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Small molecule inhibition of polo-like kinase 1 by volasertib (BI 6727) causes significant melanoma growth delay and regression *in vivo*

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Abstract

The objective of this study was to determine the therapeutic potential of polo-like kinase 1 (Plk1) inhibition in melanoma, *in vivo*. Employing Vectra technology, we assessed the Plk1 expression profile in benign nevi, malignant (stages I-IV) and metastatic melanomas. We found a significant elevation of Plk1 immunostaining in melanoma tissues. Further, a second generation small molecule Plk1 inhibitor, BI 6727, resulted in reductions in growth, viability and clonogenic survival, as well as an increase in apoptosis of A375 and Hs 294T melanoma cells. BI 6727 treatment also resulted in a G2/M-as well as S-phase cell cycle arrest in melanoma cells. Importantly, BI 6727 (*intravenous* injection; 10 and 25 mg/kg body weight) treatment resulted in significant tumor growth delay and regression *in vivo* in A375- and Hs 294T- implanted xenografts in athymic nude mice. These anti-melanoma effects were accompanied with a decreased cellular proliferation (Ki-67 staining) and induction of apoptosis (caspase 3 activation). In addition, BI 6727 treatment caused a marked induction of p53 and p21 *in vitro* as well as *in vivo*. Overall, we suggest that Plk1 inhibition may be a useful approach as a monotherapy as well as in combination with other existing therapeutics, for melanoma management.

Keywords

Melanoma; Plk1; BI 6727; Volasertib

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Conflict of Interest
None.

1. Introduction

Although melanoma is not the most common form of skin cancer, it is one of the most aggressive types. If not diagnosed early or left untreated, it can rapidly metastasize and become lethal. According to recent statistics from the American Cancer Society, 76,380 new melanoma cases and 10,130 melanoma-related deaths are predicted in the United States in 2016 [1]. Recent advancements in the understanding of melanoma driver genes have enabled a more aggressive and personalized therapeutic strategy. However, melanoma is notoriously resistant to treatment and current therapeutic approaches have not been able to effectively manage this neoplasm.

Research has shown that at least half of the diagnosed melanomas harbor a point mutation in the *BRAF* gene, typically characterized by a substitution of glutamic acid for valine at codon 600 of the BRAF kinase (BRAF^{V600E}) [2]. BRAF^{V600E} is constitutively active and drives uncontrolled cellular proliferation through the mitogen-activated protein kinase (MAPK) pathway [2]. The current standard treatment for patients with unresectable or metastatic melanoma with the BRAF^{V600E} mutation, vemurafenib, prevents the activation of the MAPK pathway through the upstream BRAF inhibitor [3]. While vemurafenib has demonstrated a significant survival advantage compared to its predecessor dacarbazine, treatment with vemurafenib ultimately results in a drug-resistant melanoma through the BRAF independent reactivation of the MAPK pathway. Therefore, new mechanism- and target- based approaches are needed for an effective management of melanoma.

In the recent past, polo-like kinase 1 (Plk1) has gained considerable attention from the research community, as a potential therapeutic target for cancer therapeutics. Plk1 is a serine/threonine kinase that plays a key role in the cell cycle by contributing to mitotic entry, progression and exit. The overexpression of Plk1 has been reported in wide range of cancer types, including melanoma, and has been linked to decreased survival rate and poor disease prognosis [4-12]. We have previously demonstrated that Plk1 is overexpressed in melanoma and its inhibition imparts anti-proliferative response in human melanoma cells *in vitro* [8]. Building on our previous *in vitro* work, the major objective of this study was to determine the therapeutic efficacy of a second generation small molecule inhibitor of Plk1, BI 6727 (volasertib) *in vivo* against melanoma. BI 6727 is one of the most specific commercially available Plk1 ATP-competitive inhibitors with an IC₅₀ of 0.87 nM [13] and has been reported as a potential therapeutic agent in multiple cancer types. We found that BI 6727 effectively inhibits melanoma growth in culture and xenograft mouse models of both BRAF^{V600E} mutant and BRAF wild-type melanomas. Further, we show that BI 6727 induces apoptosis and increases the activity and expression of p53 *in vivo*. Additionally, this study provides the first quantitative fluorescent analysis of Plk1 expression in clinical melanoma samples which has revealed an elevated expression level of Plk1 in melanoma, with significantly higher levels in metastatic melanoma when compared to benign melanocytic nevi. Overall, this study provides strong evidence that the targeted inhibition of Plk1 by BI 6727 is a viable therapeutic strategy for the treatment of metastatic melanoma and has potential implications at the clinical level.

2. Materials and Methods

2.1. Cell Culture

A375 (ATCC, Cat. CRL-1619) and Hs 294T (ATCC, Cat. HTB-140) human melanoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo, Cat. SH30243FS) supplemented with 10% fetal bovine serum (FBS; Sigma, Cat. F2442). Normal human epidermal melanocytes (Invitrogen, Cat. C0025C) were cultured in Dermal Cell Basal Medium (ATCC, Cat. PCS-200-030) supplemented with melanocyte growth kit (ATCC, Cat. PCS-200-401). Cells were maintained in a humidified incubator with 5% CO₂ at 37°C.

2.2. Cell Cycle Analysis

A375 cells (1×10^5) were grown in a 6-well tissue culture dish (TPP, Cat. 92006) and treated with BI 6727 (10 or 100 nM) (Chemietek, Cat. CT-BI6727) for 0, 6, 12, 18, 30, and 48 hours. To synchronize cells in early G1 phase, cells at 30–40% confluency were washed with PBS and starved of serum for 60 h prior to re-stimulation with media containing 10% FBS. Following treatment, cells were trypsinized for 5 min and transferred into 5 mL Falcon tubes (BD Biosciences, Cat. 352008) containing cultured media. Samples were centrifuged at 1000 RPM (233 RCF) for 2 min and the supernatant was aspirated prior to the cells being resuspended in ice-cold 100% ethanol added drop-wise with gentle mixing. Samples were stored at –20°C overnight prior to centrifugation and resuspension in 500 L propidium iodide (PI) buffer (PBS, 50 g/mL PI, 0.1 mg/mL RNase A, 0.05% Triton-X). Samples were incubated at 37°C for 40 min in dark and stored at 4°C until processed by flow cytometry in the FL-2A channel. Cell cycle analysis was done using ModFit LT software (Verity Software House).

2.3. Annexin V and PI Exclusion Assays

A375 cells (1×10^5) were plated in a 6-well tissue culture dish (TPP, Cat. 92006) and treated with BI 6727 (10–100 nM) for 24 h. Following treatment, cells were trypsinized for 5 min and transferred into 5 mL Falcon tubes containing cultured media. Samples were centrifuged at 1000 RPM for 2 min, the supernatant was aspirated, and the cells were re-suspended in 4% paraformaldehyde for fixation and subsequently stained with the FITC Annexin V and PI using a kit (Invitrogen, Cat. V13242) as per manufacturer's protocol. Flow cytometric analysis was performed on the FACScalibur (BD Biosciences) in the FL1 (FITC) and FL3 (PI) channel and analyzed using FlowJo software (FlowJo).

2.4. Western Blot Analysis

A375 or Hs 294T melanoma cells (5×10^5) were plated in a 10 cm culture dish (TPP, Cat. 93100) and treated the next day with BI 6727 (1–100 nM) or vehicle control as previously described. Following 24 h treatment, cells were trypsinized, washed with ice-cold PBS and lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) with phenylmethylsulphonyl fluoride (PMSF; Amresco, Cat. 0754) and protease inhibitor cocktail (Pierce, Cat. 78410). Protein concentration was measured with BCA Protein Assay (Pierce, Cat. 23225) as per manufacturer instructions. For immunoblot analysis, 30 g of protein was subjected to sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) using Mini-PROTEAN TGX precast gels (BioRad, Cat. 4569034) and transferred onto nitrocellulose membrane. Blots were blocked in 5% non-fat dry milk in tris-buffered saline + 0.1% tween-20 (TBST), followed by probing with desired primary antibodies (anti-p21, p53, or β -actin; Cell Signaling, Cat. 2947, 9282, 4970, respectively). Blots were then incubated with the appropriate HRP-conjugated antibodies followed by chemiluminescent detection (Pierce, Cat. 32106) using a Kodak ImageStation 4000MM (Carestream). Figures shown are representative images of three independent experiments done at least in duplicate.

2.5. MTS, Cell Titer-Glo, and Caspase 3/7 Activity

A375, Hs 294T or NHEM cells (4.0×10^3) were plated in white walled 96-half well plates (Corning, Cat. 3688) in full growth medium and treated with BI 6727 [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and Cell Titer-Glo: 0-100 nM; Caspase -Glo: 0-4000 nM] or vehicle control (0.1% DMSO) for 24 h. Following treatment, cell growth, viability, or caspase 3/7 activity was detected by using the MTS, Cell Titer-Glo, or Caspase-Glo 3/7 Assays (Promega, Cat. G5421, G7570, and G8090), respectively. All assays were performed using manufacturer's protocol. Briefly, to perform MTS assay, prepared reagent was added to cells in culture medium, incubated for 2 h at standard culture conditions described earlier, then absorbance was read at 490 nm. For Cell Titer-Glo and Caspase-Glo 3/7 assays, prepared reagent was added to cells in culture medium, mixed for 2 m (Cell Titer-Glo) or 30 s (Caspase-Glo), and incubated at room temperature for 10 m (Cell Titer-Glo) or 0.5-3 h (Caspase-Glo, 10 m kinetic reads). After incubation, luminescence was measured using the BioTek Synergy H4 microplate reader.

2.6. Colony Formation Assay

A375 or Hs 294T cells (5×10^5) were plated in a 10 cm culture dish and treated with BI 6727 (1-100 nM) or 0.1% DMSO control as previously described. Following 48 h treatment, cells were trypsinized, rinsed with PBS and assessed for viability by trypan blue exclusion assay using the Bio-Rad TC-20 automated cell counter. Next, 1×10^3 viable cells were plated in a 10 cm culture dish in full growth medium for 14 days with regular media changes every 48-72 h. Following the growth period, the plates were stained with crystal violet and colonies were manually counted per cm^2 . The median count of three replicates was used for statistical analysis and is represented graphically.

2.7. Xenograft Mouse Model

All the animal experiments described in this study were approved by the University of Wisconsin Institutional Animal Care and Use Committee (protocol M02275). A375 or Hs 294T melanoma cells (1×10^6) suspended in PBS were mixed with Matrigel (Corning, Cat. 354248; at 1:1 ratio) and injected subcutaneously into the right flank of Nu/Nu nude mice (Charles River, Strain #088, Crl:NU-Foxn1^{nu}) and allowed to grow. Once a median tumor volume of 100 mm^3 was achieved, the mice were randomly divided into three groups and scheduled for treatment with BI 6727 (10 or 25 mg/kg bodyweight) or DMSO (50% v/v, Sigma, Cat. D2438) vehicle control. Dose and delivery method of BI 6727 was derived from a previous study that demonstrated the concentrations were well tolerated in mice [14]. The

groups consisted of either 12 or 11 mice per treatment group for A375 or Hs 294T models, respectively. Treatments were administered twice weekly via tail vein injection. In our protocol corresponding with Fig. 5A and 5B, upon the control group reaching the terminal endpoint (average tumor volume $>1000 \text{ mm}^3$), the treatments were ceased and individual tumor volumes were monitored for growth rate. Treatments were discontinued following injection on day nine (four injections total) and twelve (five injections total) after the initial administration of BI 6727 for A375 and Hs 294T models, respectively. Our protocol corresponding with Fig. 5C was conducted under the same conditions with the exception that all mice were sacrificed following the first animal reaching the terminal endpoint (tumor volume $> 1000 \text{ mm}^3$). The treatments were administered to 8, 5, and 6 mice in the control, 10 mg/kg, and 25 mg/kg treatment groups, respectively. All tumors were excised, photographed, weighed and processed for immunohistochemical analysis.

2.8. Immunohistochemistry

At the termination of the experiment depicted by Fig. 5C, A375 xenografted tumors were excised from host mice, formalin fixed and paraffin embedded. Fixing, embedding, sectioning and mounting was completed by the University of Wisconsin Carbone Cancer Center Experimental Pathology Core Laboratory. For immunostaining, the slides were deparaffinized with xylenes and rehydrated in an ethanol series (100-70%). Antigens were unmasked by heat-induced epitope retrieval (HIER) for 45 min in IHC-Tek Epitope Retrieval Solution (IHC-TEK, Cat. IW-1100-1L), followed by a 20 min incubation at RT. Slides were then washed in TBS-T, blocked in 5% natural goat serum for one hour at RT followed by an overnight incubation at 4°C in a humidifying chamber with the desired primary antibodies; anti-Ki-67 (1:100), Caspase 3 (1:75), p21 (1:50), or p53 (1:50) (Santa Cruz Biotechnology, Cat. sc-15402, sc-7272; Cell Signaling, Cat. 2947, 9282, respectively). The following day, slides were washed and incubated with the appropriate HRP-conjugated secondary antibody (1:500) (Cell Signaling, Cat. 7074 and 7076) for 1 h at RT in a humidifying chamber. Tissues were then washed and exposed to DAB (3, 3'-diaminobenzidine) HRP substrate (Vector Labs, Cat. SK-4105) until desired staining intensity was achieved (3-10 min) before terminating corresponding reactions simultaneously. Slides were counterstained with modified hematoxylin, dehydrated in an ethanol series (70-100%) and cleared with fresh xylenes. Slides were imaged with an EVOS XL Core bright-field microscope (Thermo Fisher Scientific) using a 40× objective lens. Figures shown are representative images from multiple sections from each group (all the animals) as described in legend to Fig. 6.

2.9. Immunofluorescence

Immunofluorescent analysis of Plk1 expression was done at the University of Wisconsin Translational Research Initiatives in Pathology (TRIP) core facility using the Lab Vision Autostainer 360 (ThermoFisher Scientific). Plk1 primary antibody (Santa Cruz Biotechnology, Cat. Sc-17783, 1:25 dilution) and Qdot 525 secondary antibody (Invitrogen, Cat. Q22073, 1:200 dilution) were applied to a commercially available human melanoma tissue micro array with samples collected under HIPAA-compliant protocols (US Biomax, Cat. ME1004a) and imaged using the Vectra imaging system (PerkinElmer). Data was analyzed using InForm software (PerkinElmer).

2.10. Statistical Analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, Inc). All studies were done in triplicate unless otherwise stated. Statistical significance was determined by one-way ANOVA with Tukey's post hoc test or by two-tailed Student's t- test. Data is shown as mean $\pm$ SD unless otherwise specified. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Polo-like kinase 1 is overexpressed in clinical melanoma

To analyze the correlation between Plk1 expression and melanoma progression, we performed a quantitative fluorescent analysis of Plk1 expression in a patient derived tissue microarray consisting of benign nevi (24 cores), metastatic (20 cores) and malignant melanomas (56 cores). Our data demonstrates an overall increase of Plk1 expression in malignant melanoma (fold-change = 2.22) and metastatic melanoma (fold-change = 5.47) when compared to benign nevi, with metastatic tumors having significantly higher levels of Plk1 ($p < 0.05$, Fig. 1A). Furthermore, comparing the nuclear and cytoplasmic expression of Plk1, we found that in benign nevi, Plk1 is primarily expressed in the nucleus while malignant and metastatic melanomas have a pronounced cytoplasmic accumulation (Figs. 1B and S1). However, it remains to be determined if the increased cytoplasmic accumulation is due solely to increased Plk1 expression during interphase or a product of mislocalization. Additional analysis revealed no specific correlation of Plk1 levels with patient gender or age (Fig. 1C and D). Overall, these data provide quantitative evidence that Plk1 expression may have significance for melanoma progression and further support the hypothesis that Plk1 inhibition has an advantage in melanoma targeted therapy.

3.2. BI 6727 significantly reduces cell growth and viability in BRAF^{V600E} and BRAF wild-type melanoma *in vitro*

We have previously reported that the targeted depletion of Plk1 by shRNA or the small molecule inhibitor GW843682X reduces melanoma cell growth and viability.[8] In this study, to expand on our previous findings and further support our hypothesis that Plk1 is an effective therapeutic target in both BRAF^{V600E} mutant and BRAF wild-type melanomas, we assessed the anti-proliferative efficacy of second generation small molecule inhibitor BI 6727 in A375 and Hs 294T cells, respectively. In A375 cells, BI 6727 significantly reduced cell viability at low nanomolar concentrations and demonstrated a better response when compared to the standard of care treatment vemurafenib (Fig. 2A). BI 6727 treatment also significantly reduced cell viability of Hs 294T cells at low nanomolar concentrations with a better response when compared to vemurafenib, however BRAF^{V600E} mutant A375 cells demonstrated a greater sensitivity (Fig. 2B). Additionally, BI 6727 treatment was well tolerated by normal human epidermal melanocytes (NHEM) at nanomolar concentrations with no significant loss of viability until micromolar concentrations were reached (Fig. 2C). Similarly, BI 6727 also significantly reduced cell growth in both melanoma lines when compared to NHEMs in low nanomolar concentrations (25-100 nM), with A375 cells demonstrating greater sensitivity than the Hs 294T line at lower doses (Fig. 2D). Further, BI

6727 significantly reduced colony formation in both melanoma cell lines and nearly abolished it at higher concentrations (100 nM, Fig 2E).

3.3. BI 6727 inhibits Plk1 activity and induces an S-phase accumulation and G2/M arrest in melanoma cells

We have previously reported that Plk1 inhibition induces mitotic catastrophe and results in a G2/M cell cycle arrest.[8] To better characterize the arrest induced by Plk1 inhibition, we performed a cell cycle synchronization followed by a time course analysis of cell cycle progression (0-30 h, Fig. 3A). A375 cells were synchronized in the early G1 stage through serum starvation followed by a release into serum supplemented media and treated with 100 nM BI 6727 or DMSO. In the 6 to 12 h range, a marked lag in G1/S-phase progression was observed in the BI 6727 treatment group when compared to control. However, BI 6727 did not induce a G1 arrest and treated cells progressed into S-phase at a retarded rate. At eighteen to thirty hours the treated melanoma cells began to accumulate in both S and G2/M phases while the control cells returned to a more typical cell cycle distribution. These data suggested that in addition to mitotic catastrophe and G2/M arrest, BI 6727 treatment stalls DNA replication resulting in an S-phase accumulation. To determine if the interphase arrest was a result of checkpoint activation, we next analyzed the cell cycle distribution of A375 cells treated with the checkpoint kinase inhibitor, PF-0477736, in combination with BI 6727. Checkpoint kinase inhibition did not completely abrogate the S-phase accumulation, yet there was an observable shift out of S- phase (Fig. 3B). The cell cycle shift in BI 6727 treated cells induced by PF-0477736 was not associated with cytotoxicity as treatment with PF-0477736 alone did not significantly reduce cell viability when compared to the vehicle treated control, nor did it significantly ameliorate the loss of viability induced by BI 6727 when treated in combination (Fig. 3C). Although not statistically significant, 10 nM BI 6727 alone and in combination with PF-0477736 did result in a marginal loss of viability when compared to controls, with 10 nM BI 6727 + 1000 nM PF-0477736 eliciting the greatest response ($p=0.0692$). In contrast, treatment with 100 nM BI 6727 alone or in combination with PF-0477736 resulted in a significant loss of viability when compared to control ($p<0.01$) and 10 nM BI 6727 with or without the presence of PF-0477736 ($p<0.05$). These data provide evidence that in addition to the G2/M arrest associated with mitotic catastrophe, Plk1 inhibition by BI 6727 initiates the DNA damage pathway resulting in checkpoint activation and an associated S-phase accumulation.

3.4. BI 6727 induces apoptosis in BRAF^{V600E} and BRAF wild-type melanoma cells *in vitro*

To determine if the anti-proliferative effects of BI 6727 are associated with the induction of apoptosis, we determined the apoptotic population of A375, Hs 294T, and NHEM cells by flow cytometry using an annexin V-FITC conjugate coupled with PI exclusion following 24 hours of BI 6727 treatment (1-100 nM, Fig. 4A). As expected, BI 6727 treatment resulted in an induction of apoptosis in A375 and Hs 294T in a dose dependent manner, with better efficacy observed in A375 cells when compared to Hs 294T cells at the lower doses. Further, NHEMs did not show any appreciable apoptosis following BI 6727 treatment (Fig. 4A and B). To further confirm the induction of apoptosis, we employed a caspase 3/7 activity assay. Not surprisingly, caspase activity correlated to annexin V staining with both melanoma lines

showing increased caspase activity, with A375 being more sensitive, and no discernible effect in NHEMs (Fig. 4B).

3.5. BI 6727 treatment results in significant melanoma tumor growth delay and regression *in vivo*

To assess the antitumor efficacy of Plk1 inhibition against BRAF^{V600E} mutant and BRAF wild-type melanoma *in vivo*, A375 and Hs 294T derived xenograft models were used, respectively. The melanoma cells were implanted subcutaneously on the right flank of athymic nude mice (Nu/Nu) and allowed to grow. Following average tumor burden reaching 100 mm³, the mice were randomized into three groups and treated intravenously twice weekly with BI 6727 (10 mg/kg or 25 mg/kg) or DMSO. Remarkably, both xenograft models responded in a dose dependent manner with significant tumor growth delay starting at 10 mg/kg and tumor regression at 25 mg/kg (Fig. 5A and B). As predicted by our *in vitro* studies, A375 derived xenografts showed a better response when compared to Hs 294T, albeit only by a marginable difference, as BI 6727 treatment at the selected doses in both models resulted in strong antitumor activity. Additionally, termination of the treatment schedule resulted in a tumor regrowth without significant fluctuations in body weight, indicating BI 6727 treatment was responsible for the antitumor activity and the doses were well tolerated (Fig. S2). Furthermore, a second A375 derived xenograft study was performed under the same conditions with the exception that all mice were sacrificed once the target tumor burden (>1000 mm³) was reached. This protocol was used to determine the effect of Plk1 inhibition on the downstream mechanism of the observed response. This study recapitulated the previous results with a significant tumor growth delay in the 10 mg/kg treatment group and tumor regression at 25 mg/kg (Fig. 5C). These observations were supported by decreases in apparent tumor size and tumor weight in a dose dependent manner (Fig. 5D and E, respectively).

3.6. BI 6727 treatment reduces cellular proliferation and induces apoptosis in melanoma xenografts *in vivo*

Next, to assess the effect of BI 6727 treatment on melanoma cell proliferation *in vivo*, we performed immunohistochemical (IHC) staining of Ki-67 in A375 derived xenograft tumor tissues treated with 10 mg/kg and 25 mg/kg BI 6727 or DMSO control. Indicative of decreased cellular proliferation, Ki-67 expression was found to decrease in a dose dependent manner (Fig. 6A, top panel). Further, we evaluated the expression of caspase 3 as a marker for the induction of apoptosis in the tumor tissues and found a dose dependent increase in the 10 mg/kg and 25 mg/kg BI 6727 groups when compared to control (Fig. 6A, bottom panel). Taken together, these data suggest that BI 6727-mediated inhibition of Plk1 imparts a significant anti-proliferative response against human melanoma via an induction of apoptosis.

3.7. BI 6727 treatment causes an induction in p53 and p21 in melanoma xenografts

A number of studies have suggested an association between Plk1 and the tumor suppressor p53 [15-22]. To test if the observed anti-tumor effects of BI 6727-mediated inhibition of Plk1 are associated with an increase in p53 in melanoma tissue *in vivo*, we performed IHC staining of A375 derived xenograft tumor tissues treated with 10 mg/kg and 25 mg/kg BI

6727 or DMSO control. As shown in Fig. 6B, we observed a marked increase in p53 staining in both the 10 mg/kg and 25 mg/kg treated groups when compared to control. Further, the increased p53 protein levels were found to be associated with increased p53 activity as shown by an increased immunostaining of the p53 downstream target p21 (Fig. 6B, bottom panel). These data are supported by our *in vitro* western blot analyses that demonstrate BI 6727 treatment induces p53 and p21 expression in both A375 and Hs 294T cells (Fig. 6C). Densitometric analysis of the blots shown in Fig. 6C are provided in Fig. S3.

4. Discussion

The main objective of this study was to determine the therapeutic efficacy of a second generation small molecule inhibitor of Plk1, BI 6727, in both BRAF^{V600E} mutant and BRAF wild-type melanomas. Previously we have reported that targeted depletion of Plk1 by shRNA or the small molecule inhibitor GW843682X induces mitotic catastrophe and apoptosis in melanoma cells *in vitro* [8]. BI 6727 is one of the most specific commercially available Plk1 ATP-competitive inhibitor with an IC₅₀ of 0.87 nM and is currently undergoing clinical evaluation for its anticancer potential in hematological and solid malignancies [23-25]. The major finding of our study is that BI 6727 mediated Plk1 inhibition effectively and significantly reduces melanoma tumor growth *in vivo* at clinically relevant doses (Fig. 5A and B). Using a BSA based translation model, the human equivalent doses would be approximately 2.0 mg/kg and 0.81 mg/kg for the 25 mg/kg and 10 mg/kg treatments, respectively [26]. With an average human weight of 80 kg, that would roughly translate to a 320 mg/kg and 130 mg/kg weekly dose, which is within a reasonable range of the reported maximum tolerated dose of 300 mg reported in a recent phase I clinical trial [23]. In addition, we have found that BI 6727 treatment induced significant anti-proliferative response *in vitro* in both A375 and Hs 294T cell lines at low nanomolar concentrations without any appreciable effect on NHEMs (Fig. 4A and B). Further, we found that BRAF^{V600E} mutant melanoma cells have increased sensitivity to BI 6727 when compared to BRAF wild-type and BI 6727 demonstrates a favorable therapeutic profile when compared to the standard of care treatment, vemurafenib (Fig. 2A-C).

The treatment of BRAF mutant melanoma by the small molecule inhibitor vemurafenib has opened up a new era of protein specific targeted therapies. Yet, despite the initial clinical success of vemurafenib treatment in melanoma, patients inevitably develop drug-resistant metastatic melanoma through the BRAF-independent reactivation of the MAPK pathway [3]. MAPK signaling promotes uncontrolled proliferation and cell cycle progression through the induction of cyclin D [27]. Once expressed, cyclin D forms a complex with cyclin dependent kinase 4 (Cdk4), which in turn phosphorylates pRB and leads to the increased transcriptional activity of E2F1 [28]. These signaling events drive the cell cycle through G1 and past the restriction point where the cell becomes committed to replication and division. However, this pathway is not exclusive to MAPK activation. Mutations to *CDKN2A* resulting in the loss of the tumor suppressor p16 functions in a similar manner [29, 30]. Further, amplifications of cyclin D and Cdk4 are frequently reported in BRAF wild-type melanoma [31, 32].

Considering the multiple signaling pathways that coalesce into the cell cycle in early G1, it is not surprising that the targeting of single upstream effectors results in a compensatory response. To effectively disrupt the common signaling cascade induced through the previously described mechanisms, it is reasonable to consider the targeted inhibition of downstream proteins that play a regulatory role in the later stages of cell division, such as G2 and mitosis. In that regard, our data suggests targeted inhibition of Plk1 is a promising approach with a demonstrated G2/M arrest that has the potential to subvert the multiple compensatory mechanisms associated with G1 arrest (Fig. 3A). This notion is reflected by our xenograft studies that resulted in significant tumor growth delay and regression in both BRAF mutant and wild-type melanomas in response to BI 6727 treatment (Fig. 5A and B).

We have also found that BI 6727 mediated Plk1 inhibition induces the tumor suppressor p53 (Fig. 6B and C). This is an important observation because cancers that carry a high background mutation load, such as colon and lung cancers, tend to have a high p53 mutation frequency (50-70%) [32]. However, despite melanoma having a similar mutation load as colon and lung cancers, p53 is only mutated in a very small fraction of human melanoma cases [32, 33]. Even though melanomas mostly harbour the wild-type p53, this tumor suppressor is inactivated via other mechanisms such as through the dysregulation of its endogenous inhibitor MDM2 [34, 35]. Further, in addition to the loss of p16 described earlier, a common mutation of *CDKN2A* also results in the loss of the alternate reading frame (ARF) protein p14^{ARF}, which promotes MDM2 degradation and stabilizes p53 [36]. Interestingly, Plk1 is involved in many regulatory feedback loops that are associated with p53, MDM2 and p16^{INK4A}/p14^{ARF} (reviewed in depth in [37]) and with up to 84% of human melanomas expressing wild-type p53, the reactivation of p53 signaling would provide a distinct advantage in the remediation of melanomas with diverse mutational backgrounds [32].

The expression of active p53 is often associated with the p53 mediated DNA damage response (DDR). The DDR initiates DNA damage checkpoints and negatively regulates cell cycle progression through a complex signaling network that has a primary axis of ataxia telangiectasia mutated (ATM), ATM and rad-3 related (ATR), checkpoint kinases 1 and 2 (Chk1 and 2), p53 and p53 downstream targets such as p21 and p53 upregulated modulator of apoptosis (PUMA) [38]. Indeed, in addition to a G2/M arrest, we did observe an S-phase accumulation in BI 6727 treated cells (Fig 3A) that was, at least in part, ameliorated through the targeted inhibition of Chk1 and Chk2 by PF-0477736 (Fig 3B). Additionally, we found a marked upregulation of p21, further suggesting an active p53 mediated DDR (Fig 6B and C). Considering the key role of p53 in the DDR, increased p53 activity potentially mediating the reactivation of checkpoint signaling provides valuable insight into the efficacy of targeted Plk1 inhibition by BI 6727 treatment in human melanoma.

Overall, our data suggest that targeted Plk1 inhibition by BI 6727 may be an effective therapeutic strategy in both BRAF^{V600E} mutant and BRAF wild-type melanomas. Further, we have demonstrated that BI 6727 treatment results in increased expression and activity of p53 in both cell culture and xenograft mouse models. Considering the capacity of Plk1 inhibition to induce p53 expression and effectively arrest cells downstream of the most commonly occurring oncogenic mutations in melanoma, targeted Plk1 inhibition has the

potential to be a broad spectrum therapy irrespective of the driving mutation. In addition, a recent phase I clinical trial of BI 6727 in solid tumors of varying cancer types found that melanoma patients elicited a partial response to treatment [23]. Taken together, these data provide compelling reason to pursue the targeted inhibition of Plk1 in metastatic melanoma at the clinical level and to be considered as a potential candidate for a combinational strategy in conjunction with the present standard of care treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Plk1 is overexpressed in clinical melanoma samples compared to benign nevi.
- Plk1 inhibition by BI 6727 has anti-proliferative effects in human melanoma cells.
- Plk1 inhibition by BI 6727 causes G2/M & S-phase cell cycle arrests *in vitro*.
- BI 6727 treatment significantly delays melanoma growth & causes regression *in vivo*.
- Anti-melanoma effects of BI 6727 are accompanied by an increase of p53 activity

Figure 1. Plk1 is overexpressed in clinical metastatic melanoma samples when compared to benign nevi

Vectra based quantitative fluorescent analysis of Plk1 expression in a tissue micro array comprised of malignant melanoma (n=56), metastatic melanoma (n=20), and benign nevi (n=24), comparing (A) the total Plk1 expression; (B) the nuclear vs. cytosolic Plk1 expression; (C) the correlation of Plk1 with age of the patient; (D) or the level of Plk1 expression based on gender. *P = 0.05

Figure 2. BI 6727 treatment significantly reduces melanoma cell growth, viability and clonogenic survival with minimal detriment to normal human epidermal melanocytes (NHEMs)

The effects of BI 6727 and Vemurafenib (Vem) treatments on cell viability was determined using a Cell Titer-Glo assay in (A) A375, (B) Hs 294T and (C) NHEMs; The effect of BI 6727 on cell growth in A375 and Hs 294T melanoma cells and NHEMs was assessed by MTS assay (D); The effect of BI 6727 treatment on the clonogenicity of A375 and Hs 294T melanoma cells was determined by a colony formation assay (E). *** P < 0.001. Data represent three independent experiments done in triplicates.

Figure 3. BI 6727 treatment results in an accumulation of melanoma cells in G2/M and S-phases of the cell cycle

Cell cycle analysis of A375 cells synchronized in the G1 phase by serum starvation with a release into DMSO or BI 6727 (100 nM) containing media from 0-30 hours (A); Cell cycle analysis of A375 cells treated with DMSO, BI 6727 (100 nM), PF-0477736 (1000 nM), or a combination of BI 6727 and PF-0477736 (B); Relative cell viability of A375 cells, following treatments with BI 6727, PF-0477736, combination, or DMSO, as determined by trypan blue exclusion assay. **P 0.01; *** P 0.001, Data represent three independent experiments done in triplicates.

Figure 4. BI 6727 mediated Plk1 inhibition results in the induction of apoptosis in melanoma cells

The induction of apoptosis in A375 cells, Hs 294T cells and NHEMs was assessed by flow cytometric analysis of FITC conjugated Annexin V and PI staining, following BI 6727 treatment, and shown as percent apoptotic cells (A); Apoptosis was also evident by changes in caspase 3/7 activation following BI 6727 treatment using the Caspase-Glo reporter assay (B). * $P = 0.05$; *** $P = 0.001$; **** $P = 0.0001$. Data represent three independent experiments done in triplicates.

Figure 5. BI 6727 treatment results in significant tumor growth delay and regression in melanoma xenografts *in vivo*

The average volume of A375 (n = 12/group) (A) and Hs 294T (n = 11/group) (B) -derived xenografted tumors following tail vein administration of 10 mg/kg BI 6727, 25 mg/kg BI 6727 or DMSO (vehicle control); Average volume of A375 derived xenografted tumors following the described protocol with the exception of all animals being sacrificed when one tumor achieved 1000 mm³ volume (n = 8, control; 5, 10mg/kg; 6, 25 mg/kg) (C); Digital photographs (D) and average weight of excised tumors (E) from the study represented in Fig. 5C. Data shown is mean ± SEM. * P 0.05; **P 0.01; *** P 0.001

Figure 6. BI 6727 treatment decreases cellular proliferation, induces apoptosis and results in increased p53 expression and activity in xenograft mouse models

Immunohistochemical (IHC) staining of A375 derived xenograft tumor tissue for Ki-67 (top panel) and Caspase 3 (bottom panel) demonstrating a dose dependent increase in apoptosis and decrease in cellular proliferation in a dose dependent manner, respectively (A); IHC staining of A375 tumor tissue for p53 (top panel) and p21 (bottom panel) revealing a dose dependent increase in expression of both proteins following BI 6727 mediated Plk1 inhibition (B). Images are representative of three independent experiments done in at least duplicate. Western blot analysis showing a marked increase in p53 and p21 protein expression in BI 6727 treated A375 (top panel) and Hs 294T (bottom panel) melanoma cells *in vitro* (C). Blot images are representative of three independent experiments done in at least duplicate.