

RESEARCH PAPER



Identification of WEE1 as a target to make AKT inhibition more effective in melanoma

Omer F. Kuzu ^{*,a}, Raghavendra Gowda ^{*,a,f,g}, Arati Sharma ^{*,a}, Mohammad A. Noory ^a, Gregory Kardos ^a, SubbaRao V. Madhunapantula ^a, Joseph J. Drabick ^{b,f,g}, and Gavin P. Robertson ^{a,c,d,e,f,g}

^aThe Pennsylvania State University College of Medicine, Department of Pharmacology, Hershey, PA; ^bThe Pennsylvania State University College of Medicine, Department of Medicine, Division of Hematology-Oncology, Hershey, PA; ^cThe Pennsylvania State University College of Medicine, Department of Pathology, Hershey, PA; ^dThe Pennsylvania State University College of Medicine, Department of Dermatology, Hershey, PA; ^eThe Pennsylvania State University College of Medicine, Department of Surgery, Hershey, PA; ^fThe Pennsylvania State University College of Medicine, The Melanoma and Skin Cancer Center, Hershey, PA; ^gThe Melanoma Therapeutics Program, 500 University Drive, Hershey, PA

ABSTRACT

AKT3 is one of the major therapeutic targets in melanoma but clinically targeting AKT3 alone seems to be an ineffective therapeutic approach. To identify unique strategies to enhance the efficacy of targeting AKT3, a screen was undertaken where AKT3 was co-targeted with a panel of kinases important in melanoma development. The screen identified WEE1 as the most potent target that when inhibited along with AKT3 would enhance the efficacy of targeting AKT3 in melanoma. RNAi mediated inhibition of AKT3 and WEE1 synergistically inhibited the viability of melanoma cells leading to a 65–75% decrease in tumor development. This approach was effective by mechanistically modulating pathways associated with the transcription factors p53 and FOXM1. Simultaneously regulating the activity of these two transcriptionally driven pathways, cooperatively deregulated cell cycle control and DNA damage repair to synergistically kill melanoma cells. This study uniquely identifies a potential approach to improve the efficacy of targeting AKT3 in melanoma.

ARTICLE HISTORY

Received 4 March 2017
Revised 13 August 2017
Accepted 23 August 2017

KEYWORDS

AKT3; Melanoma; synergy;
WEE1


Introduction

Incidence and mortality rates of malignant melanoma continue to rise annually.¹ Advanced stage metastatic melanoma carries a poor prognosis, with a median overall survival of 2 to 8 months, and with only 5% of patients surviving beyond 5 y.¹ AKT3, a member of the AKT serine/threonine protein kinase family, is activated in up to 70% of sporadic melanomas to promote cell survival by deregulating apoptotic signaling.² Preclinical targeting of AKT3 in melanoma cells is effective in killing melanoma cells and retarding tumor development.² Regrettably, these observations have not translated clinically, and therapies targeting AKT in melanoma have not been effective.³ Hence, novel approaches are needed to improve the efficacy of agents targeting AKT signaling in melanoma.

Another major target in melanoma is the MAP (mitogen-activated protein) kinase signaling pathway that is frequently activated by ^{V600E}BRAF, which is the most frequent genetic alteration occurring in 50% of sporadic melanomas.⁴ Small molecule inhibitors targeting the over-activated MAPK pathway showed promise as effective therapeutic approaches for patients with advanced stage or unresectable melanoma.^{5–10} Unfortunately, these drugs have only a modest effect on median survival, with recurrent resistant disease developing by circumventing the point of drug inhibition.^{11,12} Activation of MAPK signaling alone is unable to drive melanoma development but requires co-operation with additional

genetic or epigenetic alterations.² This observation is supported by the fact that, 70 to 80% of melanocytic nevi carry the ^{V600E}BRAF mutation but seldom progress into melanoma.⁴ Activation of AKT signaling is a key event in BRAF mediated tumor progression. AKT promotes melanoma development by phosphorylating the ^{V600E}BRAF protein to decrease its activity to the levels that promote rather than inhibit melanocytic cell growth.² Moreover, activation of AKT signaling has also been shown to play a role in resistance development to MAPK inhibitors.^{13–16} Hence, efficacy of the combination of MAPK and AKT inhibitors are currently under investigation.^{17,18} Unfortunately, recent studies suggested that targeting AKT signaling alone or in combination with MAPK is not clinically effective.^{19,20,21} AZD5363, a new generation pan AKT inhibitor, although well tolerated, yielded a partial response in only 2 of the 92 patients with advanced solid tumors.¹⁴ Co-treatment of MEK inhibitor, trametinib, with orally bioavailable pan Akt inhibitor, GSK2141795, led to stable disease in 65% of the melanoma patients, without any partial or complete responses.²¹ Based on this background and the need to identify targets to inhibit in combination with AKT that could synergize, a set of kinases were screened to identify those that could be targeted in combination with AKT3 to synergistically inhibit melanoma tumor development. WEE1 kinase was identified as a potential target that could accomplish this objective.

CONTACT Gavin P. Robertson, Ph. D.  gproberson@psu.edu  500 University Drive, Hershey, PA 17033.

 Supplemental data for this article can be accessed on the [publisher's website](#).

*All authors are contributed equally.

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WEE1 is involved in the regulation of the cell cycle by phosphorylating and inactivating cyclin-dependent kinase-1 (CDK1).²² As a component of the G2/M checkpoint, it determines the time point for entry into mitosis and inhibits early progression through the cell cycle. It is also involved in the coordination of cellular response to DNA damage. Furthermore, WEE1 was also identified as a key signaling molecule lying downstream of ^{V600E}BRAF in the MAPK signaling cascade.²³ WEE1 levels were decreased upon genetic or pharmacological inhibition of ^{V600E}BRAF, MEK or ERK activity.²³ Genetic knockdown of WEE1 reduced tumor development in melanoma xenograft models with similar signaling alterations observed following the inhibition of ^{V600E}BRAF.²³ In this study, we show that RNAi mediated co-targeting of *WEE1* with *AKT3* can synergistically inhibit melanoma in culture as well as in tumors, and identified the unique mechanism through which it occurs.

Materials and methods

Cell lines and culture conditions

Metastatic melanoma cell lines, UACC 903 was provided by Dr. Mark Nelson (between 1995 and 1999), University of Arizona, (Tucson, AZ) and the 1205 Lu cell line (between 2003 and 2005) from Dr. Herlyn, Wistar Institute (Philadelphia, PA), both the cell lines harbor ^{V600E}B-Raf. All cell lines were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 1% GlutaMAX from Gibco (Life Technologies) and 10% FBS (HyClone, Logan, UT) in a 37°C humidified 5% CO₂ atmosphere incubator and periodically monitored for genotypic characteristics, phenotypic behavior and tumorigenic potential.

Small interfering RNA (siRNA) transfection

siRNA was introduced into melanoma cells via nucleofection using an Amaxa nucleofector with solution R / program K-17 for UACC 903 and 1205 Lu cells.^{23,24} Nucleofection efficiency was >90% with 80–90% cell viability. Following siRNA transfection, cells were plated and allowed to recover for 2 d and then replated in 96-well plates to assess viability or harvested for protein knockdown studies.²⁵ Duplexed Stealth siRNA (Invitrogen) sequences for scrambled, ^{V600E}BRAF, *WEE1*, *AURKB*, *GSK3A* and *TPK1* were as reported previously.^{23,26}

siRNA screening and synergy analysis of cultured cells to identify kinases synergizing with *AKT3*

siRNA screening was performed as described previously.^{23,26} For synergy studies, 6.25–100 picomoles of siRNA targeting *AKT3* and 5 kinases (*WEE1*, *AURKB*, *GSK3A*, *TPK1* or mutant ^{V600E}BRAF) that were identified from the screen were introduced into 1×10^6 melanoma cells alone or in combination using an Amaxa nucleofector. 2 d post transfection, cells were trypsinised and 1×10^4 cells / well in 100 μ L of serum-free media were plated into 96-well plates with 6 to 8 replicates for each siRNA and for each scrambled siRNA control. Cells were

grown for 72 hours and viability of cells was measured using an MTS assay and the decrease in viability was compared with the scrambled siRNA control. The data were subjected to Chou-Talalay analysis for determining the combination index using CalcuSyn software.

RNA-sequencing experiments

Twenty-four hours following treatment with tested agents, total RNA was extracted using the mirVana RNA isolation kit (Life Technologies). Next, using the SureSelect Strand-Specific RNA Library Preparation Kit (Agilent), cDNA libraries were constructed. For multiplexed high-throughput sequencing, unique barcode sequences were incorporated. Libraries were then denatured using the Illumina protocol, diluted with pre-chilled hybridization buffer and loaded onto TruSeq SR v3 flow cells on an Illumina HiSeq 2500 (50 cycles, single-read). Sequencing reads were extracted using Illumina CASAVA pipeline Version 1.8 and aligned to the human reference genome (hg38) using Tophat (version 2.0.9).²⁷ SeqMonk (version 0.32.0) was used for identification of differentially expressed genes. Following RNA-Seq quantitation, 75% percentile normalization was performed, and significant alterations were identified using the intensity difference test with Benjamini and Hochberg multiple testing correction (p-value < 0.05).

RPPA array experiment

UACC 903 cells were transfected with siRNAs targeting *AKT3* (50 pmole), *WEE1* (25 pmole) or their combination. siScramble (50 pmole) was used as a control. 48 hours after transfections, cell lysates were collected using protein extraction reagent (T-PER, Thermo Scientific) supplemented with 1 mM EDTA, 5 mM NaF, 2 μ M staurosporine, PhosSTOP Phosphatase Inhibitor Cocktail (Roche), and Complete Mini Protease Inhibitor Cocktail (Roche). Total protein concentration was determined by bicinchoninic acid assay (Thermo Scientific) and submitted to the Functional Proteomics Core Facility at MD Anderson for the RPPA analysis. The array consisted of 287 antibodies including 64 phospho-specific antibodies.

Western Blotting

Cell lysates were collected 48 hours after siRNA transfection or drug treatment by washing plates with PBS followed by addition of RIPA lysis buffer containing Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL, USA).²⁸⁻³³ Lysates were centrifuged (10,000 X g) for 10 minutes at 4°C to remove cell debris. Protein concentration was measured using the bicinchoninic acid assay kit (Thermo). 25 μ g of respective lysates were loaded onto 4–12% Bis-Tris NuPAGE gels (Life Technologies) and run in an XCell SureLock Mini-Cell gel apparatus (Life Technologies), transferred to polyvinylidene difluoride membranes (Pall Corporation, Port Washington, NY), probed with respective primary antibody followed by horseradish peroxidase-conjugated secondary antibody, and developed using ECL Western Blotting Substrate (Thermo Scientific) or Supersignal West Femto Chemiluminescent Substrate

(Thermo Scientific). Primary antibodies used: p21 (sc-756), p27 (sc-528), p53 (sc-6243) and ERK2 (sc-1647) from Santa Cruz Biotechnology (Dallas, TX), pAKT (9271), AKT3 (3788), Total AKT (4685), pWEE1 (4910), WEE1 (4936), p-CDC2 (Y15) (9111), pRB (S807/811) (9308), pRB (S795) (9301), pRB (S780) (9307), (9309) and p-Histone H2AX (S139) (2577) from Cell Signaling (Danvers, MA). Secondary antibodies goat anti-rabbit IgG-HRP (sc-2004) and goat anti-mouse IgG-HRP (sc-2005) were purchased from Santa Cruz Biotechnology.

In vivo analysis of synergy, cell proliferation and apoptosis in time and size matched tumors

Animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University. Tumor kinetics studies were undertaken in athymic-Foxn1^{nu} nude mice (Harlan Laboratories, Indianapolis, IN, USA). siRNA targeting *AKT3* (100 picomoles) and *WEE1* (6.25 picomoles) alone or in combination was nucleofected into 1×10^6 UACC 903 or 1205 Lu cells. Cells transfected with scrambled siRNA (106.25 picomoles) were used as a control. Since total siRNA was 106.25 picomoles for the combination group, scrambled siRNA was used to compensate for siRNA in *AKT3* or *WEE1* alone groups. After plating and allowing the cells to recover for 48 hours, 1×10^6 cells were aliquoted in 0.2 ml of 10% FBS-DMEM and then injected subcutaneously above both the left and right rib cages of 4–6 week old female mice (3–4 mice/ group). Dimensions of developing tumors were measured on alternate days up to day 17.5, using calipers by multiplying length, width and depth in mm³.

To generate tumors of the same size developing at parallel time points, 1×10^6 or 10×10^6 of UACC 903 melanoma cells were nucleofected with siScrambled, siAKT3, siWEE1 or siAKT3+siWEE1, respectively, as detailed above and injected into nude mice. Tumors were removed from euthanized mice at days 9 and 11, flash frozen in liquid nitrogen, pulverized and protein lysates collected by the addition of 600 to 800 μ L of RIPA protein lysis buffer containing Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL, USA). Protein concentration was measured using the bicinchoninic acid assay kit (Thermo) followed by Western blotting to measure the levels of AKT3, and WEE1 proteins in tumors. The band intensity was quantified by scanning the optical density of each band using ImageJ as described previously.^{23,24} Cell proliferation rates were measured using a mouse anti-human Ki-67 antibody from BD Pharmingen (BD Biosciences, San Diego, CA). Apoptosis rate was measured using the “terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)” TMR Red Apoptosis kit (Roche, Mannheim, Germany) as described previously.^{23,26} Number of Ki-67 or TUNEL stained cells were quantified as the percentage of total cells in tumors. Sections were imaged using a Nikon Eclipse 600 and quantified using IP laboratory imaging software (Scanalytics, Fairfax, VA). A minimum of 6 different tumors with 4 to 6 fields per tumor was analyzed.

Statistical analysis

Statistical analysis was performed using Prism 4.0 GraphPad Software. Data were subjected to the Chou-Talalay method for determining the combination index using CalcuSyn software and combination index (CI) values plotted against fraction affected.³⁴ Using this approach, combination index values of < 0.9 are synergistic, > 1.1 are antagonistic, and values 0.9–1.1 are nearly additive. For comparison between 2 groups, Student *t* test (2 tailed) was used. One-way Analysis Of Variance (ANOVA) was used for group wise comparisons, followed by the Tukey’s post hoc test. Results represent at least 2 to 3 independent experiments and are shown as averages \pm SEM. Results with a *P* value less than 0.05 (95% CI) were considered significant. Sample sizes and number of times experiments were repeated are indicated in the figure legends. Number of asterisks in the figures indicates the level of statistical significance as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Results

Identifying WEE1 to target in combination with AKT3 to synergistically inhibit melanoma cell survival

To identify druggable targets that enhance the efficacy of AKT3 inhibition, a screen was undertaken to identify kinases that could retard melanoma development.²³ The screen was undertaken using a pool of 3 siRNAs targeting each of the 636 kinases (totaling 1908 individual siRNAs). siRNAs were nucleofected into the UACC 903 melanoma cell line using the AMAXA 96-well shuttle transfection system and 5 d later, the viability of cells was measured by MTS assay. Results were compared with the average of high-, medium-, and low-GC content scrambled siRNA transfected cells. In this primary screen, 34 kinases reduced the viability of UACC 903 cells more significantly than the set experimental cut-off, i.e. 15% growth inhibition. The secondary screen retested the 34 kinases and validated 14 of these hits (Supplementary Table 1). A tertiary screen required that at least 2 of the 3 siRNA targeting different regions of respective mRNAs decrease UACC 903 viability. Based on these criteria, *AURKB*, *WEE1*, *GSK3A*, *TPK1*, and *BRAF* were identified as potential targets able to reduce the proliferative potential of UACC 903 melanoma cells. The potential of these targets was then confirmed in 2 additional melanoma cell lines (A375M and 1205 Lu) to show similar growth inhibitory effects. Subsequently, siRNA-mediated knockdown of these kinases was performed to measure the inhibitory effect on xenografted tumor development^{23,26} (Supplementary Table 1).

Increased AKT3 activity has been reported in up to 70% of melanomas, but inhibition of AKT3 alone in the clinic does not appear to be an effective therapeutic approach.³⁵ Thus, to achieve high-level synergistic melanoma inhibition, *AKT3* was co-targeted with the kinases identified from the screen. The viability of UACC 903 cells following siRNA-mediated knockdown of *AKT3* alone or in combination with *WEE1*, *GSK3A*, *AURKB*, *TPK1*, and *BRAF* was examined by MTS assay. The combination index (CI) values suggested strong synergism between knockdowns of *AKT3* and *WEE1* (CI value 0.095) or *GSK3A* (CI value 0.267) (Supplementary Table 2). Since synergism between targeting *AKT3*

by western blotting of size and time matched xenografted tumor lysates using an established protocol.^{25,36} Decreased levels of AKT3 and WEE1 protein expression were also observed in tumor lysates (Fig. 2C).

To dissect the cellular processes mediating enhanced tumor inhibition when targeting AKT3 and WEE1, rates of cellular proliferation and apoptosis were measured in size and time matched human melanoma xenografts.^{25,36} Formaldehyde-fixed, paraffin-embedded sections of tumors were stained with Ki-67 to measure tumor cell proliferation rates, and TUNEL to detect apoptotic cell death. Cotargeting AKT3 and WEE1 led to 4-fold inhibition in melanoma tumor cell proliferation (Fig. 2D) and 3.5-fold increase in apoptosis (Fig. 2E), compared with 1.3 and 2-fold decrease in proliferating cells and 2.5 to 1.25-fold increase in apoptotic cells, respectively when targeting each gene alone.

Targeting AKT3 and WEE1 leads to deregulation of cell cycle and DNA damage response pathways

Targeting 2 proteins together often leads to a new and novel mechanism of inhibiting tumor development, which is also hypothesized to be occurring when simultaneously targeting AKT3 and WEE1. Therefore, establishing the mechanism through which tumor inhibition occurs when targeting 2 proteins is complex and in recent years has involved the use of an

“omics” approach.³⁷ This contemporary approach was used to identify the synergistic mechanisms, through which targeting AKT3 and WEE1 inhibits melanoma tumor development. First, alterations in gene expression levels were identified using genome-wide RNA sequencing analysis of cells following genetic targeting of AKT and WEE1 kinases. Next, RPPA analysis was used to validate alterations at the protein level, which was finally confirmed by Western blotting.

RNA sequencing identified 41 significantly deregulated genes following siRNA-mediated knockdown of WEE1 in UACC 903 cells (Fig. 3A). Enrichment analyses of the 22 up-regulated genes showed the p53 protein as a significantly enriched transcriptional regulator (Table 1). As a matter of fact, 14 of the 22 genes were modulated by the p53 gene family transcription factors. Analysis of the remaining 19 down-regulated genes identified transcription factors FOXM1 and E2F4 as prominent modulators of this effect. 13 of the 19 (70%) down-regulated genes were modulated by these 2 transcription factors. Enrichment analysis of all 41 deregulated genes implicated induction of DNA damage and cell cycle deregulation (Table 1). These observations were consistent with the function of WEE1, since, as a regulator of the G2/M phase checkpoint, inhibition of WEE1 has been shown to induce DNA damage through perturbation of the cell cycle.³⁸

siRNA-mediated knockdown of AKT3 only altered 18 genes limiting the functionality of the enrichment analysis. Among these 18 genes, the most notable alteration was downregulation

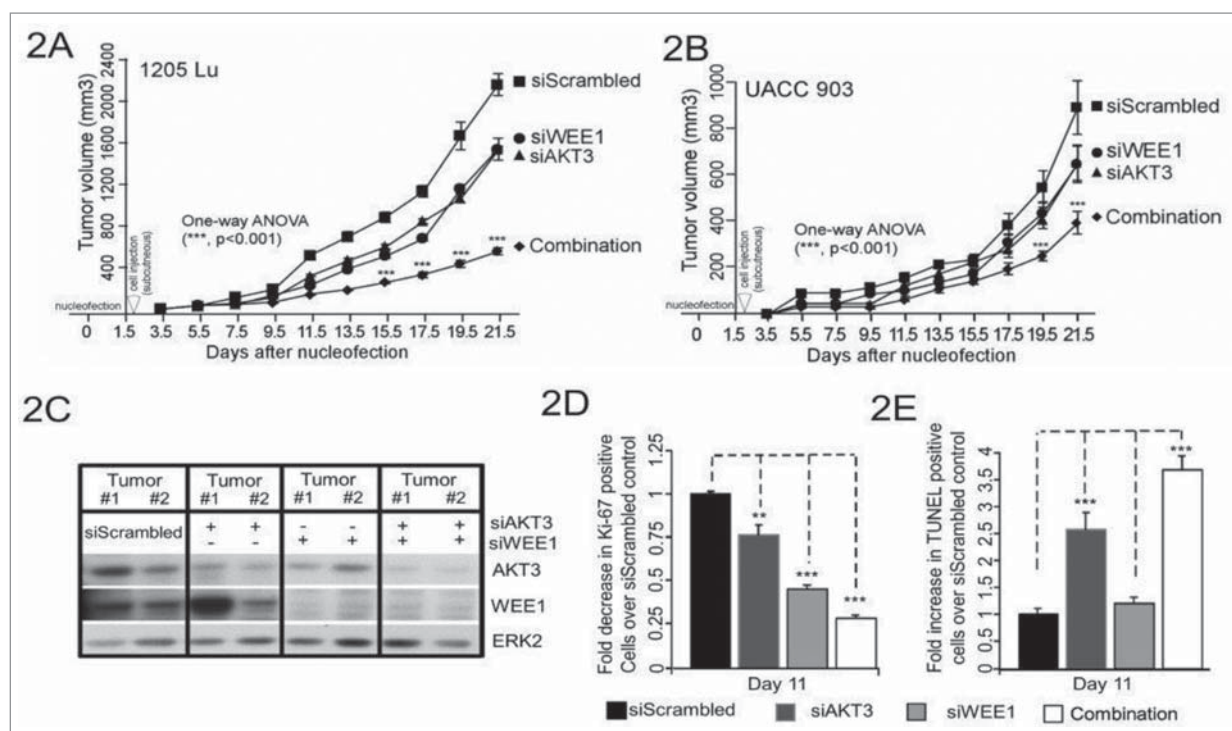


Figure 2. Targeting of AKT3 and WEE1 inhibited melanoma tumor growth. Targeting AKT3 and WEE1 synergistically inhibited melanoma tumor growth *in vivo*. UACC 903 (2A) and 1205 Lu (2B) cells were nucleofected with AKT3 and WEE1 siRNA alone or in combination and 48 hours later, viable cells were s.c. injected into left and right flanks of nude mice. Cells transfected with scrambled siRNA were used as a control. Developing tumors were measured on alternate days for 3 weeks. Significance was measured by one-way analysis of variance, followed by the post hoc test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars show SEM. Data were obtained from duplicate experiments with 3 mice per group, containing 2 tumors per mouse. C. Western blot analysis showing knockdown of AKT3 and WEE1 in tumor lysates of UACC 903 xenografts harvested at day 9. ERK2 served as a control for equal protein loading. (D) and E, Analysis of size and time matched tumors from animals injected with UACC 903 melanoma cells transfected with scrambled siRNA controls or siRNA to AKT3, WEE1 or AKT3, and WEE1. Tumor sections were immunostained for Ki-67 (2D) or TUNEL (2E) to measure the proliferation and apoptosis, respectively. Bar graphs show the fold change in Ki-67 or TUNEL-positive cells compared with the scrambled siRNA control. Data were obtained from 3 to 4 tumors with 4 to 5 fields averaged per tumor. Significance was measured by one-way analysis of variance, followed by the post hoc test, ** $P < 0.01$, *** $P < 0.001$, NS; Non-significant. Columns, mean; error bars, SEM.

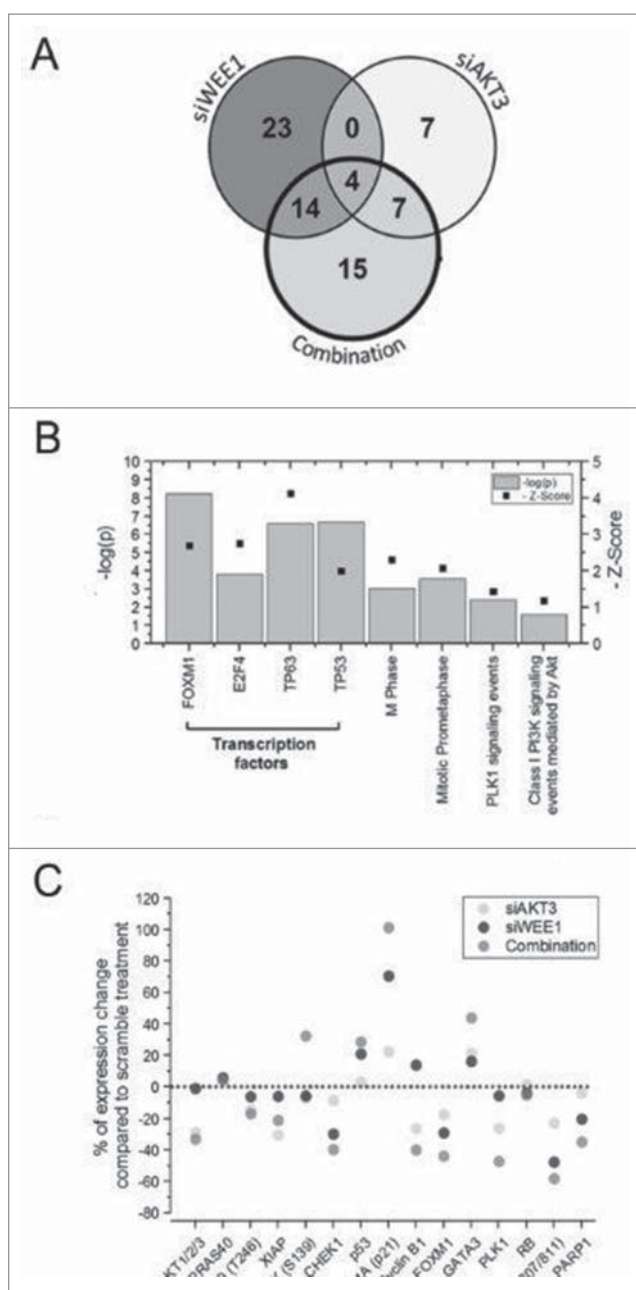


Figure 3. Alteration in gene expression and protein levels through genome-wide RNA sequence and RPPA analysis respectively. A, Venn diagram showing significantly altered genes identified following RNA-Seq analysis of UACC 903 cells transfected with siRNAs targeting *AKT3*, *WEE1* or both. B, Enrichment analysis of 40 genes significantly altered by the combination. C, RPPA array analysis showing some of the altered proteins in UACC 903 melanoma cells transfected with siAKT3, siWEE1 or the combination. In RNA-Seq experiments, significant genes were determined using the intensity difference test with a p-value threshold of 0.05 and Benjamini and Hochberg multiple testing correction.

of cyclin-dependent kinase 6 (CDK6), which is an important effector of AKT signaling.³⁹ In contrast to targeting AKT3 alone, siRNA-mediated cotargeting of AKT3 with WEE1 deregulated 40 genes, of which 14 were upregulated and 26 were downregulated (Fig. 3A). PLK1 signaling modulating the FOXM1 transcription factor was enriched only during the combination treatment (Fig. 3B and Table 1). FOXM1 and E2F transcription factors were enriched among the downregulated genes while GATA3 was enriched among the upregulated ones (Table 1).

To validate the signaling alterations at the protein level, RPPA analysis was used (Fig. 3C). Confirming the RNA sequencing results, knockdown of WEE1 enhanced the expression of DNA damage marker phospho H2AX, as well as p53 and downstream p21 levels. Furthermore, FOXM1 levels were decreased concomitantly with PLK1 levels (Fig. 3C).⁴⁰ Knockdown of AKT3 increased p21 and phospho H2AX levels while reducing total AKT, pPRAS40, XIAP, PLK1, FOXM1 and phospho-Rb levels (Fig. 3C). Targeting the 2 kinases synergistically increased GATA3, phospho H2AX, p53 and downstream p21 levels while leading to a significant reduction in PLK1, FOXM1, Cyclin B1 and phospho-Rb levels. These observations were in concordance with the RNA sequencing analysis suggesting that, WEE1 inhibition leads to DNA damage that activates p53/p21 signaling, while AKT inhibition modulates this process through the PLK1/FOXM1 axes (Fig. 3C).

The results obtained from RPPA analyses were confirmed by Western blotting studies. Knockdown of *WEE1* kinase led to a dose-dependent decrease in the phosphorylation of its substrate CDK1 (Figs. 4A and B). Decreased phosphorylation of CDK1 was predicted to increase its activity leading to the bypass of the G2/M checkpoint and induction of DNA damage.³⁸ As expected, a dose-dependent increase in the phosphorylation of H2AX was observed. Consequently, this resulted in increased p53, p21 as well as p27 levels, which are known to be inhibitory to cell proliferation⁴¹ (Figs. 4A and B). FOXM1 and phosphorylated RB levels were also dose-dependently decreased by *WEE1* knockdown. Decreased phosphorylation of RB and enhanced expression of p21 were observed with the AKT3 knockdown. Co-targeting of *AKT3* and *WEE1* was more effective at reducing FOXM1 and phosphorylated RB1 levels while enhancing cellular levels of p53 (Figs. 4A and B).

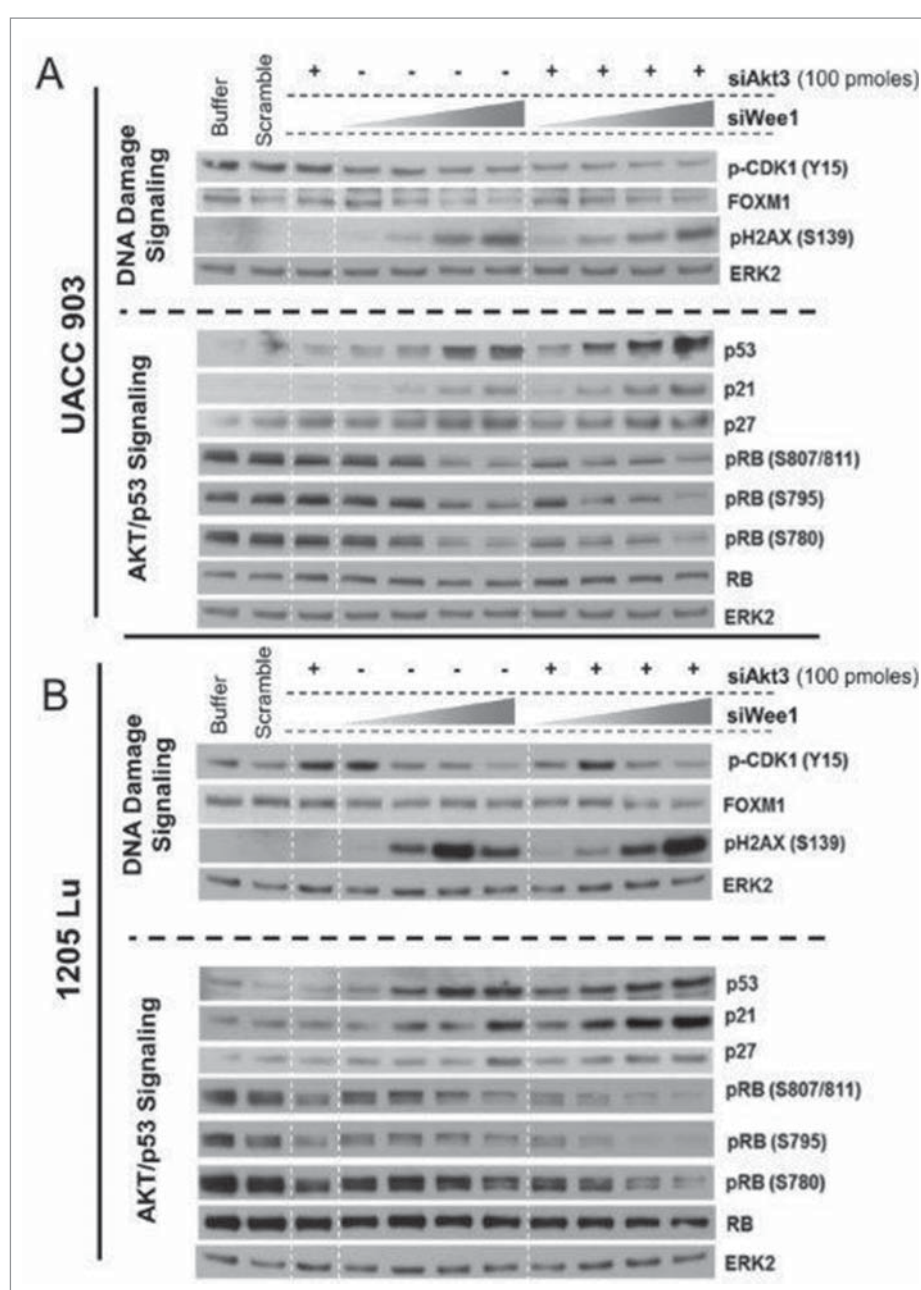
Discussion

The AKT3 signaling cascade is a major target in melanoma, activated in up to 70% of the patients.² Loss of functional PTEN phosphatase triggers AKT3 activation, which is the most concurrent event occurring along with BRAF mutation.² One of the functions of AKT3 is to phosphorylate mutant V^{600E}BRAF protein to decrease its activity to levels that promote rather than inhibit melanocytic cell growth.² Moreover, over-activated AKT signaling also positively regulates cell cycle while negatively regulating cell death cascades.⁴²⁻⁴⁴ In melanoma, over-activated AKT abrogates an efficient DNA damage response allowing cells to continuously divide without being eliminated by apoptosis.⁴⁵ Furthermore, activation of AKT signaling is also implicated in drug resistance to mutant BRAF targeted therapies.^{16,17} Hence, targeting AKT3 has a significant chemotherapeutic potential to suppress melanoma tumor growth, as well as to reverse melanoma drug resistance.^{17,35}

Unfortunately, targeting AKT3 alone in melanoma patients remains ineffective, requiring the identification of targets with which it can synergize for better therapeutic efficacy.^{2,46} This study identified WEE1 as a potential target to inhibit in combination with AKT3. Cotargeting *AKT3* and *WEE1* kinases synergistically reduced cellular proliferation and induced apoptosis. Based on the results of RNA sequencing, RPPA array and Western blotting

Table 1. Gene set enrichment analysis. Enrichment analysis of gene sets identified by RNA sequencing of genetically targeting of AKT and WEE1 pathways in UACC 903 cells. For the full list, please see Supplementary Table 4.

	Treatment	Subgroup	Pathway/Transcription Factor	p-Value	Z Score	Combined Score	Tool
1	siWEE1	DOWN	FOXM1	5.00E-14	-2.70	68.11	CHEA
2	siWEE1	DOWN	E2F4	1.40E-06	-2.84	26.12	CHEA
3	siWEE1	UP	TP63	3.89E-09	-4.14	61.69	CHEA
4	siWEE1	UP	TP53	4.30E-12	-2.00	41.32	CHEA
5	siWEE1	ALL	M Phase	1.82E-03	-2.30	5.61	REACTOME
6	siWEE1	ALL	DNA Damage Response	2.00E-03	-1.83	6.80	WIKIPATHWAYS
7	siWEE1	ALL	TP53 Network	2.93E-03	-1.45	5.38	WIKIPATHWAYS
8	siWEE1+siAKT3	DOWN	FOXM1	5.73E-09	-2.70	36.85	CHEA
9	siWEE1+siAKT3	DOWN	E2F4	1.63E-04	-2.76	15.13	CHEA
10	siWEE1+siAKT3	UP	GATA3	2.19E-03	-1.85	4.05	ENCODE
11	siWEE1+siAKT3	ALL	RB in Cancer	1.38E-03	-1.92	5.44	WIKIPATHWAYS
12	siWEE1+siAKT3	ALL	M Phase	9.25E-04	-2.30	6.86	REACTOME
13	siWEE1+siAKT3	ALL	Validated transcriptional targets of TP63 isoforms	2.89E-05	-1.70	11.82	NCI NATURE
14	siWEE1+siAKT3	ALL	Direct p53 effectors	2.19E-03	-1.59	5.94	NCI NATURE
15	siWEE1+siAKT3	ALL	PLK1 signaling events	3.91E-03	-1.44	4.95	NCI NATURE
16	siWEE1+siAKT3	ALL	class I PI3K signaling events mediated by Akt	2.56E-02	-1.19	2.34	NCI NATURE

**Figure 4.** Targeting AKT3 and WEE1 increased p53 while reducing FOXM1 and CDK1 signaling. (A) and (B). Knockdown of *WEE1* kinase led to a dose-dependent decrease in the phosphorylation of its substrate CDK1. Dose-dependent increase in the phosphorylation of H2AX was observed. Consequently, this resulted in increased p53, p21 as well as p27 levels, which are known to be inhibitory to cell proliferation. ERK2 served as a control for equal protein loading.

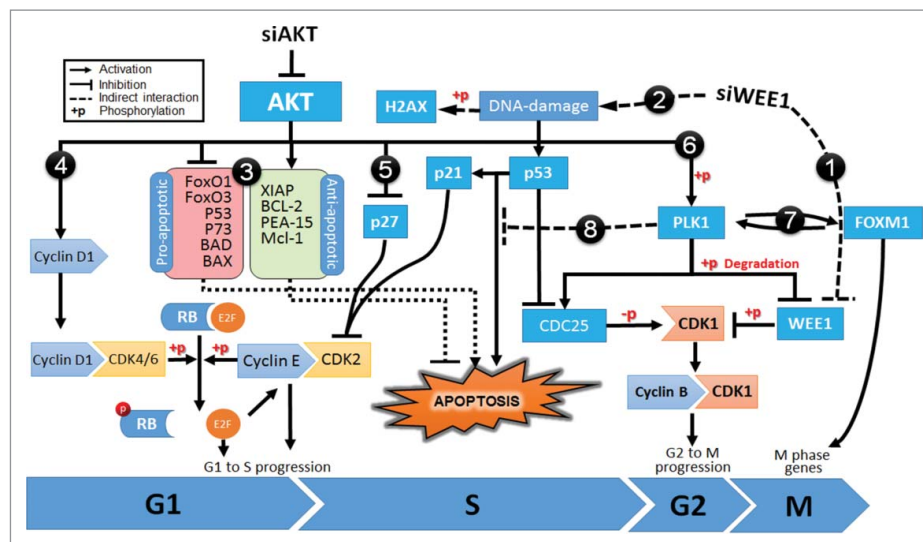


Figure 5. Diagram showing the mechanism of synergism for co-targeting AKT and WEE1 signaling pathways. Inhibition of siWEE1 (1) suppresses inhibitory phosphorylation of CDK1 leading to early-G2/M progression. This leads DNA damage (2) and activates p53 signaling. p53 inhibits cell cycle progression by induction of p21, allowing DNA damage repair. If the DNA damage is not repairable, p53 induces apoptosis. However, in many cancer cells, apoptotic cascades are suppressed by oncogenic alterations. Over-activated AKT inhibits pro-apoptotic factors while inducing antiapoptotic factors (3). AKT signaling also enhances cell cycle progression by CyclinD1 mediated phosphorylation of RB (4) and inhibition of p27 (5). Furthermore, AKT phosphorylates and induces Polo-like kinase 1 (PLK1) (6), which in turn inhibits pro-apoptotic functions of p53 and its family members, p63 and p73 (7). In addition, PLK1 also induces FOXM1 activity and M-phase progression (8). Proteins that were validated by Western blotting are shown in bold.

experiments, a potential signaling map that mediate synergistic tumor inhibition is illustrated in Fig. 5. Accordingly, WEE1 is an important regulator of cell cycle progression and controls the G2/M checkpoint by catalyzing the inhibitory phosphorylation of CDK1 kinase at Tyr15 (Fig. 5#1).²² WEE1 mediated regulation of the mitotic cell cycle ensures that the cell has completed DNA replication and is ready for mitosis. WEE1 inhibition leads to DNA damage and mitotic catastrophe due to premature progression into the cell cycle (Fig. 5#2).⁴⁷ Indeed, in this study, DNA damage induced by genetic targeting of WEE1 was observed as an increase in phospho H2AX, and resulted in a concomitant increase in p53-p21 signaling. Upregulation of p21 is known to halt S-phase progression by blocking the activity of CDK2.⁴⁸ As a consequence inhibition of CDK2 leads to decreased phosphorylation of RB1 causing inhibition of E2F family of transcription factors required for the cell cycle progression.⁴⁸ In this study we also observed a dose-dependent reduction of phosphorylated RB1 levels following the knockdown of WEE1. Taken together, targeting WEE1 induces cell cycle arrest through induction of DNA damage and transcriptional activity of p53.

In steady-state conditions, DNA damage activates repair mechanisms. However, persistent DNA damage triggers apoptosis to eliminate damaged cells.⁴⁹ In cancer cells, besides promoting cell cycle, over activated AKT bypasses apoptotic signals through multiple pathways (Fig. 5#3 to #5).⁵⁰ Moreover, activated AKT also disrupts the DNA damage response by modulating p53 activity (Fig. 5#6). AKT not only induces ubiquitin-mediated degradation of p53 but also interferes with its function through stimulating the catalytic activity of PLK1.⁵¹⁻⁵⁴ As a proto-oncogene frequently overexpressed in

tumor cells, PLK1, inhibits the pro-apoptotic functions of p53 via physical interaction and phosphorylation (Fig. 5#7).^{55,56} In fact, studies have shown that the loss of PLK1 activity can induce pro-apoptotic pathways and inhibit tumor growth.⁵⁵ PLK1 also promotes the activity of FOXM1, a proto-oncogene transcription factor that regulates G2/M phase progression as well as maintenance of chromosomal segregation and genomic stability (Fig. 5#8).^{56,57} Inhibition of AKT significantly hinders PLK1 activity and delay metaphase to anaphase transition by reducing FOXM1 activity.⁵⁴ Indeed, genes that are deregulated by cotargeting of AKT with WEE1 were significantly enriched in the FOXM1 and PLK1 transcription factor networks as well as M/G1 transition and prometaphase genes.

Taken together, cotargeting of AKT and WEE1 kinases could modulate cell cycle and DNA damage responses through a complicated network that involves PLK1 and FOXM1 proteins. Since WEE1 has been identified as downstream of BRAF in the MAPK signaling cascade, this combinatorial approach might also be useful for overcoming acquired resistance to mutant BRAF inhibitors.²³ Targeting PI3K-AKT and MAPK signaling is proposed as a strategy to reduce resistance to mutant BRAF inhibitors.^{9,58} Currently efficacy of WEE1 inhibitor, MK-1775, is being evaluated in clinical trials for the treatment of advanced solid tumors (NCT01748825, NCT02095132 and NCT01827384). Based on the results in this report, further studies to investigate the therapeutic potential of combining MK-1775 together with AKT or PI3K inhibitors are warranted for the treatment of melanoma to make AKT3 targeting more effective in the clinic.



Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Grant support

National Institutes of Health R01 CA-136667, R01 CA-1138634 (to Gavin P. Robertson). The Foreman Foundation for Melanoma, The Geltrude Foundation, The Penn State Chocolate Tour Cancer Research Fund.

ORCID

Omer F. Kuzu  <http://orcid.org/0000-0001-8715-4800>
 Raghavendra Gowda  <http://orcid.org/0000-0003-2312-8384>
 Arati Sharma  <http://orcid.org/0000-0001-7178-949X>
 Mohammad A. Noory  <http://orcid.org/0000-0002-8376-0704>
 Gregory Kardos  <http://orcid.org/0000-0001-9706-6652>
 SubbaRao V. Madhunapantula  <http://orcid.org/0000-0001-9167-9271>
 Joseph J. Drabick  <http://orcid.org/0000-0002-7630-2721>
 Gavin P. Robertson  <http://orcid.org/0000-0003-0152-2997>

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