatment with vehicle, RIDR-PI-103 (2.5 μ M, 5 μ M, and 10 μ M) or PI-103 (5 μ M and 10 μ M), the media was substituted with 0.5 mg/mL MTT solution (Sigma Aldrich, St. Louis, Missouri, USA) for 4 h and absorbance was read at 570 nm using SPECTRAMax PLUS Microplate Spectrophotometer Plate Reader (Molecular Devices Incorporation, San Jose, California, USA) and expressed as the mean of quadruplicate relative to vehicle (DMSO) control together with SEM. For experiments with glutathione, briefly 1×10^4 cells/wells were seeded in 96-well plates in quadruplicate. Cells were treated with glutathione (100 μ M) alone or in combination with RIDR-PI-103 (2.5 μ M, 5 μ M, and 10 μ M) for 72 h, and proliferation was assessed as mentioned above. For experiments with NAC, cells were plated and treated with NAC (50 mM) alone or in combination with RIDR-PI-103 (2.5 μ M, 5 μ M, and 10 μ M) for 72 h and proliferation was assessed as mentioned above. For experiments with TBHP, cells were plated and treated with TBHP (WM115 TDR and WM983B TDR – 20 mM and A375 TDR – 50 mM) alone or in combination with RIDR-PI-103 (2.5 μ M) for 72 h and proliferation was assessed as mentioned above. In other experiments, equal number of WM115 TDR cells were plated and transfected with control empty vector plasmid (pECE), constitutively active AKT plasmid (myrAkt delta4-129), control empty vector plasmid (LZRS-Rfa), constitutively active MEK plasmid (L1E-1) for 24 h and treated with 5 μ M RIDR-PI103 for another 24 h and MTT assay was performed as described above. The experiment was repeated twice and data were plotted as mean values with respect to that of control plasmids.

BrdU cell proliferation assay

Cell proliferation of WM115 TDR, WM983B TDR, A375 TDR cells was analyzed using BrdU cell proliferation assay kit from Cell Signaling Technology (cat# 6813; Danvers, Massachusetts, USA). Briefly, equal number of TDR cells (2×10^4 cells/well) were plated and treated with 0–60 μ M RIDR-PI 103 for 48 h. After 48 h, media was removed and 10X BrdU solution was added to the wells and incubated for 1 h in the incubator. Then, 100 μ L/well Fixing solution was added and cells were incubated at room temperature for 30 min. Further, 1X detection antibody solution and 1X horse radish

peroxidase (HRP)-conjugated secondary antibody solution were added as per manufacturer's protocol. In the final step, 100 μ L TBP solution was added followed by stop solution, and the plates were read at 450 nm using SPECTRAMax PLUS Microplate Spectrophotometer Plate Reader (Molecular Devices Incorporation) and the values were expressed as the mean of triplicate relative to vehicle (DMSO) control together with SEM.

Crystal violet assay

TDR cells were seeded density of 2×10^4 cells/well in 12-well plates (Falcon) in triplicates. Complete media containing vehicle or RIDR-PI-103 (5 μ M and 10 μ M) was replenished every 2 days. On day 15, cells were stained with 0.5% crystal violet. The cultures were exposed to crystal violet solution for 2 min and washed with water to remove excess stains. The intensities were measured using an Odyssey infrared system (LI-COR, Lincoln, Nebraska, USA). The experiments were repeated at least twice. For low-dose RIDR-PI-103 experiments, 1.5×10^4 cells/well were seeded in a 24-well plate in quadruplicate. Complete media containing vehicle or RIDR-PI-103 (1 μ M, 2.5 μ M, and 5 μ M) was replenished every 2 days. On day 15, cells were stained as mentioned above.

Matrigel growth assay

Three-dimensional (3D) growth assays were performed in growth factor-reduced Matrigel (cat# CB-40230; BD Biosciences, San Jose, California, USA). Ninety-six-well plates were coated with 70 μ L of Matrigel/well in duplicate. TDR cells (2×10^4 /well) were plated and incubated at 37°C for 24 h. Cells were treated with vehicle (DMSO) or RIDR-PI-103 (2.5 μ M, 5 μ M, and 10 μ M) every alternate day. After 21 days of incubation, cells were visualized, and photographs were captured from five random fields under phase contrast microscope (10x magnification, Nikon, Melville, New York, USA). The areas of the cells were measured by ImageJ and represented as mean areas normalized to DMSO control.

Western blot analysis

Cells were lysed in RIPA buffer (Cat# BP-115; Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitors (Cat# 88669; Thermo Fisher Scientific). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Cat# 23225; Thermo Scientific) according to manufacturer's instructions. A minimum of 15 μ g of protein was used for each sample. Immunoblotting was performed using the following primary antibodies from Cell Signaling Technology: p-AktS473 (Cat# 4060), Akt (Cat# 9272), p-S6(240/244) (Cat# 2215), p-S6(235/236) (Cat# 4858), S6 (Cat# 2217), and β -Actin (Cat# 4970). The primary antibody (1:1000 dilution) was added to the nitrocellulose and incubated overnight at 4°C with gentle agitation. Blots were then washed with 1x Tris Buffered Saline with Tween (TBST)

(three washes, 10 min each) and incubated with anti-rabbit IgG-HRP (cat# SC-2357; 1:2000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. Blots were then washed with 1X TBST (three washes, 10 min each). Protein signals were detected using Pierce ECL Western blotting substrate (Cat# 32106; Thermo Fisher Scientific). At least two independent repeats were performed.

Cell migration assay

The transwell migration chambers were soaked in complete media for 4 h prior to start of the experiment. Complete media was added to the lower chambers and upper chambers were placed in the wells. 2×10^4 cells/well of WM115 TDR, A375 TDR, and WM983B TDR were counted and added to the upper chambers of the transwells in serum-free media along with vehicle or RIDR-PI-103 (2.5 μ M, 5 μ M, and 10 μ M) and incubated for 24 h. FBS (10%) was used as chemoattractant and added in lower chambers. After 24 h, migrated cells in the lower chamber were stained with 0.5% crystal violet and images were captured from five areas under phase contrast microscope at 10x magnification. The intensities from the captured areas were measured using ImageJ and expressed as % of control and represented graphically.

Trypan blue exclusion assay

Equal number of TDR cells were seeded and treated with vehicle or RIDR-PI-103 (2.5 μ M, 5 μ M, and 10 μ M) for 48 h in 60 mm plates. Cells were collected and centrifuged to get a cell pellet. The cell pellet was resuspended in 1 mL of 1X Phosphate Buffered Saline (Cat# 21-040-CV; Thermo Fisher Scientific). From this, 0.1 mL of the cell suspension was added to 0.1 mL of 0.4% trypan blue solution and incubated for 5 min at room temperature. The cells were immediately counted and cell viability was determined using the formula:

$$\% \text{ viable cells} = \left[1.00 - \left(\frac{\text{Number of dead cells}}{\text{Number of total cells}} \right) \right] \times 100$$

Statistical analysis

Data are shown as the mean $\pm$ SEM and representative of at least three independent experiments unless otherwise indicated. One-way analysis of variance followed by Tukey's test for post hoc analysis was used for comparisons between more than two groups. Statistical analysis was performed using GraphPad Prism software. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

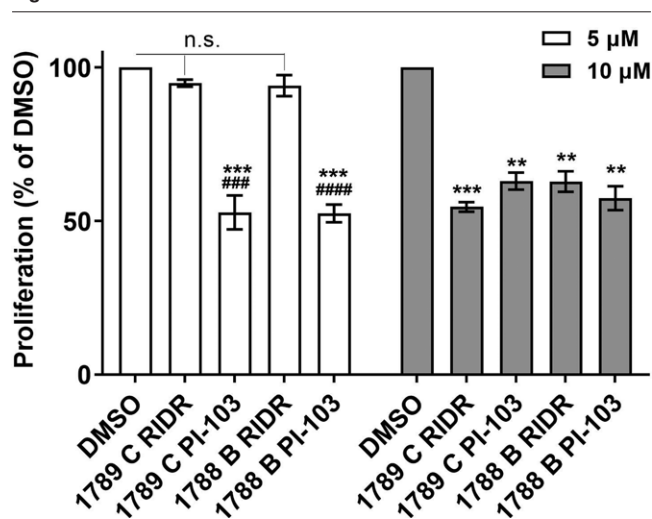
ROS-induced drug release-PI-103 inhibits growth of BRAF-mutant TDR cells

We evaluated the effects of RIDR-PI-103 on cell proliferation in WM115 TDR, WM983B TDR, A375 TDR, 1789C melanocytes, and 1788B melanocytes

at 5 μ M and 10 μ M concentration using 72-h MTT assay. Additionally, we assessed the effects of PI-103 on 1789C and 1788B melanocytes at 5 μ M and 10 μ M. Type C melanocytes were obtained from lightly pigmented skin while type B melanocytes were obtained from a dark pigmented skin. We assessed the effects of RIDR-PI-103 only on TDR cell lines since these resistant cells demonstrated significant increase in ROS levels compared to the drug-sensitive parental cells [20]. RIDR-PI-103 at 5 μ M did not significantly inhibit proliferation of melanocytes compared to DMSO group. PI-103 significantly inhibited proliferation in 1789C and 1788B melanocytes at 5 μ M compared to melanocytes treated with the same concentration of RIDR-PI-103. When melanocytes were treated with 10 μ M of RIDR-PI-103 or PI-103, both the parent molecule PI-103 and pro-drug RIDR-PI-103 inhibited the proliferation of melanocytes (Fig. 1).

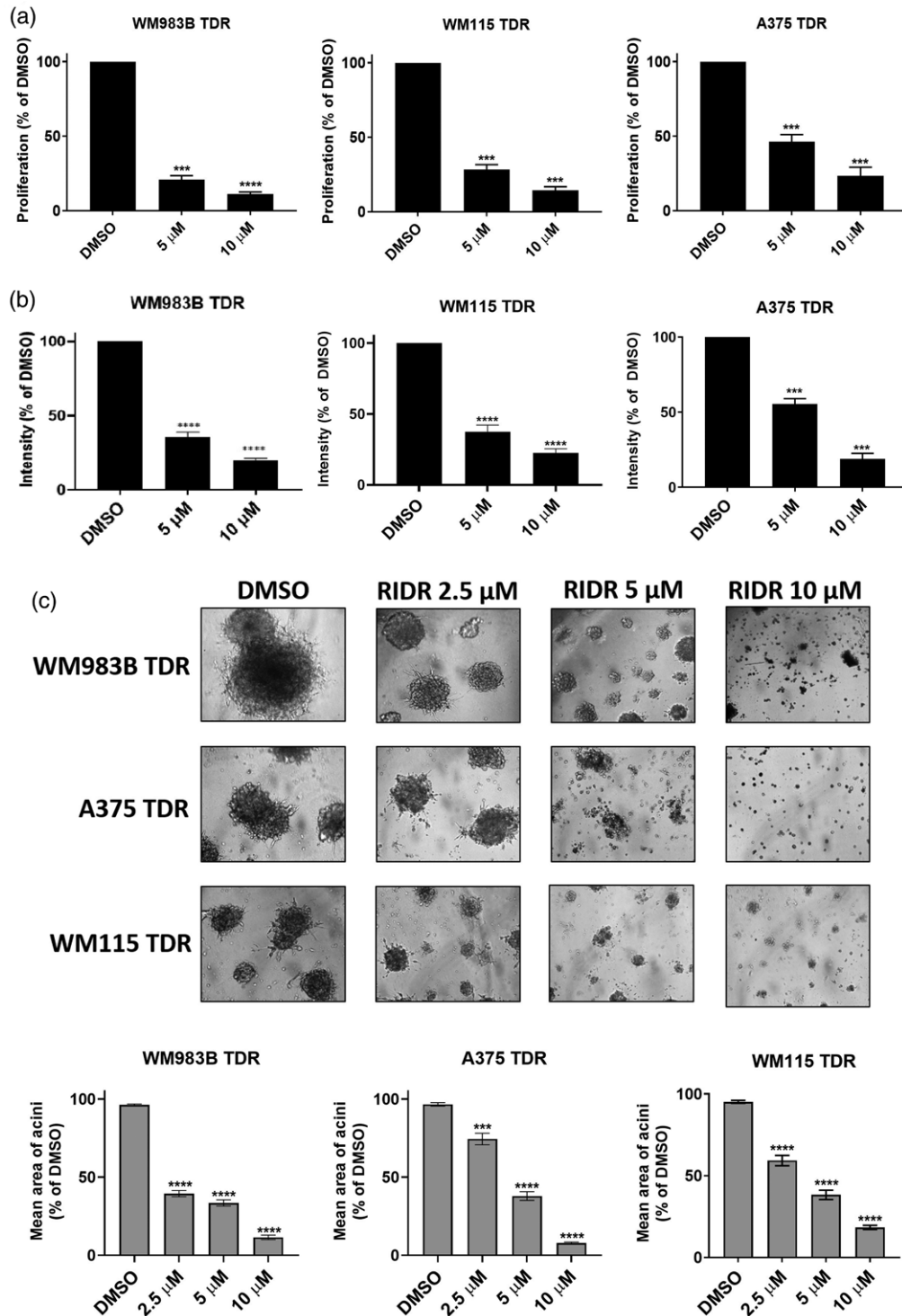
TDR cells treated with RIDR-PI-103 had significantly inhibited proliferation at 5 μ M and further inhibition was observed at 10 μ M (Fig. 2a). These data indicate that RIDR-PI-103 has increased specificity in TDR cells relative to melanocytes. RIDR-PI-103 significantly reduced cell growth in TDR cells in a dose-dependent manner compared to DMSO (Fig. 2b and S1, Supplemental Digital Content 1, <http://links.lww.com/ACD/A491>) as measured by crystal violet staining. RIDR-PI-103 was effective at concentration as low as 2.5 μ M in WM115 TDR and WM983B TDR cells (Fig. S2, Supplemental Digital

Fig. 1



Effects of RIDR-PI-103 and PI-103 on melanocytes. 1789C and 1788B melanocytes were seeded in 96-well plates and treated with PI-103 or RIDR-PI-103 (RIDR) at 5 μ M/10 μ M for 72 h. Cells were treated with MTT and absorbance was read at 570 nm. Error bars: SEM. ** $P < 0.01$, *** $P < 0.001$ vs. DMSO; ### $P < 0.001$, #### $P < 0.0001$ vs. ANOVA, analysis of variance; RIDR-PI-103 treatment group (one-way ANOVA followed by Tukey's test for post hoc analysis). RIDR, ROS-induced drug release; ROS, reactive oxygen species.

Fig. 2



RIDR-PI-103 inhibits growth of TDR cells. (a) WM983B TDR, WM115 TDR, and A375 TDR cells were seeded in 96-well plates and treated with increasing concentrations of RIDR-PI-103 (RIDR) for 72 h. Cells were treated with MTT and absorbance was read at 570 nm. Data reflect three independent repeats and are presented as mean $\pm$ SEM. Error bars: SEM. **** P <0.0001 vs. DMSO (one-way ANOVA followed by Tukey's test for post hoc analysis). (b) TDR cells were plated and treated with media containing DMSO or RIDR (5 μ M or 10 μ M). Media containing drugs was replenished every second day and stained with 0.5% crystal violet on Day 15. Quantitation of intensities for the wells is represented as mean. Error bars: SEM. *** P <0.001, **** P <0.0001 vs. DMSO (one-way ANOVA followed by Tukey's test for post hoc analysis). (c) TDR cells were seeded on a Matrigel basement membrane and treated with indicated treatment every second day. Pictures of wells were captured on day 21 (Top panel) at 10 $\times$ magnification. Quantification of mean area of acini (Bottom panel). Error bar: SEM. *** P <0.001, **** P <0.0001 vs. DMSO (one-way ANOVA followed by Tukey's test for post hoc analysis). ANOVA, analysis of variance; RIDR, ROS-induced drug release.

Content 2, <http://links.lww.com/ACD/A492>). We next examined the growth potential of TDR cells in presence of RIDR-PI-103 at 2.5 μ M, 5 μ M, and 10 μ M on a basement membrane of matrigel. WM983B TDR cells treated with DMSO exhibited aggressive growth. Treatment with RIDR-PI-103 inhibited growth on matrigel basement membrane in a dose-dependent manner (Fig. 2c). We also observed a decrease in cell viability for WM115 TDR and A375 TDR cells at 48 h using increasing concentration of RIDR-PI-103 using cell viability using the trypan blue exclusion assay (Fig. S3, Supplemental Digital Content 3, <http://links.lww.com/ACD/A493>). In addition, we analyzed the effect of RIDR-PI-103 (0–60 μ M) on BRAF and MEK inhibitor-resistant melanoma cell lines cell proliferation using BrdU assay. Our data indicated that RIDR-PI-103 inhibited WM115 TDR, WM983B TDR, and A375 TDR cell proliferation in a dose-dependent manner (Fig. S4, Supplemental Digital Content 4, <http://links.lww.com/ACD/A494>). We observed a more pronounced effect of RIDR-PI-103 in the MTT, crystal violet, and Matrigel growth assays compared to the BrdU cell proliferation which could indicate RIDR-PI-103 primary effect is on cell viability and not cell division.

ROS-induced drug release-PI-103 inhibits PI3K signaling and downstream S6 signaling

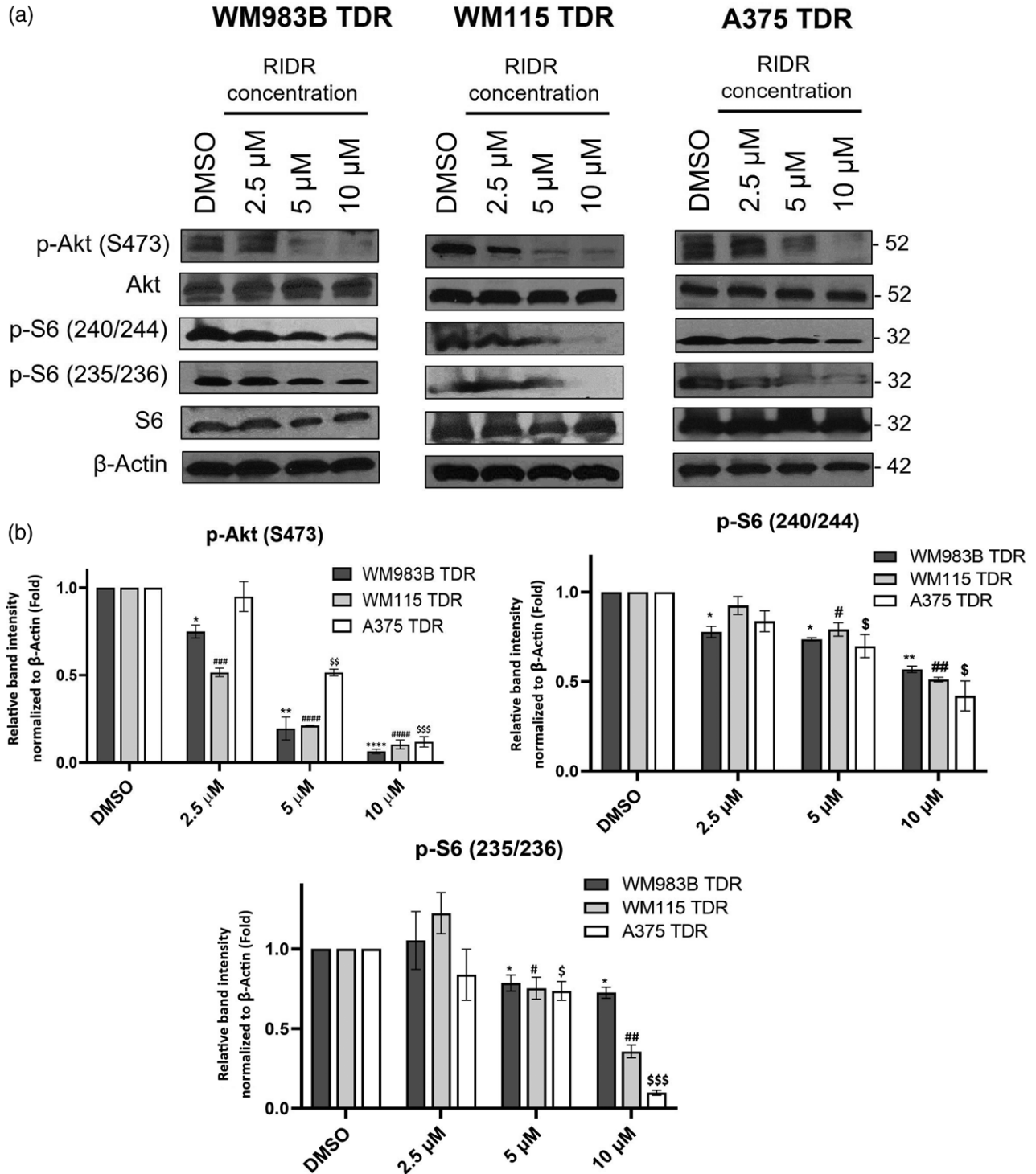
We examined the effect RIDR-PI-103 at 2.5 μ M, 5 μ M, and 10 μ M on signaling pathway in TDR cells by performing a series of western blots. We have previously established that TDR cells maintain p-Akt levels compared to their drug-sensitive counterparts [10]. In line with those results, we observed the presence of p-Akt in TDR cells treated with DMSO control. In A375 TDR cells, we did not observe a statistical reduction in phosphorylation of Akt (S473) at 2.5 μ M RIDR-PI-103, while we see a dose-dependent decrease in p-Akt (S473 levels) with higher concentrations of the prodrug. For WM115 TDR and WM983B TDR cells, we observed a prominent reduction in p-Akt (S473) levels starting at 2.5 μ M and p-Akt was inhibited further at 5 μ M and 10 μ M of RIDR-PI-103. Ribosomal protein S6 is a component of the 40S small ribosomal subunit and plays an important role in protein translation, ribosome biogenesis, cell proliferation and growth, apoptosis, glucose homeostasis, and DNA damage in cancer cells [31–33]. Activation of S6 has been implicated in various malignancies such as melanoma, breast cancer, glioblastoma, head and neck squamous cell carcinoma, and non-small cell lung cancer [34]. It has been observed that melanoma cells resistant to BRAF and MEK inhibitors maintain phosphorylated S6 levels [35]. We observed a dose-dependent inhibition of p-S6 at sites Ser240/244 and Ser235/236 when all three TDR cells were treated with RIDR-PI-103 (Fig. 3). Since RIDR-PI-103 targets the PI3K enzyme and downstream the AKT pathway, we examined if constitutive activation of AKT signaling could overcome the inhibitory effect of

RIDR-PI-103 in melanoma cells. In addition, we were also interested to check the effect of MEK constitutive activation on RIDR-PI-103 mediated signaling. Since only WM115 TDR cell line was amenable to transient transfection as indicated by increased p-Akt and p-Erk (Fig S5 A, C, Supplemental Digital Content 5, <http://links.lww.com/ACD/A495>), we performed an MTT assay where WM115TDR cells transfected with using AKT or MEK constitutively active constructs were treated with 5 μ M RIDR-PI-103 as described in materials and method section. The data showed that RIDR-PI-103 significantly inhibited cell growth in WM115TDR cell lines transfected with control plasmids, respectively. This effect was rescued in presence of constitutively active AKT but not MEK constitutively active plasmid (Fig S5, Supplemental Digital Content 5, <http://links.lww.com/ACD/A495>).

Addition of glutathione to ROS-induced drug release-PI-103 rescues cell proliferation in TDR cells

Glutathione is an antioxidant tripeptide consisting of cysteine, glycine, and glutamic acid. We examined the effects of glutathione alone or in combination with RIDR-PI-103 on TDR cell proliferation using the MTT assay. Glutathione alone had no significant effect on proliferation of all three TDR cell lines. RIDR-PI-103 alone inhibited cell proliferation in TDR cells in a dose-dependent manner. We observed a significant rescue in TDR cell proliferation when glutathione was combined with RIDR-PI-103 at 5 μ M compared to RIDR-PI-103 (5 μ M) only group. For WM983B TDR cells we observed the rescue in cell proliferation induced by RIDR-PI-103 even at 10 μ M RIDR-PI-103 while this effect was not observed in A375 TDR and WM115 TDR cells (Fig. 4). We also assessed the effect of another antioxidant, NAC alone or in combination with RIDR-PI-103 on these TDR cells. NAC alone did not have any effect on the proliferation of A375 TDR while it significantly inhibited proliferation in WM115 TDR and WM983B TDR cells compared to control group. We observed rescue in cell proliferation for A375 TDR cells when NAC was combined with RIDR-PI-103 at 5 μ M concentration as compared to A375 TDR cells treated with RIDR-PI-103 alone. While NAC itself inhibited cell proliferation in WM983B TDR cells, we also observed a significant inhibition in cell proliferation between NAC treatment group vs NAC + RIDR-PI-103 (10 μ M) group. A significant rescue in WM983B TDR cell proliferation was observed in NAC + RIDR-PI-103 (10 μ M) group compared to RIDR-PI-103 group (10 μ M). Since NAC strongly inhibited proliferation in WM115 TDR cells, interpretation of data for this cell line is difficult (Fig. S6, Supplemental Digital Content 6, <http://links.lww.com/ACD/A496>). These data indicated that endogenous ROS levels affect the activation of RIDR-PI-103.

Fig. 3



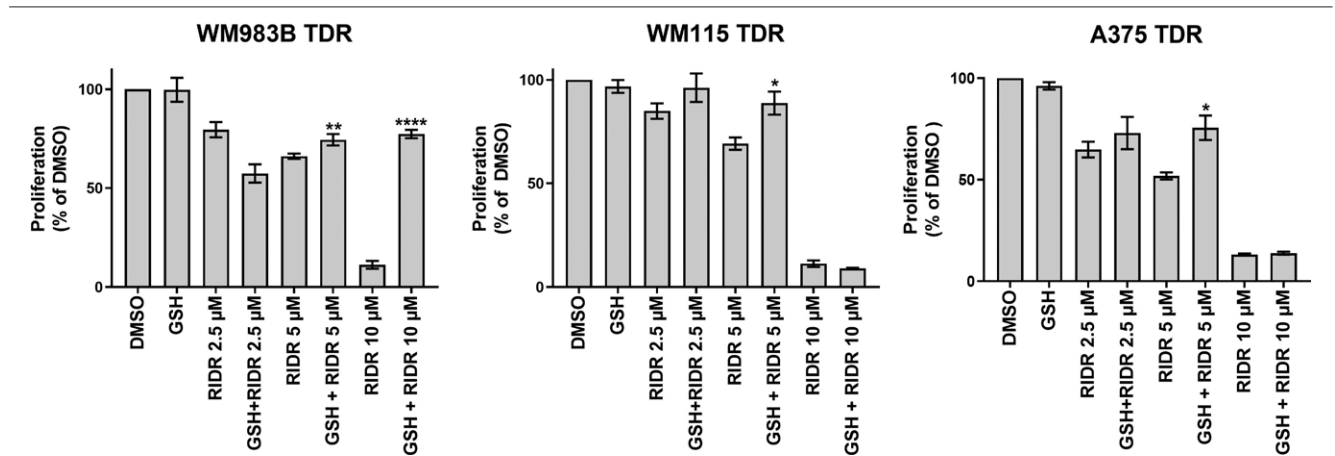
RIDR-PI-103 inhibits p-Akt and p-S6 in a dose-dependent manner. (a) Immunoblots of WM983B TDR, WM115 TDR, and A375 TDR cells were treated for 24 h with RIDR-PI-103 (RIDR; 2.5 μ M, 5 μ M or 10 μ M) or vehicle (DMSO). Whole-cell lysates from the cell lines were analyzed for western blot using indicated antibodies. Actin served as the loading control. (b) Band intensities for p-Akt (S473), p-S6 (240/244), and p-S6 (235/236) were quantified using ImageJ software and normalized to intensity for total protein. Two independent repeats were performed. Error bars: SEM. * P <0.05, P <0.01, P <0.001, P <0.0001 vs. WM983B TDR DMSO; # P <0.05, ## P <0.01, ### P <0.001, #### P <0.0001 vs. WM115 TDR DMSO; \$ P <0.05, \$\$ P <0.01, \$\$\$ P <0.001 vs. A375 TDR DMSO (one-way ANOVA followed by Tukey's test for post hoc analysis). ANOVA, analysis of variance; RIDR, ROS-induced drug release; ROS, reactive oxygen species.

TBHP in addition to ROS-induced drug release-PI-103 inhibits cell proliferation in TDR cells

TBHP, an organic compound is used as ROS inducer. Since TBHP is more stable than hydrogen peroxide, we used TBHP in our experiments instead of hydrogen peroxide to augment ROS levels. We examined the effects of TBHP alone or in combination with RIDR-PI-103 on TDR cells using MTT cell proliferation assay. TBHP alone was not toxic to TDR cells and did not significantly inhibit proliferation in all

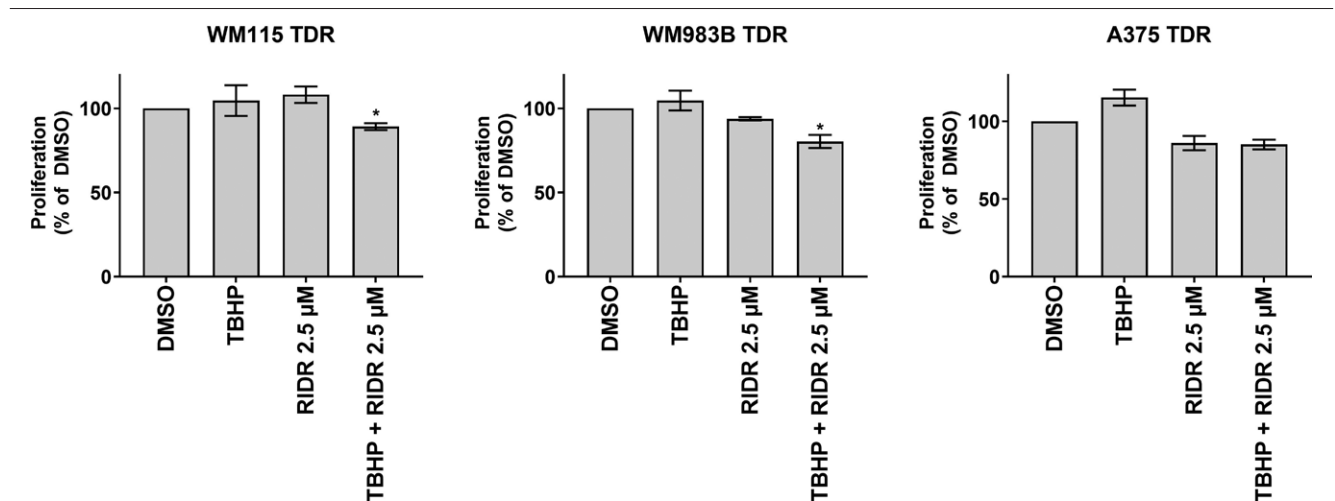
three TDR cell lines. We observed a significant inhibition in WM115 TDR and A375 TDR cell proliferation when TBHP was added with RIDR-PI-103 at 2.5 μ M compared to cells treated only with RIDR-PI-103. For WM983B TDR cells, we did not observe any inhibition in cell proliferation when TBHP was added with 2.5 μ M RIDR-PI-103 (Fig. 5). This data demonstrates that augmentation of ROS levels in TDR cells inhibits cell proliferation in two out of the three TDR cell lines.

Fig. 4



RIDR-PI-103 in combination with glutathione rescues TDR cell proliferation. TDR cells (WM983B, WM115, and A375) plated for 24 h and treated with DMSO, RIDR-PI-103 (RIDR; 2.5 μ M, 5 μ M, or 10 μ M) treated alone or in combination with glutathione (100 μ M) and treated with MTT. The graphs are represented as mean. Error bars: SEM. * P < 0.05, ** P < 0.01, and **** P < 0.0001 vs. the group treated with RIDR-PI-103 only at the corresponding concentration (one-way ANOVA followed by Tukey's test for post hoc analysis). ANOVA, analysis of variance; RIDR, ROS-induced drug release; ROS, reactive oxygen species.

Fig. 5



RIDR-PI-103 in combination with TBHP inhibits TDR cell proliferation. TDR cells (WM983B, WM115, and A375) were plated for 24 h and treated with DMSO, RIDR-PI-103 (RIDR; 2.5 μ M) treated alone or in combination with TBHP (WM115 and WM983B TDR – 20 mM; A375 TDR – 50 mM) and treated with MTT. The graphs are represented as mean. Error bars: SEM. * P < 0.05 vs. RIDR 2.5 μ M (one-way ANOVA followed by Tukey's test for post hoc analysis). ANOVA, analysis of variance; RIDR, ROS-induced drug release; ROS, reactive oxygen species.

ROS-induced drug release-PI-103 suppresses TDR cell migratory potential

We assessed the cell migration of TDR cells when treated with increasing concentrations of RIDR-PI-103. The TDR cells and RIDR-PI-103 (2.5 μ M, 5 μ M, and 10 μ M or control) were added to the upper chambers of the transwell under serum starvation and allowed to migrate for 24 h to the lower chambers containing 10% FBS. Migration of TDR cells treated with 2.5 μ M, 5 μ M, and 10 μ M of RIDR-PI-103 was significantly reduced at 24 h (Fig. 6). We also performed cell migration for 48 h and no differences were observed between 24 h and 48 h migration when treated with RIDR-PI-103 (data not shown).

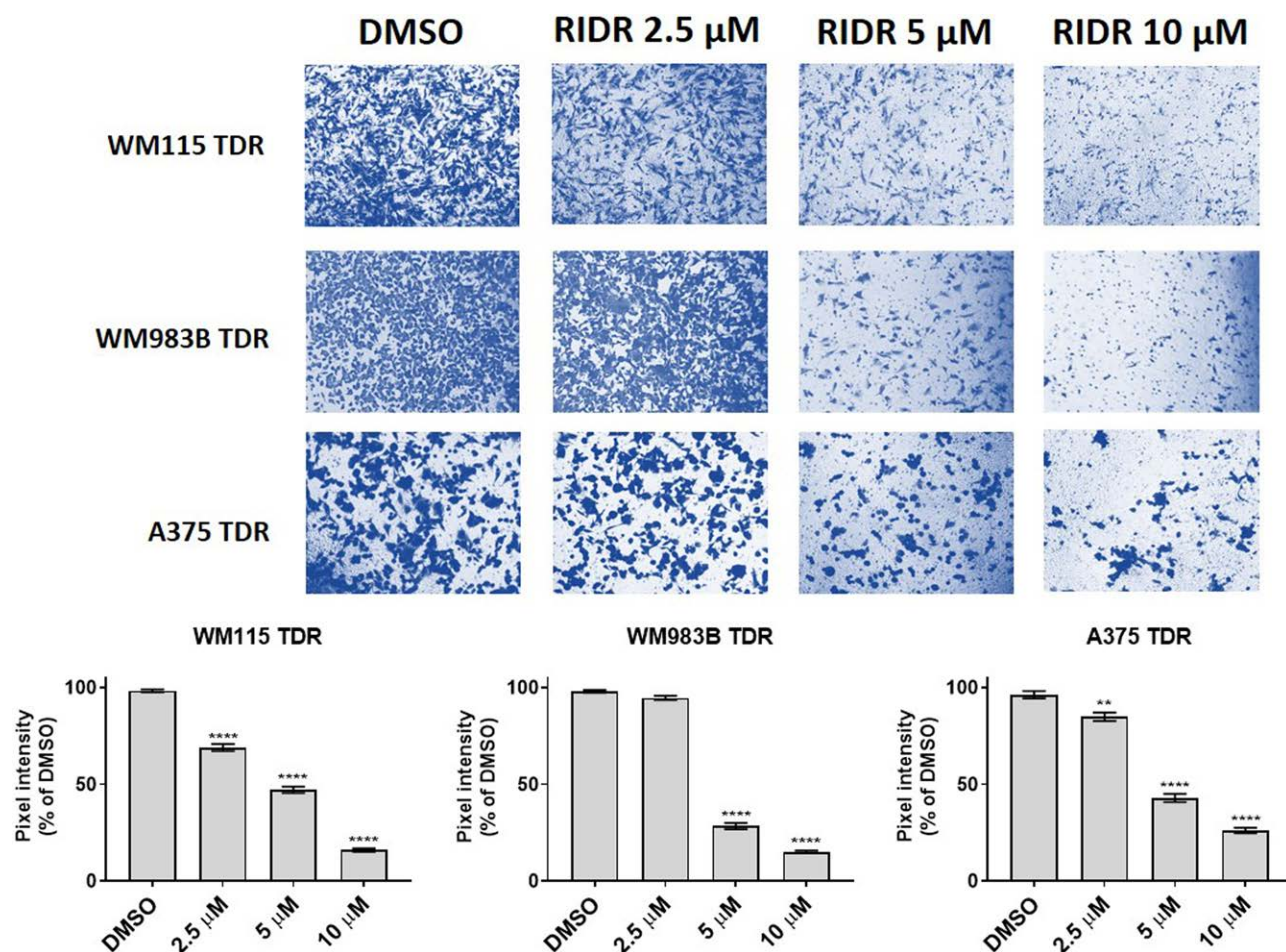
Discussion

The PI3K/Akt pathway serves as a therapeutic target in drug-resistant melanoma [36]. The two most common

events that attribute to PI3K/Akt pathway activation in melanoma are mutations in the N-Ras oncogene (15–20%) and loss of expression or function in phosphatase and tensin homolog (PTEN) (20–30%). There is a strong association between complete loss of PTEN and BRAF mutations [37–39]. Only five PI3K inhibitors – Copanlisib (Aliqopa), Idelalisib (Zydelig), Alpelisib (PIQRAY), Duvelisib (COPIKTRA), and Umbralisib (Ukoniq) have been FDA approved to date [26]. Despite numerous PI3K inhibitors in preclinical and clinical trials, only a handful have been granted FDA approval as there are several factors that have limited the successful development of these inhibitors.

The PI3K pathway plays a role in basic cellular processes throughout the body including autophagy, protein translation, and metabolism. Owing to the ubiquitous role of this pathway, drugs designed to inhibit PI3K pathway

Fig. 6



RIDR-PI-103 inhibits migration in TDR cells. TDR cells (WM983B, WM115, and A375) treated with DMSO, RIDR-PI-103 (RIDR; 2.5 μ M, 5 μ M or 10 μ M) were seeded in upper chamber of transwell in serum-free media for 24 h. 10% FBS was added as chemoattractant in the lower chamber. After 24 h, the migrated cells were stained, and images were captured (Top panel) at 10x magnification. The images were quantified using Image J and presented as bar graphs (Bottom panel). Error bars: SEM. $P < 0.01$, $P < 0.0001$ vs. DMSO (one-way ANOVA followed by Tukey's test post hoc analysis). ANOVA, analysis of variance; FBS, fetal bovine serum; RIDR, ROS-induced drug release; ROS, reactive oxygen species.

are associated with toxicities [40]. A major challenge in PI3K pathway inhibitor development is that drugs fail to achieve optimal target blockade in tumors. Common toxicities observed with pan-PI3K inhibitors are fatigue, hyperglycemia, and rash. Some toxicities are also isozyme specific. While PI3K δ inhibitors are associated with myelosuppression, gastrointestinal effect, and transaminitis, the PI3K α inhibitors are associated with side effects such as rash and hyperglycemia. Due to these toxicities, it is challenging to combine PI3K inhibitors with other small molecule inhibitors [41]. PI3K inhibition blocks the action of insulin, which in turn prevents glucose uptake in adipose tissue and stimulates glycogen breakdown in the liver. This ultimately leads to hyperglycemia, since genes encoding for glycolytic enzymes are transcribed by PI3K/Akt [42]. PI-103 is also associated with these toxicities and hence we have designed a prodrug, RIDR-PI-103 for this pan-PI3K inhibitor, which is activated in the presence of high oxidative stress in the cells. The goal of this prodrug is to circumvent toxicities observed with systemic inhibition of PI3K signaling.

The phenol in the structure of PI-103 was used to attach a self-cyclizing molecule by an ethereal linkage to make the prodrug. The TDR cells have demonstrated high ROS levels as compared to their drug-sensitive counterparts using 2',7'-dichlorofluorescein diacetate and MitoSOX assay [20]. In the presence of high level of oxidative stress in the tumor milieu, the prodrug undergoes self-cyclization to release PI-103, which inhibits the conversion of PIP₂ to PIP₃. This leads to blockade of downstream Akt [29]. Endogenously generated ROS has been demonstrated to bring about oxidation of PTEN, a tumor suppressor and results in sustained activation of the signaling pathway [23]. H₂O₂ also interacts with PTEN and oxidizes and inactivates the tumor suppressor by forming disulfide bonds between Cys¹²⁴ residue and Cys⁷¹ in oxidized PTEN [43]. Utilizing the increase in physiological ROS levels could be an effective treatment to overcome resistance to BRAF and MEK inhibitors in BRAF-mutant melanoma.

RIDR-PI-103 in combination with ROS inducers such as doxorubicin or cytosine arabinoside demonstrated efficacy *in vitro* against acute myeloid leukemia cells. RIDR-PI-103 has reduced affinity for PI3K and withstands liver microsomes. RIDR-PI-103 undergoes oxidation and release PI-103 in the cells. The studies assessing the conversion of the prodrug to its active components were performed using HPLC and mass spectrometry [29]. In breast cancer cells RIDR-PI-103 in combination with doxorubicin inhibited cell proliferation, impaired PI3K signaling, and activated DNA damage [30]. Use of RIDR-PI-103 in cord blood cells and fibroblasts did not demonstrate toxicity [29,30]. Similarly in our study, we observed that RIDR-PI-103 had increased specificity in TDR cells over melanocytes at 5 μ M (Figs. 1 and

2). Previous studies used ROS inducers including the chemotherapeutics doxorubicin or cytosine arabinoside to activate RIDR-PI-103. In our study, we did not utilize ROS inducer and still observed activation of the prodrug. We also observed the activity of RIDR-PI-103 at a concentration as low as 5 μ M in the BRAF mutant-resistant cells. On the basis of our *in-vitro* data, we establish RIDR-PI-103 at 5 μ M as the optimal concentration for treatment of BRAF-mutant resistant melanoma cells since 10 μ M dose of RIDR-PI-103 was toxic to melanocytes. We still proceeded to test the 10 μ M dose of RIDR-PI-103 on TDR cell lines to observe whether the higher concentration elicit effects similar to 5 μ M of the prodrug. This bolsters our findings of the study that 10 μ M RIDR-PI-103 used does not aberrantly re-activate the PI3K/Akt pathway in TDR cells but is toxic to cells. We observed some inconsistent data for WM983B TDR cell line in proliferation assays (Fig. 2) compared to cell viability assay using trypan blue (Fig. S3, Supplemental Digital Content 3, <http://links.lww.com/ACD/A493>). In Fig. 2, we are measuring the proliferation of TDR cells in the presence of RIDR-PI-103 using 2D and 3D assays, in Fig. S3, Supplemental Digital Content 3, <http://links.lww.com/ACD/A493>, we are measuring cell viability using the trypan blue exclusion assay. The variance in the results can be attributed to assay differences. For Fig. S3, Supplemental Digital Content 3, <http://links.lww.com/ACD/A493>, we have measured cell viability using trypan blue exclusion assay for up to 72 h. WM983B is derived from a metastatic melanoma whereas A375 and WM115 are primary melanomas [44–46]. The lack of effect of RIDR-PI-103 on cell viability using the trypan blue exclusion assay in WM983B TDR compared to other TDR cells could be attributed to the metastatic nature of WM983B cells and activation of other pro-survival signaling pathways in response to RIDR-PI-103 which antagonize the effect of this prodrug.

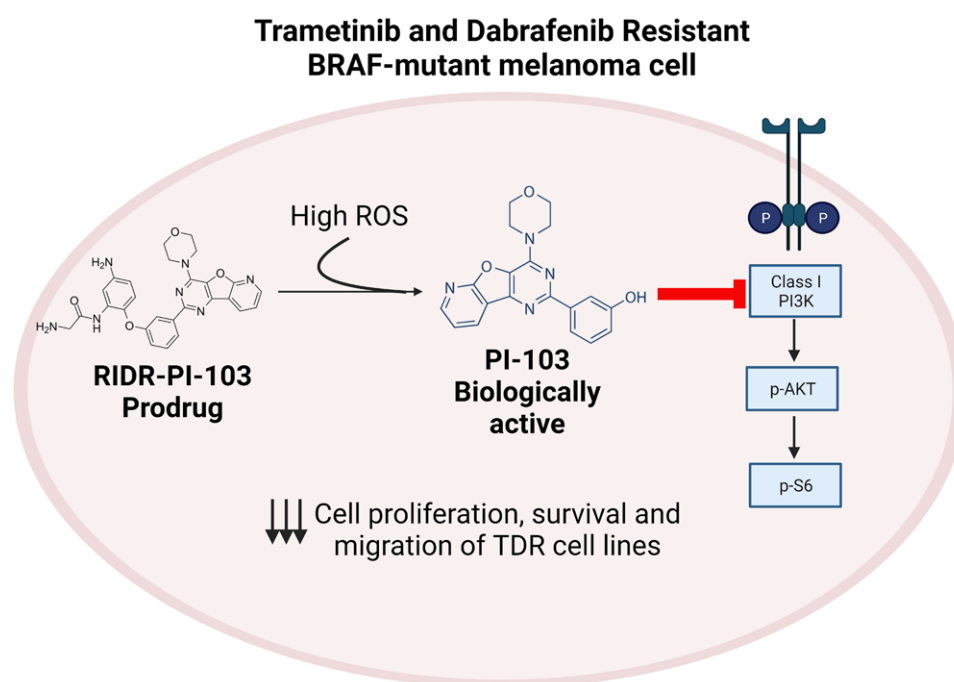
Our data show that RIDR-PI-103 inhibits its target, the PI3K/Akt pathway. We observed inhibition of p-AktS473 in WM115 TDR, A375 TDR, and WM983B TDR cells with 24 h of RIDR-PI-103 treatment in a dose-dependent manner. The inhibitory effect of RIDR-PI-103 via PI3K signaling was confirmed using a constitutively active AKT plasmid where transient transfection of WM115TDR cell using AKT constitutive active plasmid prior to RIDR-PI-103 treatment rescued the inhibitory effect induced by it. However, we could not confirm a similar mechanism in WM983TDR or A375TDR cell lines as they were not amenable to transient transfection. S6 is a protein that is downstream of Akt. Blockade of p-Akt using PI3K inhibitors also blocks p-S6 [47,48]. We observed blockade of p-S6 upon treatment with RIDR-PI-103 in a dose-dependent manner at residues Ser240/244 and Ser235/236 (Fig. 3). We did not observe attenuation in the mTOR1/2 pathway

proteins in treatment with increasing concentrations of the prodrug (data not shown). In order to determine activation of RIDR-PI-103 relies on ROS levels, we treated cells with glutathione, ROS scavenger with or without RIDR-PI-103. Glutathione exists in a reduced and oxidized (GSSG) state in cells. Redox status of the cells is determined by the ratio of glutathione to GSSG. An excess of ROS is ultimately converted to H_2O_2 by the cells. In presence of H_2O_2 , glutathione gets converted to GSSG and results in formation of water and oxygen, which in turn reduces oxidative stress in the cells [49,50]. When TDR cells were co-treated with glutathione, glutathione can quench ROS, and owing to decreases in ROS levels, the prodrug would have reduced activity. This was indicated by the rescue in cell proliferation that we observed in the groups treated with glutathione and RIDR-PI-103 compared to cells treated with RIDR-PI-103 alone (Fig. 4). Addition of NAC to RIDR-PI-103 rescued cell proliferation induced by RIDR-PI-103 in A375 TDR and WM983B TDR cell lines. Since NAC inhibited cell proliferation in the WM115 TDR cell line, interpretation of data for this cell line is difficult (Fig. S6, Supplemental Digital Content 6, <http://links.lww.com/ACD/A496>). Glutathione is composed of three amino acids – glycine, cysteine, and glutamic acid while NAC is the precursor for glutathione. A head-to-head study comparing the effects of NAC

and glutathione on melanoma cells has not been conducted; we anticipate that glutathione might be a more stable molecule compared to NAC. A study examining glutathione levels in TDR cells will help to delineate if endogenous glutathione aids in the rescue of cell proliferation along with the addition of exogenous glutathione in these cell lines.

A corollary of using ROS scavenger was to use ROS inducer and assess the effect of ROS inducer in combination with RIDR-PI-103. When TDR cells are co-treated with RIDR-PI-103 and TBHP, the TBHP will increase ROS levels in the cells. More activation of RIDR-PI-103 is expected when treated with the combination opposed to RIDR-PI-103 alone which can be demonstrated by inhibition of cell proliferation. Combination of RIDR-PI-103 and TBHP significantly inhibited cell proliferation in WM115 TDR and WM983B TDR cells while we did not observe this for A375 TDR cell line (Fig. 5). This could possibly be attributed to differences in endogenous ROS levels in A375 TDR cells compared to WM115 TDR and WM983B TDR cells. A study simultaneously examining the ROS levels in all three TDR cell lines will provide further insights. Fig. 7 summarizes the efficacy of RIDR-PI-103 in BRAF and MEK inhibitor-resistant BRAF-mutant melanoma.

Fig. 7



Summary of the efficacy of RIDR-PI-103 in BRAF and MEK inhibitor-resistant BRAF-mutant melanoma. RIDR-PI-103 (a prodrug of PI-103) is activated in a high ROS environment in TDR cells and releases biologically active PI-103. PI-103 is a pan-PI3K inhibitor and inhibits the activation of Akt and S6. RIDR-PI-103 inhibits cell proliferation, growth, and migration in TDR cells. Figure generated using biorender.com. ANOVA, analysis of variance; RIDR, ROS-induced drug release; ROS, reactive oxygen species.

Single-dose pharmacokinetics of RIDR-PI-103 revealed the half-life of this drug to be around 9h based on a one-compartment model [30]. Our study has limitations. In-vivo studies using RIDR-PI-103 in resistant melanomas are beyond the scope of our current work and are a limitation of this study. It is important to assess the conversion of RIDR-PI-103 to PI-103 *in vitro* and *in vivo*. Since RIDR-PI-103 is a novel compound, there are challenges in terms of how to safely administer the prodrug and its efficacy *in vivo*. Pharmacokinetics studies focusing on single and repeated doses and dose escalation of RIDR-PI-103 will be carried out in the future. This will give a thorough understanding of the prodrug dosage and frequency. Future studies will be focused on developing the optimal dosage forms that can be utilized to administer the prodrug and circumvent toxicities and first-pass metabolism. In this study, we provide insight into the efficacy of RIDR-PI-103 *in vitro* in drug-resistant melanomas. Further studies investigating the safety and efficacy of RIDR-PI-103 are warranted on cancers resistant to BRAF and MEK inhibitors in BRAF-mutant melanoma.

Conclusion

In this study, we have tested the efficacy of ROS-activated prodrug (RIDR-PI-103) on BRAF-mutant TDR cells. We show that this inhibits cell growth and migration in a dose-dependent manner in these cells. Our data indicated that RIDR-PI-103, in a dose-dependent manner inhibits the activation of Akt and downstream S6 signaling. Therefore, utilizing ROS-based therapies could be an effective strategy in BRAF-mutant-resistant melanomas and warrants further investigation.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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Article

IGF1R/IR Mediates Resistance to BRAF and MEK Inhibitors in BRAF-Mutant Melanoma

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Simple Summary: Melanoma accounts for only 4% of skin cancer, but is the major cause of skin cancer related deaths. The use of dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor), two FDA approved drugs to treat patients with BRAFV600E melanoma, is limited in the clinic due to the development of resistance. The IGF family of receptors is known to play a crucial role in cancer progression. In our in vitro screening, we identified that the activation of Insulin-like growth factor 1 receptor (IGF1R) and Insulin Receptor (IR) mediates resistance to dabrafenib and trametinib. Patients with high levels of IGF1R and IR have worse survival outcomes compared to patients with low levels of these receptors. We demonstrate that combining dabrafenib and trametinib with an IGF1R/IR inhibitor, BMS-754807, in vitro and in vivo, is efficacious and inhibits proliferation and tumor growth. This research opens up avenues for the development of novel and potent IGF1R/IR inhibitors for patients with BRAF-mutant melanoma.



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Abstract: The use of BRAF and MEK inhibitors for patients with BRAF-mutant melanoma is limited as patients relapse on treatment as quickly as 6 months due to acquired resistance. We generated trametinib and dabrafenib resistant melanoma (TDR) cell lines to the MEK and BRAF inhibitors, respectively. TDR cells exhibited increased viability and maintenance of downstream p-ERK and p-Akt as compared to parental cells. Receptor tyrosine kinase arrays revealed an increase in p-IGF1R and p-IR in the drug resistant cells versus drug sensitive cells. RNA-sequencing analysis identified IGF1R and INSR upregulated in resistant cell lines compared to parental cells. Analysis of TCGA PanCancer Atlas (skin cutaneous melanoma) showed that patients with a BRAF mutation and high levels of IGF1R and INSR had a worse overall survival. BMS-754807, an IGF1R/IR inhibitor, suppressed cell proliferation along with inhibition of intracellular p-Akt in TDR cells. Dual inhibition of IGF1R and INSR using siRNA reduced cell proliferation. The combination of dabrafenib, trametinib, and BMS-754807 treatment reduced in vivo xenograft tumor growth. Examining the role of IGF1R and IR in mediating resistance to BRAF and MEK inhibitors will expand possible treatment options to aid in long-term success for BRAF-mutant melanoma patients.

Keywords: melanoma; BRAF; resistance; IGF1R; IR



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1. Introduction

Melanoma, the most aggressive form of skin cancer, originates in melanin producing melanocytes of the skin [1]. The five-year survival rate for patients diagnosed with metastatic melanoma is around 20% [2]. The mitogen-activated protein kinase pathway (MAPK), comprised of downstream effector proteins Ras, MEK, and ERK, plays a pivotal role in the proliferation and mutagenesis in melanoma [3]. The PI3K/Akt pathway and the loss of tumor suppressor PTEN contribute to melanoma tumorigenesis [4–6]. Up to 90% of melanomas contain BRAF^{T1799A} transversion, which encodes for constitutively active BRAF^{V600E} oncoprotein. This is critical for the survival and proliferation of melanoma

cells and results in hyperactivation of the MAPK pathway [7]. Co-targeting BRAF and MEK with dabrafenib and trametinib, respectively, demonstrated a long term benefit and dramatically improved response rates in about 70% patients harbouring the BRAF mutation as compared to standard chemotherapy and single agent BRAF inhibitors [8,9].

Chronic treatment with these inhibitors has met challenges as patient responses begin to drop due to the development of acquired resistance. Resistance to BRAF inhibitor vemurafenib or dabrafenib monotherapy occurs in 50% of patients within 6–8 months of drug treatment [10,11]. Disease progression for BRAF-mutant metastatic melanoma patients treated with a combination of dabrafenib and trametinib occurs within 9–10 months due to the development of resistance [12–14]. Resistance to BRAF and MEK inhibitors include, but are not limited to, alterations in BRAF splicing [15], mutations in NRAS [16], BRAF copy number amplification [17], activation of PI3K/Akt pathway, and hyperactivation of receptor tyrosine kinases (RTK), cell surface receptors that activate the MAPK and PI3K/Akt pathways [18–20].

IGF1R and IR are RTKs that belong to the insulin-like growth factor (IGF) family of receptors. These receptors are commonly overexpressed and their role in tumor cell proliferation and survival is well established in various malignancies [21–25], including melanoma [26]. Overexpression leads to alterations in the levels of regulators of these receptors, including ligands IGF1, IGF2, insulin, and serum insulin-like growth factor binding proteins (IGFBP 1–6). Activation of these receptors mediates downstream MAPK, PI3K/Akt, or pathways associated with cell contraction and motility by recruiting various adaptor proteins such as insulin receptor substrate 1 (IRS1), IRS2, and Src homology domain containing (Shc) [27,28]. Dysregulation of the IGF1R pathway acts as an oncogenic signal in initial tumorigenesis as well as resistance to targeted therapies [29].

In this study, we generated dabrafenib and trametinib resistant melanoma cell lines and characterized the cells. We observed that ERK and Akt were constitutively active in resistant cells as compared to the parental counterparts. We demonstrate that IGF1R and IR are activated in response to chronic dabrafenib and trametinib resistance in BRAF-mutant melanoma and are differentially expressed in parental and resistant cell lines. We utilized BMS-754807, a potent IGF1R/IR inhibitor with limited off targets effects [30,31]. We observed that the MAPKi resistant melanoma cells treated with BMS-754807 decreased the short and long-term proliferation along with the inhibition of phosphorylation of Akt. In vivo in a subcutaneous xenograft model, dabrafenib and trametinib resistant cells demonstrated increased tumorigenicity. Administration of BMS-754807 along with dabrafenib and trametinib significantly suppressed tumor growth compared to vehicle or dabrafenib and trametinib treatment. These findings provide mechanistic insight and a rationale for targeting MAPKi resistance BRAF-mutant melanoma with IGF1R/IR inhibition.

2. Materials and Methods

2.1. Reagents

Dabrafenib and trametinib were purchased from LC Laboratories. BMS-754807, Linsitinib, BMS-536924, and GSK1838705A were purchased from MedChemExpress. SCH772984 was obtained from Selleckchem (Houston, TX, USA). All drugs were dissolved in DMSO (Fisher Scientific(Waltham, MA, USA)) at described concentrations and stored at -20°C . IGF2 (cat#100-12) was purchased from Peprotech (Cranbury, NJ, USA).

2.2. Cell Lines and Culture Conditions

A375 and WM115 cells were purchased from American Type Cell Culture (ATCC, Manassas, VA, USA). WM983B cells were purchased from Rockland Immunochemicals, Inc (Limerick, PA, USA). A375 cells were cultured in DMEM (Corning, cat#MT15017CV); WM115 cells were cultured in MEM (Corning, cat#MT10009CV); WM983B cells were cultured in RPMI-1640 medium (Corning, cat#MT10040CM) supplemented with 10% (vol/vol) FBS (VWR, cat# 89510-188) and 1% Penicillin–streptomycin solution 100 $\times$ (Corning, cat#30-

002-CI). Cultured cells were incubated in a humidified incubator at 37 °C with 5% (vol/vol) CO₂ and 95% (vol/vol) air.

2.3. Establishing Resistant Lines

A375, WM983B, and WM115 trametinib and dabrafenib resistant lines (TDR cells) were generated by treating parental cells with increasing concentration of dabrafenib and trametinib starting over a period of 9–11 months until cells could grow in the presence of a specified concentration of dabrafenib and trametinib. A375 TDR cell lines were maintained in 250 nM dabrafenib and 12.5 nM trametinib. WM983B TDR cells were maintained in 2.4 µM dabrafenib and 500 nM trametinib. WM115 TDR cells were maintained in 800 nM dabrafenib and 200 nM trametinib. The TDR cells and corresponding parental cells were tested for mycoplasma contamination and authenticated by STR profiling by ATCC every six months.

2.4. RTK Array

Parental and TDR cells treated with DMSO or a combination of dabrafenib and trametinib were lysed and 400 µg proteins were used for Proteome Profiler Human Phospho-RTK Array Kit (R&D Systems, cat#ARY001B (Minneapolis, MN, USA)) as per the manufacturer's instructions. The intensities on the array were quantified using a negative control on the array and Image J software 1.8.0 (NIH) (Bethesda, MD, USA).

2.5. Cell Proliferation Assay and Long Term Growth Assays

2.5.1. MTT Assay

Growth kinetics of A375, A375 TDR, WM983B WM983B TDR, WM115, and WM115 TDR cells were determined by the 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 1×10^4 cells/well were seeded in 96-well plates in triplicates. For Figure 1B, a total of 10 concentrations of dabrafenib and trametinib were used in combination. The concentrations of dabrafenib and trametinib for WM115 were vehicle, 0.1 nM, 0.5 nM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, and 1 µM. The concentrations of dabrafenib and trametinib for WM983B were vehicle, 0.5 nM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 µM, and 5 µM. The concentrations of dabrafenib and trametinib for A375 were vehicle, 0.1 nM, 0.5 nM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, and 1 µM. For Figure 1C, a total of 10 concentrations of dabrafenib and trametinib used were as follows: vehicle, 0.1 nM, 0.5 nM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, and 1 µM. After 72 h of treatment with dabrafenib and trametinib, the medium was substituted with 0.5 mg/mL MTT solution (Sigma Aldrich) for 4 h and absorbance was recorded at 570 nm using SPECTRAmax PLUS Microplate Spectrophotometer Plate Reader (Molecular Devices Corporation, San Jose, CA, USA) and expressed as the mean of triplicates relative to vehicle (DMSO) control together with standard error of mean (SEM). IC₅₀ values were generated using the GraphPad Prism 7 software, version 7.01 (San Diego, CA, USA). In separate experiments, dabrafenib and trametinib were withdrawn from the TDR cells for 21 days and an MTT assay was performed with parental, TDR, and TDR cells with drugs withdrawn from them in the presence of dabrafenib and trametinib with the same conditions as mentioned above. Growth kinetics of TDR cells in the presence of 3, 5, and 10 µM BMS-754807 were assessed as mentioned above.

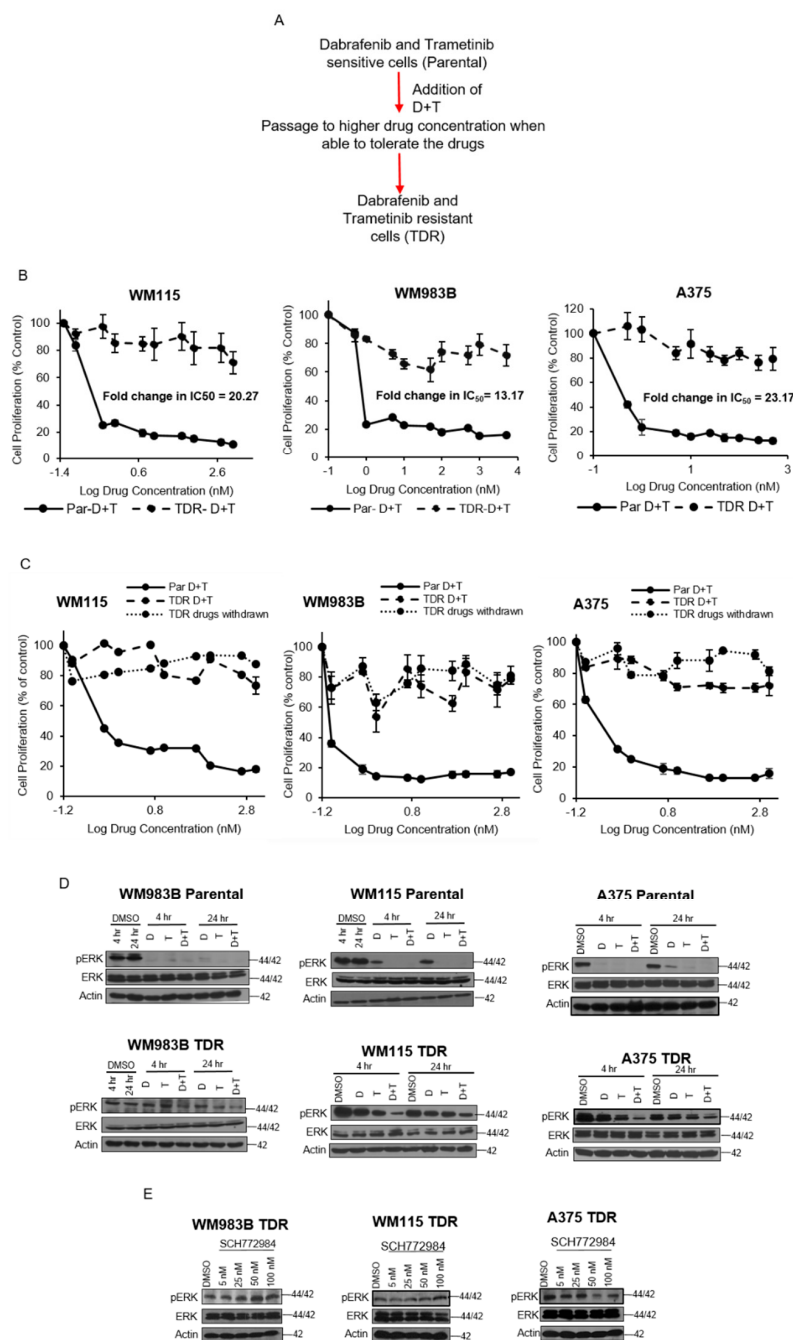


Figure 1. Growth kinetics and characteristics of TDR cell lines. **(A)** Schematic of TDR cell generation. **(B)** WM115, WM983B, and A375 parental and TDR cells (1×10^4 cells/well) were seeded in 96-well plates and treated with increasing concentrations of dabrafenib (D) and trametinib (T) (0.1 nM–5 μ M) for 72 h. Cells were treated with MTT for 4 h and absorbance was read at 570 nm. Data reflect three independent experiments and are presented as mean $\pm$ SEM. **(C)** Dose response MTT proliferation assay for parental and TDR cell lines maintained in drug-free conditions for 21 days. Cells were plated for 24 h and treated with 72 h with increasing concentrations of dabrafenib (D) and trametinib (T). Cells were treated with MTT. Data reflected as mean $\pm$ SEM. **(D)** Immunoblots of WM983B, WM115, and A375 parental and TDR cells treated for 4 and 24 h with dabrafenib (D), trametinib (T), or combination of dabrafenib and trametinib (D + T). Whole cell lysates from the cell lines were analyzed by Western blot using indicated antibodies. Actin served as the loading control. **(E)** Immunoblots for TDR cells treated with increasing concentrations of SCH772984 (5–100 nM) for 24 h. Actin was used as the loading control.

2.5.2. Crystal Violet Assay

TDR cells were seeded at a density of 2×10^4 cells/well in 12 well plates (Falcon) in triplicates. Complete media containing BMS-754807 (5 μ M), dabrafenib, and trametinib was replaced every 2 days. On day 21, cells were stained with 0.5% crystal violet. The cultures were exposed to crystal violet solution for 2 min and washed off with water to remove the excess stain. The intensities were measured using an Odyssey infrared system (LI-COR, Lincoln, NE, USA). The Experiments were repeated at least twice.

2.5.3. Matrigel Growth Assay

Three-dimensional (3D) growth assays were performed in growth factor-reduced matrigel (BD Biosciences, cat#CB-40230 (San Jose, CA, USA)) where 96-well plates were coated with 70 μ L of matrigel/well. TDR cells (2×10^4 /well) were plated and incubated at 37 °C for 24 h. Cells were treated with vehicle (DMSO), BMS-754807 (5 μ M) and/or dabrafenib + trametinib every alternate day. After 21 days of incubation, cells were visualized and photographs were captured from 5 random fields under microscope (Nikon, Melville, NY, USA) at 10 $\times$ magnification. The areas of the cells were measured by ImageJ and represented as mean areas normalized to DMSO control.

2.6. Western Blot Analysis

Cells were lysed in RIPA buffer (Thermo Fisher Scientific, Cat#BP-115) supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, Cat#88669). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Cat#23225) according to manufacturer's instructions. A minimum of 15 μ g of protein was used for each sample. Primary antibodies used for immunoblotting are described in Table S1. The primary antibody (1:1000 dilution) was added to the nitrocellulose and incubated overnight at 4 °C with gentle agitation. Blots were then washed with 1 $\times$ TBST (3 washes, 10 min each) and incubated with anti-rabbit IgG-horse radish peroxidase (HRP) (Santa Cruz Biotechnology, cat#SC-2357; 1:2000 dilution) for 1 h at room temperature. Blots were then washed with 1 $\times$ TBST (3 washes, 10 min each). Protein signals were detected using Pierce ECL Western blotting substrate (Thermo Fisher Scientific, Cat# 32106). For serum starvation experiment, TDR cells were serum starved for 24 h and IGF2 (10 μ g/mL) was added at specified time points. In a separate set of experiments, cells were serum starved for 24 h, followed by addition of BMS-754807 and IGF2. No blots were stripped and reprobed.

For lysing mice tumors, the tumors were thawed on ice and RIPA buffer supplemented with 3 times concentration of protease and phosphate inhibitors was added for 20 min. The tumor was homogenized using a homogenizer for 5 min, briefly spun in centrifuge, and the supernatant was collected. Protein concentration was determined and Western blot analysis was performed. No blots were stripped and reprobed.

2.7. RNA Extracztion and RT-qPCR

Total RNA was extracted from parental and TDR cells treated with DMSO or dabrafenib + trametinib using the RNA extraction mini kit (Qiagen, cat#74104, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was determined using a NanoDropTM (ThermoScientific, Waltham, MA, USA) spectrophotometer. Two-step RT-qPCR was performed to assess the mRNA levels. First strand cDNA was synthesized using iScriptTM cDNA Synthesis Kit (BioRad, cat#1708891, Hercules, CA, USA). The mRNA levels were quantified by qPCR using primers purchased from Integrated DNA Technologies. qPCR was set up using CFX96 Real-Time System (BioRad). The relative mRNA levels were calculated by the $2^{-\Delta\Delta C_t}$ method. Actin was used as internal control. Primers used for the experiment are listed in Table S2.

2.8. Knockdown of IGF1R and INSR

TDR cells were transfected with 20 nM of siRNA, specifically targeting ON-TARGETplus Human IGF1R siRNA-SMARTpool IGF1R (Horizon Discovery) (Sequences: GAACAAG-GCUGCCGAGAGU; AAACGAGGCCCGAAGAUAUU; ACGGAGACCUGAAGAGUGCUA; GCAGGUCCCUUGGCCAUGU) and/or ON-TARGETplus Human INSR siRNA-SMARTpool (Horizon Discovery) and ON-TARGETplus Non-targeting Pool (Horizon Discovery, Cambridge, England) (Sequences: GGAAGCACCCUUUAAGAAU; GGACUCAGUACGCCGU-UUA; AAAUACGGAUCACAAGUUG; AGUGAGAUCUUGUACAUUUC) as control as instructed by manufacturer's protocol. After 72 h, cells were harvested for proliferation and Western Blot analysis.

2.9. RNA Sequencing

Total RNA was extracted using the RNA extraction mini kit (Qiagen, cat#74104) according to the manufacturer's instruction. The RNA quality was determined by Bioanalyzer (Agilent, Santa Clara, CA, USA). To isolate the polyA RNA, NEBNext Poly (A) mRNA Magnetic Isolation Module (New England BioLabs, Ipswich, MA, USA, cat#E7490S) was used with a total of 1 µg of good quality total RNA as input. The NEBNext Ultra II Directional RNA Library Prep Kit (New England BioLabs, cat#E7760S) was used for library preparation, which is dUTP-based stranded library. After library quality control and quantification, individually indexed and compatible libraries were proportionally pooled and sequenced using the Illumina HiSeq 1000 sequencing platform. Under the sequencing setting of single read 1 × 51 bp, about 25 million pass filter reads per sample were generated. Data were analyzed using iGEAK [32] (Interactive gene expression analysis kit; Cincinnati Children's Hospital and Medical Center, Cincinnati, OH, USA).

2.10. In Vivo Xenograft Study

Institutional Animal Care and Use Committee (IACUC), University of Cincinnati ethically approved the in vivo mouse experiment (UC IACUC approval: 19-04-19-01; Approval date: 14 April 2021). Male athymic nude mice were purchased from Jackson Laboratories at the age of 3–4 weeks (cat# 007850 Homozygous Foxn1nu). Approximately 1×10^6 cells WM115 TDR cells resuspended in $1 \times$ PBS and growth factor-reduced matrigel (BD Biosciences, cat#CB-40230) (1:1 ratio, total volume injected 200 µL) were injected s.c. into right flank of the mice. Formice injected with WM115 parental and TDR cells, the cells were prepared as mentioned above and WM115 parental were injected into the left flank and WM115 TDR cells were injected into the right flank of nude mice. Tumor volume was determined using the formula: tumor volume = length × width²/2. For WM115 TDR tumors, when the tumors reached 200 mm³, mice were randomized into three groups: (i) vehicle (PEG400:water 1:1 and 0.5% HPMC and 0.2% Tween; (ii) dabrafenib (30 mg/kg daily) + trametinib (0.3 mg/kg daily) resuspended in 0.5% HPMC and 0.2% Tween and (iii) BMS-754807 (10 mg/kg twice daily; resuspended in PEG400: water 1:1 ratio) and dabrafenib (30 mg/kg daily) and trametinib (0.3 mg/kg daily) resuspended in 0.5%HPMC and 0.2% Tween. The drugs were administered by p.o. route using an oral gavage needle. The volume of drug administered was 100 µL. The duration of treatment was 15 days. Vehicle and dabrafenib + trametinib were administered once a day in the morning. Two doses of BMS-754807 administered in a day were at least 8 h apart, once in the morning and once in the evening. Tumor size was recorded 2 to 3 times a week post randomization. The last dose of drugs was given 1 h prior to sacrificing mice. Tumors were collected and snap-frozen in liquid nitrogen and placed in −80 °C and then used for Western blot analysis.

2.11. Statistical Analysis

Data are shown as the mean ± standard error of mean (SEM) and representative of at least three independent experiments unless otherwise indicated. Statistical analysis among groups was performed using the two-tailed Student's *t*-test, and one-way analysis

of variance; $p < 0.05$ was considered statistically significant. Logrank Test p -value for patient data was generated by cBioPortal.

3. Results

3.1. Phospho-ERK Levels Are Maintained in Response to Chronic Dabrafenib and Trametinib Resistance In Vitro

We exposed BRAF mutant melanoma cell lines A375, WM115, and WM983B to increasing concentrations of the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib (Figure 1A). The BRAF status of these cell lines along with PTEN and CDKN2A status are summarized in Table 1.

Table 1. BRAF, PTEN, and CDKN2A status in A375, WM983B, and WM115 BRAF-mutant melanoma cell lines [33].

Cell Line	BRAF Status	PTEN Status	CDKN2A Status
A375	V600E	WT	p.E10, p.E18
WM983B	V600E	WT	p.P63L
WM115	V600D	Loss of heterozygosity [34]	Homozygous Deletion [35]

We generated trametinib- and dabrafenib-resistant (TDR) WM115, A375, and WM983B cell lines via a gradual dose escalation of each drug and found a shift in IC_{50} values for resistant cells (Figure 1B). When parental cells were treated with dabrafenib, trametinib, or a combination of both inhibitors, we observed a striking reduction in acini size as compared to TDR cells (Figure S1). We observed that with three weeks of drug withdrawal, drug resistant cells maintained their drug resistant phenotype, (Figure 1C). The parental drug sensitive cell lines demonstrated reduced p-Erk in the presence of dabrafenib and/or trametinib, whereas drug resistance cells maintained p-Erk in the presence of drug (Figure 1D). Furthermore, drug resistant cells maintained p-Erk in the presence of the Erk1/2 inhibitor SCH772984 (Figure 1E). These data indicate that the drug resistant cells maintain p-Erk in the presence of drug and preserve their drug resistance phenotype after three weeks of drug withdrawal.

3.2. IGF1R and IR Are Activated and Upregulated in BRAF-Mutant Melanoma TDR Cell Lines

We next examined if there was differential phosphorylation of receptor tyrosine kinases (RTK) between the parental and drug resistant cells by performing RTK arrays which examine the phosphorylation status of 49 different RTKs. We observed that drug resistant cells treated with drugs had elevated levels of phosphorylated EGFR, HER3 (ErbB3), IR, IGF1R, Axl, and Dtk (Figure 2A). We quantified the signal density and observed that phosphorylated HER3 was increased in drug resistant WM115 cells treated with vehicle DMSO or dabrafenib and trametinib treatment (Figure 2B). Phosphorylated Axl was increased in drug resistant WM115 cells treated with vehicle DMSO and in drug resistant WM983B cells treated with vehicle DMSO or dabrafenib and trametinib treatment. Phosphorylated EGFR was increased in drug resistant WM983B cells treated with vehicle DMSO or combination of dabrafenib and trametinib. Phosphorylated Dtk was increased in drug resistant WM983B cells treated with vehicle DMSO. Phosphorylated IGF1R was increased in drug resistant WM115 and A375 cells treated with vehicle DMSO or dabrafenib and trametinib treatment. Strikingly, phosphorylated IR was increased in all three drug resistant cells treated with vehicle DMSO or dabrafenib and trametinib treatment. Next, to validate the RTK array results, we performed a Western blot analysis (Figure 2C). Phosphorylated HER3 was increased in drug resistant WM115 cells treated with vehicle DMSO or dabrafenib and trametinib treatment compared to parental DMSO treated cells. Phosphorylated Axl was increased in drug resistant WM115 and WM983B cells, correlating with RTK array data. Phosphorylated EGFR was increased in drug resistant WM983B cells treated with vehicle DMSO or dabrafenib and trametinib. Addition of dabrafenib and trametinib to WM115, WM983B, and A375 parental cells had no effect on p-IGF1R/p-IR levels compared to parental cells treated with DMSO. In accordance with the results

from RTK array, WM115 and A375 TDR cells treated with DMSO or dabrafenib and trametinib exhibited an increase on phosphorylation at p-IGF1R at site Y1131 and p-IR at site Y1146, while these levels were maintained in WM983B TDR cells. These data indicate that drug resistant cells display differential phosphorylation of RTKs with notably increased phosphorylated IGF1R/IR.

3.3. Differential Expression of IGF1R and INSR in Parental and TDR Cells

We performed RNA-seq to examine differences between BRAF pathway inhibitor drug sensitive and drug resistant cells at the gene level. We found that IGF1R is upregulated in all three drug resistant cells compared to parental cells (Figure 3A). INSR, the gene that encodes IR, was increased in drug resistant A375 and WM115, but not in WM983B cells. IGF1 and IGF2, the high affinity ligands to IGF1R, were increased in drug resistant WM115 cells only. Addition of dabrafenib and trametinib to parental cells led to the upregulation of INSR in all three parental cell lines. IGF1R was only upregulated in A375 parental cells treated with dabrafenib and trametinib and not in WM983B and WM115 cell lines. Treatment with dabrafenib and trametinib for WM983B and WM115 parental cells increased the levels of IGF1 and IGF2. A375 parental cells treated with dabrafenib and trametinib exhibited downregulation of their ligands, IGF1 and IGF2. The differences in gene expression across the three cell lines could be attributed to their characteristics, i.e., WM983B is a metastatic cell line whereas A375 and WM115 are primary melanoma cell lines along with the presence of various mutations described in Table 1. As a separate way to quantify RNA levels, we performed real-time quantitative PCR for IGF1R, IGF1, IGF2, and INSR (Figure 3B). Consistent with RNA-seq data, we found that IGF1R increased in all TDR compared to parental cells. INSR was increased in drug resistant A375 and WM115, but not in WM983B cells, correlating with RNA-seq data. We found that IGF1 and IGF2 levels increased in drug resistant WM115 cells only, but not in WM983B and or A375 cells. Treating WM115 and WM983B parental cells with dabrafenib and trametinib significantly decreased the mRNA expression of IGF1R and INSR, while the expression of IGF1R was increased for A375 parental cells under the same condition. IGF1 expression was increased when WM983B parental cells were treated with a combination of dabrafenib and trametinib. Addition of dabrafenib and trametinib to A375 parental cells augmented IGF2 levels by two folds. We observed an increase in mRNA expression for Axl, Protein S, and Gas6 in TDR cell lines (Figure S2). We next examined that survival curves of melanoma patients from the TCGA data set for patients with a BRAF mutation along with high or low levels of INSR or IGF1R. Patients with high levels of INSR had a statistically significant worse survival compared to patients with low levels of INSR (Figure 3C). Comparison of survival for patients with high levels of IGF1R versus low levels of IGF1R approached significance with a *p*-value of 0.0723. Overall, these data indicate that the BRAF pathway inhibitor resistant cells have elevated IGF1R at the mRNA level and melanoma patients with elevated IGF1R and INSR have a worse survival compared to patients with low levels of IGF1R.

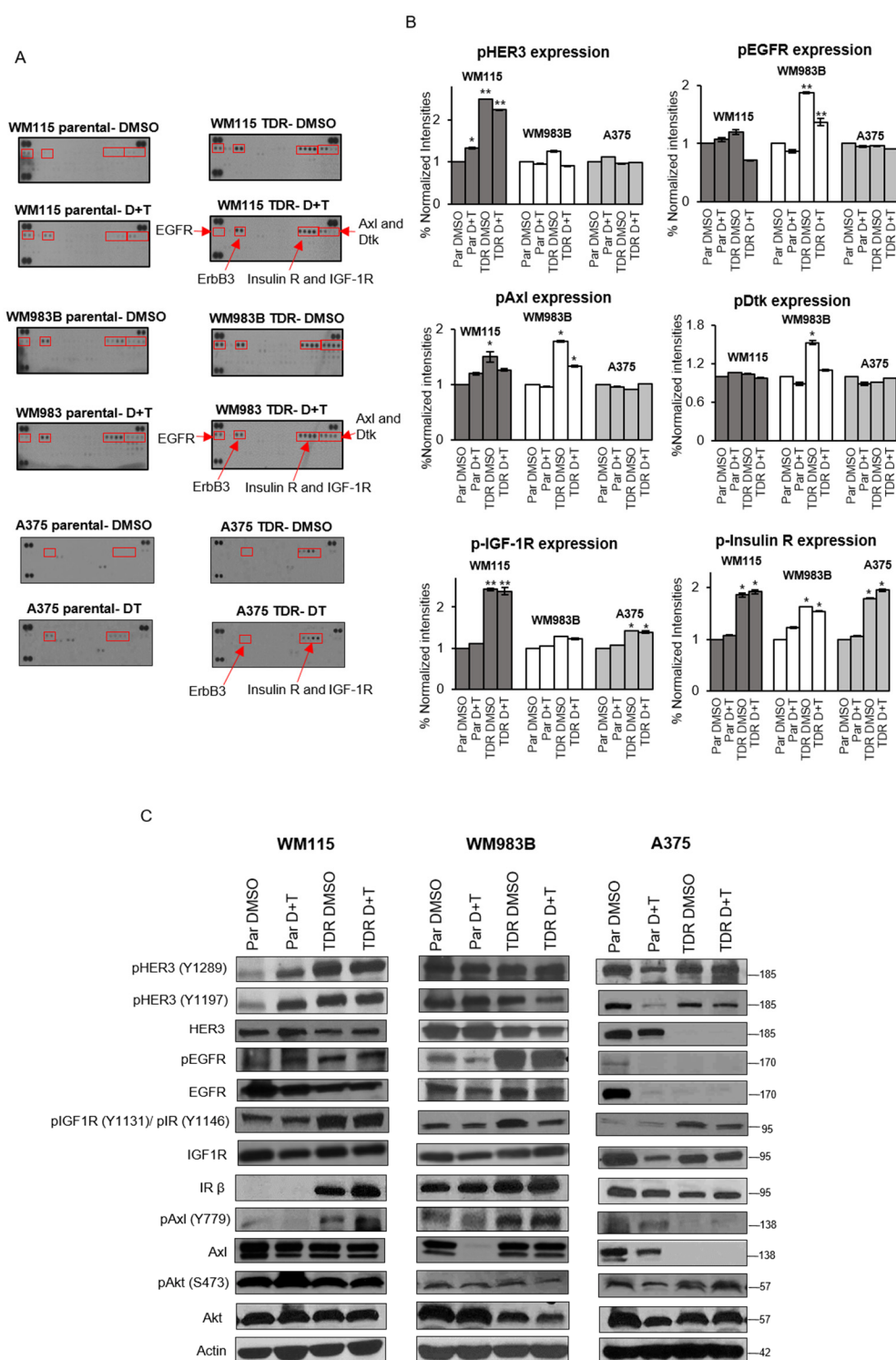


Figure 2. Upregulation of IGF1R and IR kinase activity in BRAF-mutant melanoma cells resistant to BRAF and MEK inhibitors. **(A)** Parental and TDR cells (WM115, WM983B, and A375) either treated with DMSO or combination of dabrafenib (D) and trametinib (T) (WM115: D: 800 nM, T: 200 nM; WM983B: D: 2.4 μ M, T: 500 nM; A375: D: 250 nM, T: 12.5 nM) for 24 h. The red boxes indicate the receptors with altered tyrosine kinase activity. Each RTK spotted in duplicate with positive controls in the corner. **(B)** The average signal density of spots normalized to parental DMSO group; *p*-values were computed using GraphPad Prism 7.0. * *p* < 0.05; ** *p* < 0.001 by two-tailed Student's *t*-test. Data are presented as mean $\pm$ SEM. **(C)** Immunoblot analysis of parental and TDR cells treated with DMSO or combination of dabrafenib and trametinib (D + T) for 24 h. Whole cells were analyzed using Western blot with indicated antibodies. Actin served as the loading control.

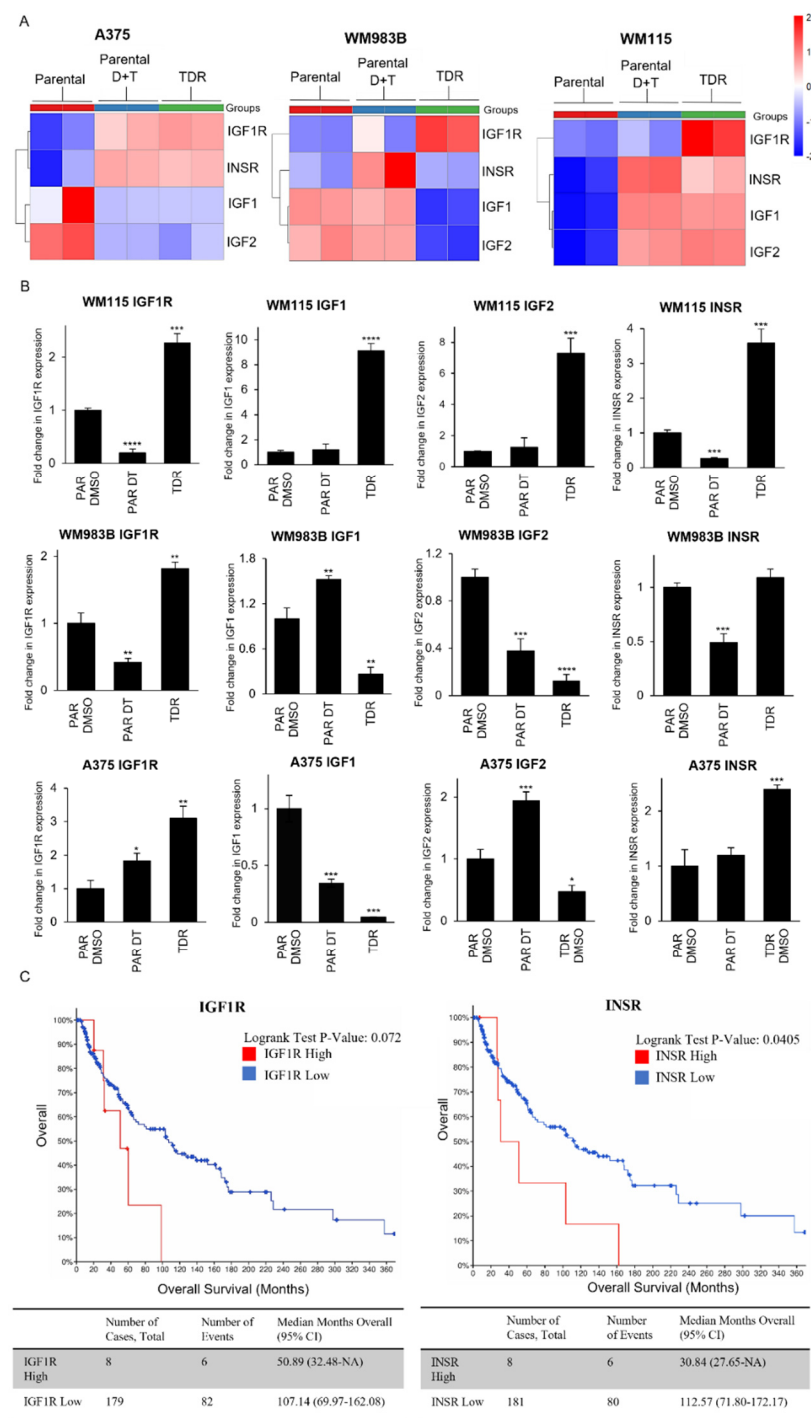


Figure 3. IGF1R and INSR are differentially expressed in TDR cells. **(A)** Heatmap of differentially expressed genes in parental cells, parental cells treated with combination of dabrafenib (D) and trametinib (T) (WM115: D: 800 nM, T: 200 nM; WM983B: D: 2.4 μ M, T: 500 nM; A375: D: 250 nM, T: 12.5 nM), or TDR cells. **(B)** Quantification of Actin-normalized mRNA levels using real-time quantitative PCR for IGF1R, IGF1, IGF2, and INSR for parental cells treated with DMSO or dabrafenib + trametinib and TDR following 24 h of treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ by two-tailed Student's *t*-test. Data are presented as mean $\pm$ SEM. **(C)** Kaplan Meier survival curve of skin cutaneous melanoma patients (TCGA, PanCancer Atlas) with data stratified for BRAF mutation along with IGF1R expression z-score > 2 ($n = 8$) and IGF1R expression z-score < 2 ($n = 179$) (Left panel); and INSR expression z-score > 2 ($n = 8$) and INSR expression z-score < 2 ($n = 181$) (Right panel).

3.4. BMS-754807 Inhibits Cell Proliferation and Intracellular Akt Signaling in TDR Cells

We probed the effect of pharmacological inhibition of IGF1R and IR in our BRAF pathway inhibitor resistant cells using BMS-754807, a reversible inhibitor of IGF1R and IR. While the addition of BMS754807 to dabrafenib and trametinib resulted in a reduction in proliferation (Figure 4A), Linsitinib, BMS-536924, and GSK1838705A failed to inhibit proliferation in combination with dabrafenib and trametinib in TDR cells (Figure S3). BMS-754807 as a single agent did not inhibit cell proliferation and p-IGF1R/p-IR expression in TDR cells (Figure S4). A 21-day crystal violet assay revealed similar results (Figure 4B). We next plated BRAF pathway inhibitor resistant cells on a basement membrane of matrigel and treated with vehicle, dabrafenib and trametinib or dabrafenib and trametinib and BMS-754807. We observed a striking reduction in size of acini in cells treated with dabrafenib and trametinib and BMS-754807 (Figure 4C). We assessed the effect that BMS-754807 has on signaling in drug resistant cells. After stimulation with the ligand IGF2, BMS-754807 treatment reduced p-IGF1R, p-IR, and p-Akt, but did not affect p-Erk levels (Figures 4D and S5). These data indicate that pharmacological inhibition of IGF1R and IR reduces the growth of BRAF pathway inhibitor resistant cells and reduces p-IGF1R, p-IR, and p-Akt within these cells.

3.5. Knockdown of IGF1R/INSR Regulates TDR Cell Viability

We next assessed the effect of genetic inhibition of IGF1R and IR in our BRAF pathway inhibitor resistant cells. We found that siRNA targeting IGF1R reduced IGF1R in all three cell lines (Figure 5A). INSR siRNA reduced IR levels and the combination of IGF1R and INSR siRNA reduced both IGF1R and IR in all three cell lines. We performed an MTT cell proliferation assay on cells after transfection with siRNA targeting IGF1R, INSR, or both siRNAs. We observed a reduction in the proliferation for cells transfected with siRNA targeting IGF1R and INSR compared to siRNA control. siRNA targeting INSR reduced cell proliferation of A375 TDR cells (Figure 5B). These data indicate genetic inhibition of IGF1R and IR reduces the growth of BRAF pathway inhibitor resistant cells.

3.6. IGF1R/IR Blockade Decreases Tumor Growth in WM115 TDR Xenograft

We assessed the in vivo growth potential of WM115 parental and BRAF pathway inhibitor resistant cells. Athymic nude mice were injected on the right flank with WM115 TDR and the left flank with parental WM115 cells (Figure 6A). We observed that drug resistant cells grew over time whereas the parental drug sensitive cells did not grow in our hands (Figure 6B). These data indicate the growth potential in vivo of the drug resistant cells. We next performed a drug efficacy study in WM115 TDR tumors. Once tumors were greater than 200 mm³, mice were randomly allocated to either vehicle, dabrafenib and trametinib, or dabrafenib and trametinib and BMS-754807. At day 15 of treatment, mice treated with dabrafenib and trametinib and BMS-754807 had a statistically significant reduction in tumor growth compared to either vehicle or dabrafenib and trametinib treated groups (Figure 6C). We did not observe a significant change in weight of mice treated with dabrafenib and trametinib and BMS 754807 (Figure 6D). Mice were given drug one hour before sacrifice at day 16, sacrificed, and tumors were harvested for analysis. Western blot analysis of xenografts revealed a reduction in p-IGF1R/p-IR in tumors treated with dabrafenib and trametinib and BMS-754807 compared to vehicle or dabrafenib and trametinib treated tumors (Figure 6E). Downstream of p-IGF1R/p-IR, we observed a reduction in p-Akt in two out of three tumors treated with dabrafenib and trametinib and BMS-754807 compared to vehicle or dabrafenib and trametinib treated tumors. These data indicate that inhibition of IGF1R/IR could be an effective strategy to overcome resistance to BRAF pathway inhibitor resistant tumor. All original images from Western blots are included in Figures S5–S11.

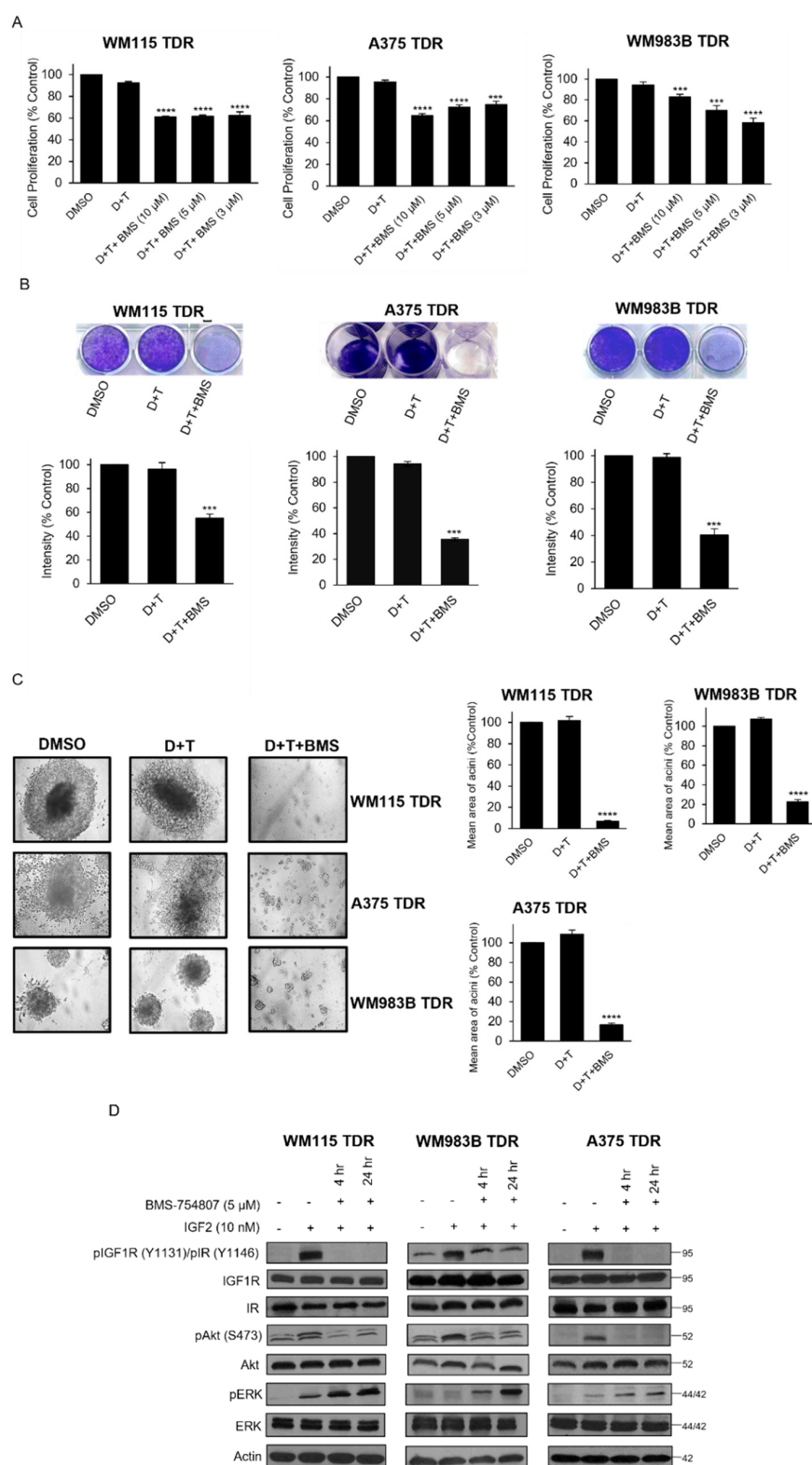


Figure 4. BMS-754807 in combination with dabrafenib and trametinib inhibits cell proliferation and inhibits intracellular Akt (A) TDR cells (WM115, WM983B, and A375) plated for 24 h and treated with DMSO, dabrafenib (D), and trametinib (T) (WM115: D: 800 nM, T: 200 nM; WM983B: D: 2.4 μM, T: 500 nM; A375: D: 250 nM, T: 12.5 nM) treated alone or in combination with BMS-754807 (3, 5, and 10 μM) and treated with MTT. The intensities are represented as mean. Error bars: SEM *** $p < 0.001$; **** $p < 0.0001$ by two-tailed Student's t -test compared to DMSO group.

(B) TDR cells were plated and treated with media containing DMSO, dabrafenib (D) and trametinib (T) (WM115: D: 800 nM, T: 200 nM; WM983B: D: 2.4 μ M, T: 500 nM; A375: D: 250 nM, T: 12.5 nM), or BMS-754807 (5 μ M) in combination with dabrafenib and trametinib (D + T + BMS). Media containing drugs were replenished every second day and stained with 0.5% crystal violet on Day 21. Representative images of the well for each treatment (Top panel). Quantification of intensities (Bottom panel) for the wells is represented as mean. Error bars: SEM ($n = 2$ independent experiments performed in triplicates). *** $p < 0.001$ by two-tailed Student's *t*-test compared to DMSO group. (C) TDR cells were seeded on matrigel basement membrane and treated with indicated drugs every second day. Pictures of each well were captured on day 21 (left panel) at 10 $\times$ magnification. Quantification of mean area of acini (right panel). **** $p < 0.0001$ by two-tailed Student's *t*-test compared to DMSO group. (D) TDR cells were serum starved for 24 h and treated with BMS-754807 (5 μ M) along with dabrafenib (D) and trametinib (T) (WM115 TDR: D: 800 nM, T: 200 nM; WM983B TDR: D: 2.4 μ M, T: 500 nM; A375 TDR: D: 250 nM, T: 12.5 nM) for 4 or 24 h followed by IGF2 (10 nM) stimulation for 1 h. Whole cell lysates were analyzed by Western blot using indicated antibodies. Actin was used as loading control.

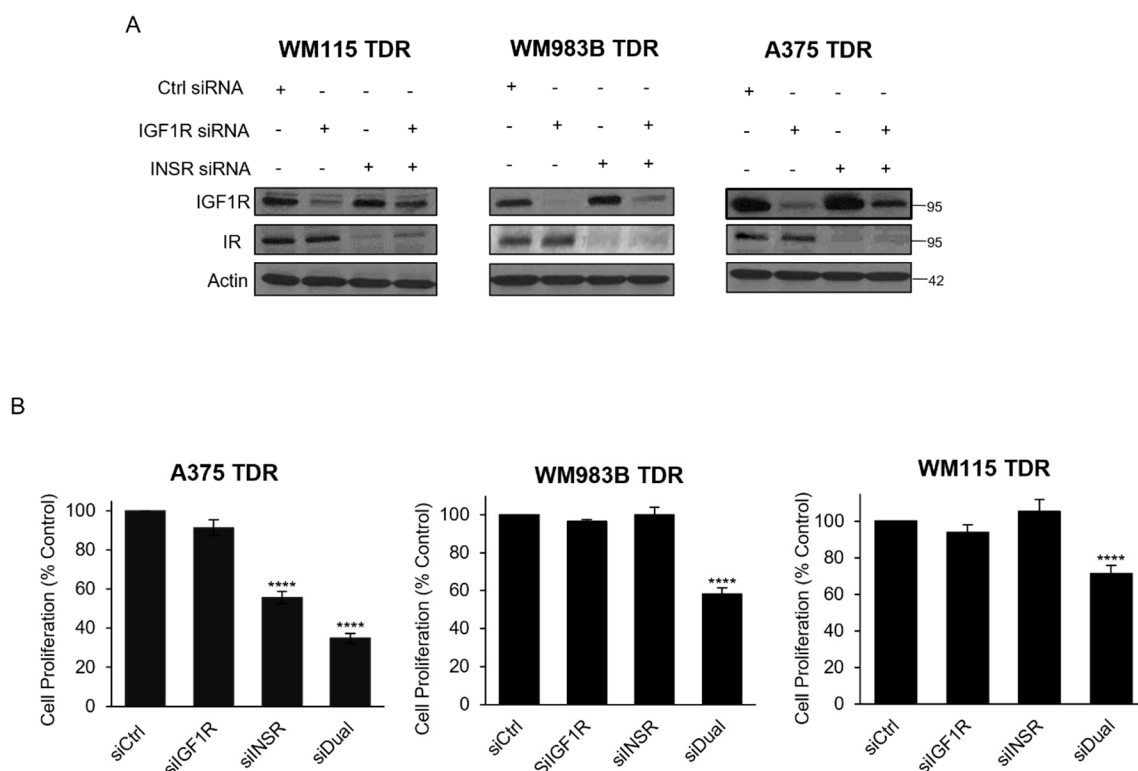


Figure 5. Dual knockdown of IGF1R and INSR reduces proliferation in TDR cells. (A) TDR cells were transfected with Ctrl siRNA, IGF1R siRNA, INSR siRNA, or combination of IGF1R and INSR siRNA for 72 h. Cells were collected, lysed, and analyzed for indicated antibodies. Actin was used as loading control. (B) TDR cells were transfected as described above, and plated and analyzed for MTT assay. **** $p < 0.0001$ by two-tailed Student's *t*-test compared to siCtrl.

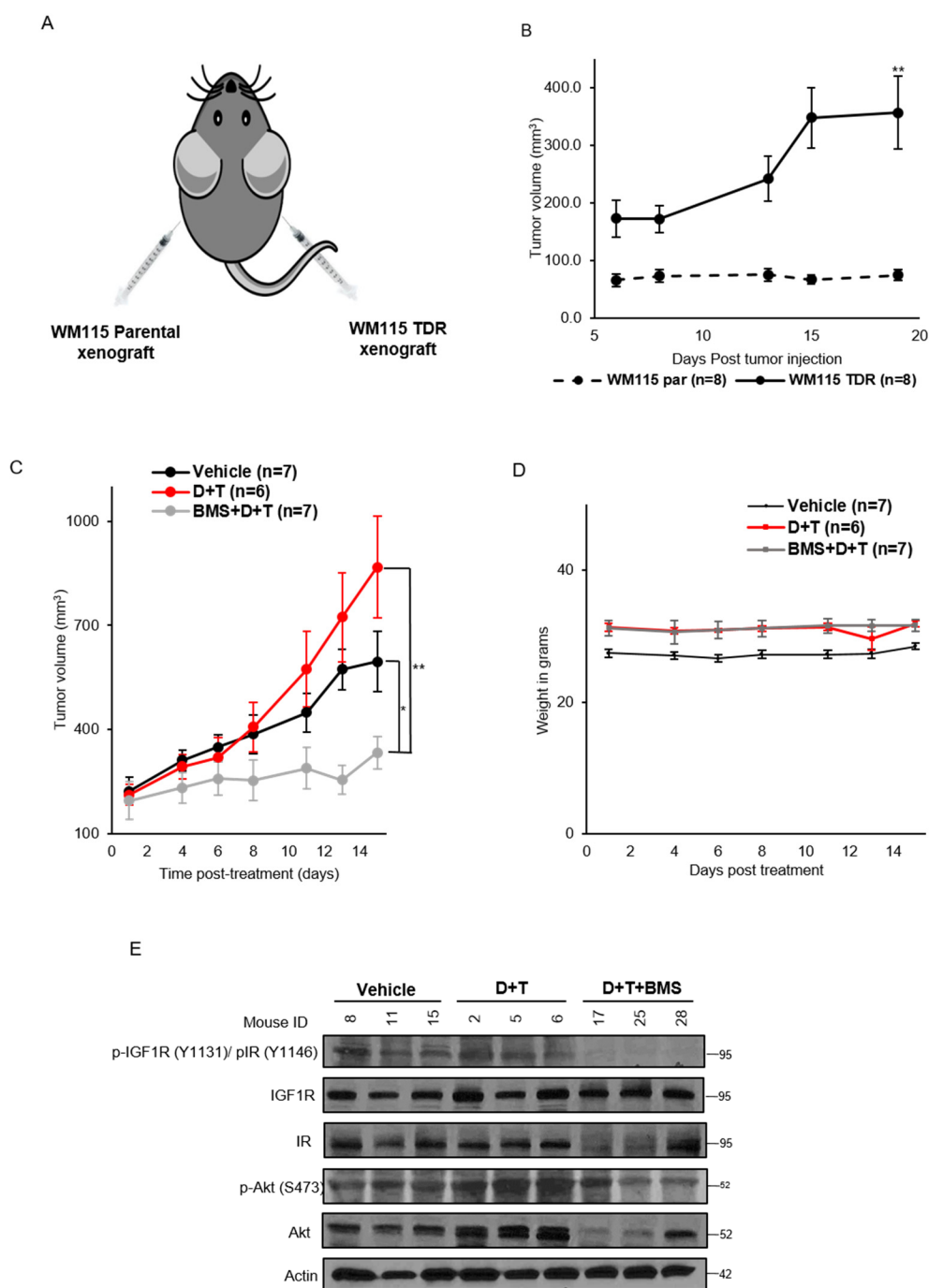


Figure 6. BMS-754807 inhibits tumor growth in WM115 TDR xenograft model. **(A)** Image of WM115 parental and TDR xenograft injection in nude mice. **(B)** Tumor growth kinetics of WM115 parental ($n = 8$) and TDR xenograft ($n = 8$) for 20 days post tumor injection. **(C)** Nude mice were injected with WM115 TDR cells and treated with vehicle, dabrafenib + trametinib (D + T), BMS-754807 (BMS), or the combination (BMS + D + T). Treatment was administered for 2 weeks by oral route. Tumors were measured thrice a week with calipers. Each data point represents the mean tumor volume $\pm$ SEM ($n = 6-7$). All data plotted as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.005$ calculated using Student's t -test. **(D)** Mice weight was recorded post treatment with BMS-754807 in combination with dabrafenib and trametinib and represented. **(E)** p-IGF1R/p-IR and p-Akt expressions were assessed in the tumor lysate by Western blot. Actin served as loading control.

4. Discussion

Acquired resistance to dabrafenib and trametinib occurs in most patients with BRAF-mutant metastatic melanoma and the majority of these patients fail the targeted therapy

regimen. About 20% of these acquired resistant melanomas harbor alterations that stimulate and activate downstream MAPK and PI3K/Akt signaling pathways [36]. While BRAFV600E amplification along with non-MAPK alteration tend to co-occur along with other genetic changes within resistant tumors, mutations in MEK1/2, BRAF, and NRAS occur in isolation [7,12,37]. In order to mimic the resistance setting in vitro, we generated trametinib and dabrafenib resistant cell (Figure 1A). The final concentration of dabrafenib and trametinib used to generate the TDR cells was dictated by the sensitivity of the parental cell lines to the combination, which could be attributed to the type of BRAF mutation present in the cell line along with other mutations such as CDKN2A and PTEN status. It is well established that MAPK reactivation occurs in resistance to the combination of dabrafenib and trametinib in BRAF-mutant melanoma [38,39]. Likewise, we observed that p-Erk levels were maintained in TDR cells in response to dabrafenib and/or trametinib (Figure 1D). SCH772984, an ERK1/2 inhibitor, failed to show any effect on TDR cells (Figure 1E), which incited us to explore other mechanisms of resistance.

IGF1R and IR are frequently expressed at high levels in various malignancies such as breast cancer [40–42], colorectal cancer [43,44], and NSCLC [45,46]. Dysregulation of IGF1R/IR axis acts as an oncogenic signal in initial tumorigenesis as well as mediating resistance to targeted therapies [47,48]. The IGF2/IGF1R/IR axis mediates adaptive resistance to erlotinib by undergoing an IGF1R/IR phenotypic switch in cholangiocarcinoma [49]. A recent study identified that a single nucleotide polymorphism (SNP) of IGF1R may have a protective effect for melanoma risk [50] in support that differential IGF1R signaling is involved in melanoma development. A phosphoproteomic screen identified IGF1R and IR as a new therapeutic opportunity for driver inhibition while simultaneously preventing the development of acquired resistance [51,52]. Paired tissue samples from BRAF inhibitor relapsed patients exhibited increased IGF1R expression [53]. A recent study has also shown that upregulation of IGF1R in BRAF and MEK inhibitor resistant cells involves the activation of the MEK5-ERK5 pathway [54]. IGF1R, along with IR, was activated and upregulated in TDR cells as compared to the parental cells and this was confirmed by RTK array analysis (Figure 2A–C). Upregulation (WM115 and A375TDR) or maintenance (WM983B TDR) of p-IGF1R and p-IR levels could be a function of basal levels of these proteins present in parental cells. Parental A375 and WM115 cells are primary melanoma cell lines whereas WM983B is a metastatic cell line. Hence, acquiring resistance could escalate the activation of signaling mechanisms such as p-IGF1R and p-IR, which could render the A375 and WM115 TDR cells with more metastatic characteristics. It is well established that there are genetic and transcriptomic changes that are acquired while on dabrafenib and trametinib regimen and these changes promote resistance [37,55]. Likewise, our data indicated differential expression of IGF1R and INSR and their high affinity ligands IGF1 and IGF2 (Figure 3A,B).

In this study, we have used BMS-754807, a small-molecule TKI targeting IGF1R and IR in combination with dabrafenib and trametinib on TDR cells. Initially, TKIs only specifically targeting IGF1R were developed. However, owing to high level of structural and functional homology between IGF1R and IR especially in the kinase domain, these tend to target both receptors [56,57]. BMS-754807 combined with dabrafenib and trametinib strikingly reduced the growth of TDR cells (Figure 5A–C). IGF2 is a high affinity ligand for IGF1R and IR compared to IGF1 and insulin [58]. IGF2 ligand can be present in the mature form and unprocessed forms (pro and big) [30]. The unprocessed and mature form of IGF2 displays similar binding potential to IGF1R/IR. IGF2 has the ability to activate IGF1R and IR by autocrine and paracrine mechanisms [59,60]. There are reports showing that BMS-754807 is a more potent inhibitor of the PI3K/Akt over the MAPK pathway [30,61]. Similarly, when TDR cells were exposed to BMS-754807 in the presence of IGF2, we observed an attenuation of p-Akt, but not of p-Erk (Figure 4D). Another likely mechanism for this p-Erk maintenance could be that BMS-754807 stimulates p70S6K1 activity via MEK1/2 and promotes survival as previously reported [62]. We observed a compensatory upregulation in IR and IGF1R when TDR cells were treated with siIGF1R and siINSR, respectively (Figure 6A). Dual

knockdown of IGF1R and INSR reduced the growth of TDR cells, providing a rationale for targeting both IGF1R and IR to circumvent the upregulation of the other receptor, which may promote further oncogenesis.

We observed that in WM115 TDR xenograft, BMS-754807 in combination with dabrafenib and trametinib inhibited local tumor growth (Figure 6C). Immunoblotting data from three representative tumors (Figure 6E) correlated with p-IGF1R/IR and p-Akt inhibition as reported in previous data (Figure 4D). Consistent with the literature [63,64], we also observed drug addiction of WM115 TDR tumors to dabrafenib and trametinib, which augmented tumor volume for this group as compared to the vehicle group. Dysregulation of glucose homeostasis would be a major concern for using BMS-754807 in the clinic, as inhibition of the insulin receptor could lead to hyperglycemia. In the short term treatment of BMS-754807, we did not observe signs of toxicity. However, long term tolerance of BMS-754807 in combination with dabrafenib and trametinib remains uncertain. A limitation of our study is that we did not obtain blood glucose levels of mice to assess these effects. In patients with metastatic melanoma, resistant to dabrafenib and trametinib with Type 2 diabetes mellitus, precaution must be taken while dosing and a suitable dose must be established for this patient population. Our results suggest that BMS-754807 along with dabrafenib and trametinib was efficacious in vitro and in vivo. Therefore, further studies investigating the safety and efficacy of combining an IGF1R inhibitor with BRAF and MEK inhibitors are warranted in cancers harboring BRAF mutations and resistant to targeted therapies.

5. Conclusions

In this study, we have characterized trametinib and dabrafenib resistant cells and observed that IGF1R and IR were activated in resistant cell lines. We show differential expression of IGF1R, INSR, along with their ligands IGF1 and IGF2 across the parental and TDR cells. BMS-754807, an IGF1R and IR inhibitor, inhibited proliferation along with p-Akt downstream. Dual genetic knockdown of IGF1R and IR inhibited cell proliferation as compared to siRNA targeting only IGF1R and IR. In vivo examination of combination dabrafenib and trametinib along with BMS-754807 suppressed tumor growth and supported our findings in vitro. This study provides a rationale for targeting IGF1R and IR. Therefore, we propose that combining dabrafenib, trametinib, and an IGF1R/IR inhibitor in patients with BRAF-mutant melanoma who have relapsed or dabrafenib and trametinib therapy regimen could be an effective strategy and warrants further investigation in the clinic.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/cancers13225863/s1>, Figure S1: Dabrafenib and trametinib inhibit growth of parental cells but not TDR cells, Figure S2: mRNA expression of AXL, HER3, EGFR and their ligands in parental and TDR cells, Figure S3: Effect of Linsitinib, BMS-536924 and GSK1838705A on TDR cell proliferation, Figure S4: BMS-754807 does not inhibit proliferation and p-IGF1R/p-IR expression in TDR cells, Figure S5: IGF2 dependent activation of IGF1R and IR in TDR cells. Figure S6: Whole blots for Western blots in Figure 1D, Figure S7: Whole blots for Western blots in Figure 1E, Figure S8: Whole blots for Western blots in Figure 2C, Figure S9: Western blots for Western blots in Figure 4D, Figure S10: Whole blots for Western blots in Figure 5A, Figure S11: Whole blots for Western blots in Figure 6E, Figure S12: Whole blots for Western blots in Figure S4, Figure S13: Whole blots for Western blots in Figure S5, Table S1: List of antibodies, Table S2: List of RT-PCR primers.

Author Contributions: Conceptualization, H.P., R.M. and J.T.G.; methodology, H.P. and J.T.G.; resources, J.T.G.; data curation, H.P., M.K.K. and N.Y.; writing—original draft preparation, H.P., R.M. and J.T.G.; writing—review and editing, H.P., R.M., N.Y., S.A., M.K.K. and J.T.G.; supervision, J.T.G.; funding acquisition, J.T.G. All authors have read and agreed to the published version of the manuscript.

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