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Inhibition of melanoma development in the *Nras*^{(Q61K)::Ink4a}^{-/-} mouse model by the small molecule BI-69A11

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Abstract

Despite advances in developing specific inhibitors to *BRAF* mutant melanomas, to date there are no effective therapies for tumors bearing *NRAS* mutations, present in approximately 15–20% of human melanomas. Here, we extend earlier studies, demonstrating that the small molecule BI-69A11 inhibits the growth of melanoma cell lines in vitro and in vivo. Gene expression microarray analysis of BI-69A11-responsive melanoma cells revealed the induction of interferon- and cell death-related genes that were associated with responsiveness to BI-69A11. Strikingly, the administration of BI-69A11 inhibited melanoma development in genetically modified mice bearing an inducible form of activated *Nras* and a deletion of the *Ink4a* gene (*Nras*^{(Q61K)::Ink4a}^{-/-}). Biweekly administration of BI-69A11 starting at 10 weeks or as late as 24 weeks after the induction of mutant *Nras* expression inhibited melanoma development (100% and 36%, respectively). BI-69A11 treatment did not inhibit the development of histiocytic sarcomas, which comprise about 50% of the tumors in this model. Immunofluorescent staining analyses of CD45 revealed increased levels of immune cell infiltration in BI-69A11-treated tumors. Gene expression profiling of BI-69A11-resistant *Nras*^{(Q61K)::Ink4a}^{-/-} tumors revealed the upregulation of functional gene networks associated with the cytoskeleton, DNA damage response, and small molecule transport. The ability to attenuate development of *NRAS* mutant melanomas by BI-69A11 even when administered at a late stage of the tumor development, support its further development and clinical assessment.

Malignant melanoma is a rapidly metastasizing tumor that is notoriously unresponsive to common therapies (Bhatia et al., 2009; Flaherty, 2012). Thanks to the wealth of data from genetic studies carried out over the past decade, we have begun to unravel the complexity of melanoma and categorize the disease subtypes according to their genetic mutations (Lin et al., 2011; Woodman et al., 2012). By current estimates, approximately 50%, 15–20%, and <5% of melanomas have been shown to harbor mutations in *BRAF*, *NRAS*, and *c-KIT*, respectively (Albino et al., 1984; Curtin et al., 2006; Davies et al., 2002). Other mutations characterize different subtypes of melanoma, such as mutations in the G protein α subunit *GNAQ* and its paralogue *GNAI1* in uveal melanoma (Harbour, 2012). The list of mutations present in melanomas continues to grow (for recent reviews see (Dutton-Regester and Hayward, 2012; Walia et al., 2012), although deciphering their significance for melanoma development and progression will require considerable effort.

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The finding that melanomas harbor mutations in specific kinases has provided an opportunity to develop enzyme inhibitors as potential therapies, as has been demonstrated for *BRAF* mutant melanomas (Ribas and Flaherty, 2011). Clinical studies have provided encouragement that such inhibitors can effectively block melanoma development, although resistance invariably develops. Today, third generation *BRAF* inhibitors and inhibitors of other components of the MAPK pathway (e.g. MEK; (Paraiso et al., 2010)) are being evaluated in clinical trials, often in combination with inhibitors of other signaling pathways (i.e. AKT/PI3K; (Paraiso et al., 2011; Villanueva et al., 2010) or more general pathways (i.e. HDAC; (Boyle et al., 2005) that are deregulated in melanoma. Despite the prevalence of *NRAS* mutant melanomas, specific treatments for these tumors have not been developed as for *BRAF*-bearing melanomas. Here, we describe a small molecule that inhibits melanoma development in the genetic *Nras*^(Q61K)::*Ink4a*^{-/-} mouse model, while extending earlier characterization of BI-69A11 in culture and in vivo.

The small molecule BI-69A11 was identified initially in a screen for inhibitors of AKT with IC₅₀ of 2.5 μM (Forino et al., 2005). Further screening against 315 kinases confirmed that BI-69A11 inhibited all three AKT isoforms as well as sphingosine kinase. Inhibition of sphingosine kinase by BI-69A11 was shown to affect the NF-κB pathway (Feng et al., 2011), demonstrating that BI-69A11 targets two key anti-apoptotic pathways that are commonly upregulated in melanoma and other tumors. In cell culture, the addition of BI-69A11 resulted in inhibition of AKT enzymatic activity and, at higher concentrations, also affected AKT stability by interfering with its association with HSP90 (Gaitonde et al., 2009). BI-69A11 was also shown to block NF-κB transcriptional activity through its upstream regulatory component IκB (Feng et al., 2011). Of note, BI-69A11 was able to prevent growth of melanoma cells that were resistant to *BRAF* inhibitors (Feng et al., 2011). In the present study, we first compared the inhibitory effects of BI-69A11 on 2 mutant *BRAF* (Lu1205/WM793) and 2 mutant *NRAS* (WM1346/WM1366) melanoma cell lines grown in vitro. All 4 melanoma cell lines exhibited a dose-dependent response to BI-69A11 with potent toxicity, within the range of 0.5–2 μM (Fig 1a), consistent with earlier studies in melanoma, breast and prostate cancer cell lines (Gaitonde et al., 2009).

To further characterize the possible effects elicited by BI-69A11 on melanoma in vitro and in vivo, we set to evaluate changes in gene expression that might highlight pathways that can be associated with responsiveness to BI-69A11. Gene expression profiling was performed on control- and BI-69A11-treated mutant *BRAF* (WM793/Lu1205) compared with mutant *NRAS* (WM1346/WM1366) cell lines. This analysis identified 223 genes that exhibited significant fold-changes in expression (> 1.5, p-value < 0.05) uniquely in the Lu1205/WM793 cell lines, compared to 208 genes uniquely in the WM1346/1366 cell lines, after BI-69A11 treatment (Fig 1b and data not shown). However, to identify functional pathways that might be associated with the responsiveness of melanoma cells to BI-69A11, we examined the genes that were altered in common in both *NRAS* and *BRAF* responsive groups. Our analysis identified 95 genes that were altered in response to BI-69A11 in both the Lu1205/WM793 group and the WM1346/WM1366 group (Fig 1b). Notably, IPA analysis of these 95 genes has confirmed a signature of inflammation/interferon- and cell death-related genes as the top 2 functional clusters. The 95 specific genes and their respective fold-changes are listed in Table S1. Quantitative PCR analysis of 7 representative genes from the interferon and cell death clusters, [4 upregulated (MX1, IFIT1, ISG15, IF44L) and 3 downregulated (HIST1H4A, IGFBP5, VCAN)], confirmed the changes identified in the gene expression microarray data (Table S2). These findings suggest that responsiveness to BI-69A11 is associated with altered inflammation/interferon- and cell death-related pathways.

In vivo, BI-69A11 effectively inhibited the growth of UACC903, which harbors mutations in both *BRAF* and *PTEN* in melanoma xenografts, when administered by IP (0.5 mg/kg), or orally (30-100mg/kg) (Gaitonde et al., 2009). The ability of BI-69A11 to block melanoma development was confirmed using the mutant *Nras* bearing SW1 mouse melanoma cell line in syngeneic C3H mice (Feng et al., 2011).

Pharmacokinetic (PK) analysis revealed that BI-69A11 displays good exposure after IP dosing at 2.22 mg/kg (AUC= 2,424 ng·hr/mL), with a reasonable maximal plasma concentration (~ 1 μ M) and moderate half-life (1.6 h; Fig S1). This PK profile fully supports the effects seen in the mouse xenograft models. Notably, BI-69A11 also displayed moderate clearance after intravenous (IV) administration (plasma clearance rate (CL_p) = 17 mL/min/kg) with half-life of over 8 h. We therefore sought to determine if IV administration of BI-69A11 would be effective in reducing tumor development. Here, we demonstrated that UACC903 melanoma regression could also be achieved after IV administration of BI-69A11 (Fig 1c).

Genetic models are powerful systems for assessing efficacy of potential anti-cancer compounds, because they better mimic the genetic changes seen in human tumors while allowing efficacy to be tested in the context of the tumor microenvironment. Therefore, we next examined the effect of BI-69A11 in *Nras*^(Q61K)::*Ink4a*^{-/-} mice, which express a doxycycline-inducible form of activated *Nras* on an *Ink4a*-deficient background. Melanoma develops slowly and with low penetrance in this model, with about 50% of the animals developing tumors ~20–50 weeks after induction of mutant *Nras* expression. Approximately 50% of the tumors are melanomas (25% penetrance; confirmed by pathological and IHC analyses for melanoma markers S100 and DCT; Fig S2) and ~25% are histiocytic sarcomas (Kamijo et al., 1999; Shah et al., 2010).

To test the effect of BI-69A11 at early and late phases of melanoma development, we administered BI-69A11 starting at 10 or 24 weeks following mutant *Nras* induction. Prior to treatment initiation, none of the 10-week-old mice had melanoma but 31 animals in the 24-week-old group had developed melanoma; these were excluded from the analysis and only animals without evidence of tumors were treated. Significantly, melanoma development was abolished (0/16 animals with melanoma compared with 3/17 in the control group; Fig 2a), by initiating BI-69A11 administration (biweekly IP injection of 0.5 mg/kg) 10 weeks after mutant *Nras* induction. Remarkably, initiating BI-69A11 treatment as late as 24 weeks after mutant *Nras* induction still resulted in 36% inhibition of melanoma development (7/58 animals compared with 11/59 in the control group; Fig. 2b). Of interest, latestage BI-69A11 administration to the *Nras*^(Q61K)::*Ink4a*^{-/-} animals did not attenuate the development of histiocytic sarcoma (23/58 animals compared with 24/59 in the control group), which are characteristic of *Ink4a*-deficient animals (Kamijo et al., 1999; Shah et al., 2010). Similarly, administration of BI-69A11 starting at 10 weeks did not affect the frequency of histiocytic sarcoma development (5/16 in BI-69A11 group and 6/17 in the control group). Collectively, these findings demonstrate that administration of BI-69A11 to animals that are genetically prone to develop *Nras*-driven melanomas selectively inhibited melanoma development by 36 to 100%, depending on the time at which BI-69A11 treatment was initiated.

Toxicological studies were performed on animals administered BI-69A11 for 4 to 6 months. Standard serum analyses, including liver function tests and biochemistry panels, revealed no differences between the control and BI-69A11-administered animals (Table S3). Pathological assessment of liver sections confirmed the absence of BI-69A11-related toxicity (Fig S3), consistent with lack of evidence for weight or clinical signs of toxicity. As mentioned, histiocytic sarcoma are characteristic of the *Ink4a*^{-/-} animals and, as expected, were observed in both treated and control groups.

We next examined the possible effects of BI-69A11 on the immune cell infiltration of the tumors of vehicle-treated *Nras*^(Q61K)::*Ink4a*^{-/-} mice or the tumors that developed despite treatment with BI-69A11 (resistant tumors, within the late treatment group). To this end, we performed immunofluorescence staining analysis for the leukocyte marker, CD45. BI-69A11-treated tumors exhibited increased levels of CD45-positive cells (~14%), compared to vehicle-treated tumors (Fig 3a). Immunostaining analyses for CD3 (T cell marker)- and IBA1 (macrophage marker)-positive staining cells revealed a similar trend of increased infiltration upon BI-69A11 treatment (data not shown). Notably the increased immune cell infiltration in BI-69A11-treated tumors was insufficient to block the progression of the non-responsive tumors.

To further assess changes elicited by BI-69A11 treatment, we have monitored changes in proliferation and apoptosis between the tumors of the vehicle- or the BI-69A11-treated *Nras*^(Q61K)::*Ink4a*^{-/-} mice. Immunofluorescent staining of the vehicle or BI-69A11-treated *Nras*^(Q61K)::*Ink4a*^{-/-} melanoma tumors revealed a decrease in the proliferation marker Ki67, but no significant changes in TUNEL staining, indicative of cell death, in response to BI-69A11 (Fig 3b, c). Taken together with the immune cell findings above, these results point to the effect of BI-69A11 on proliferation and inflammation, which were likely attenuated by the resistance of the tumors to the cell death program in the group subjected to the delayed administration of BI-69A11.

To determine the possible mechanisms underlying resistance to BI-69A11 we performed microarray analysis on the BI-69A11-non-responsive *Nras*^(Q61K)::*Ink4a*^{-/-} murine melanoma tumors. These analyses identified 2,593 genes that were significantly altered in expression (fold-change ≥ 2, *p* < 0.05) in response to treatment with BI-69A11.

Ingenuity network analysis (IPA) of the 2,593 genes altered in the *Nras*^(Q61K)::*Ink4a*^{-/-} tumors identified the following two functional clusters within the top 10% of significantly altered genes (including both up- and downregulated genes): cytoskeleton/motility/small molecule transport (164 genes) and cell cycle/development (94 genes) (Table S4). Quantitative PCR analysis of vehicle- or BI-69A11-treated *Nras*^(Q61K)::*Ink4a*^{-/-} tumors confirmed altered expression of 7 representative genes selected from the individual functional clusters (3 upregulated genes: GRB7, KRT10, TACSTD2; 4 downregulated genes: CSN3, GPX7, UBE2M, TSPO), thereby providing initial validation to our microarray findings (Table S5). Notably, 3 upregulated and 3 downregulated functional clusters were identified within the top 10% of genes that were altered in response to BI-69A11. The top 3 most upregulated functional networks identified were associated with cytoskeletal organization, DNA repair, and small molecule transport/lipid metabolism (Fig 3D; two representative network maps for the upregulated cytoskeletal organization and the DNA repair networks identified are shown as examples in Fig S4). Three of the most downregulated functional networks were associated with Cop9 signalosome/trafficking/Fos, ubiquitin machinery/glutathione metabolism, and cell death/mitochondrial dysfunction (Fig 3D; representative functional network maps of the two most downregulated functional clusters—Cop9 signalosome/protein trafficking/Fos and ubiquitin machinery/glutathione metabolism—are shown in Fig S5).

The functional networks identified in our gene expression profiling have been implicated in tumorigenesis and/or chemoresistance. For instance, of the upregulated networks, altered cytoskeletal organization-related gene expression, (i.e. various cytokeratins, vimentin, or as RhoA), have been associated with chemotherapeutic/multidrug resistance (Bichat et al., 1997; Kang et al., 2005). Enhanced DNA repair mechanisms have also been demonstrated to facilitate the malignancy and chemoresistance of several cancer types including melanoma and glioma (Frosina, 2009; Hatton et al., 1995; Redmond et al., 2008). Recent studies point

to the role of altered lipid metabolism in cancer progression (Santos and Schulze, 2012). Likewise, mitochondrial dysfunction, the downregulation of which was associated with resistance to BI-69A11, is associated with cell death (Luo et al., 2011). Notably, the increase in COP9 signalosome and ubiquitination/glutathione metabolism pathways been associated with tumorigenesis (Luo et al., 2011; Singh et al., 2012), suggesting that their downregulation in the BI-69A11-treated tumors could represent tumor-suppressive effects that are, albeit, insufficient to suppress the tumors.

Although these exciting data demonstrate that BI-69A11 treatment can inhibit the development of *NRas* mutant melanomas, when administered sufficiently early during the course of melanoma development, the data also raise a number of questions. While BI-69A11 elicits a marked effect on melanoma development when administered at the early time points, it is of interest to determine why it only affected 36% of the animals when administered late during the course of tumor development.

Identification of molecular changes allowing us to distinguish the responsive from non-responsive groups will enable better understanding of requirements for efficient response to BI-69A11. The possibility that at such late stage in melanoma development, BI-69A11 effect primarily delayed the development of the slower appearing tumors cannot be ruled out. These observations also point to the need to assess the effect of BI-69A11 in combination with other therapies, possibly those directly affecting the MEK/MAPK pathways, when administered at late stage of tumor development. Identification of markers that can be used to distinguish early from advanced stages in melanoma development (i.e. 10 vs. 24 week groups used in the present study) would further advance our ability to select adjuvant-based therapies, of which BI-69A11 might be one candidate to consider. Notably, our gene expression array analyses identified interferon- and inflammatory-related genes, as well as genes associated with cell death as pathways that are associated with the responsiveness to BI-69A11. In contrast, melanoma tumors that were refractory to BI-69A11 exhibited upregulated cytoskeletal changes, DNA repair, and small molecule biochemistry/lipid metabolism and downregulated Cop9 signalosome/protein trafficking, ubiquitination machinery/glutathione metabolism, and cell death/mitochondrial dysfunction.

Other than the mode and dose of BI-69A11 administration, one needs to consider its effect on the tumor microenvironment and the immune system. BI-69A11-treated *Nras*^(Q61K)::*Ink4a*^{-/-} tumors exhibited increased immune cell infiltration, as indicated by increased infiltrating cells that immunostained positively for CD45. Consistent with these observations is the notion that BI-69A11 is administered at very low doses (0.5 mg/kg), which is expected to affect tumor microenvironment and immune components. That the expression of inflammatory/immune-related gene networks did not rank significantly in our gene expression analysis of BI-69A11-treated *Nras*^(Q61K)::*Ink4a*^{-/-} tumors but did rank highly in the analysis of the BI-69A11-treated responsive cell lines in vitro indicates that although BI-69A11 elicits effects on the innate immune system in the context of *Nras*^(Q61K)::*Ink4a*^{-/-} tumors, such effects are insufficient to induce the inflammation/immune-associated lethality observed in the cell lines. The selective effect of BI-69A11 is demonstrated by lack of its impact on histiocytic sarcomas developed in the experimental groups studied here. The efficacy of BI-69A11 in the mutant *Nras* genetic melanoma model raises the possibility that BI-69A11 might be a candidate for adjuvant-like treatment of a select group of human melanomas that still await effective therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Despite significant advances in the treatment of *BRAF* mutant melanomas, we lack therapies targeting the approximately 50% of melanomas that do not bear this mutation. Here, we demonstrate the ability of the small molecule BI-69A11 to delay or inhibit the development of melanomas harboring an activating *Nras* mutation, when tested in the genetic *Nras*^(Q61K)::*Ink4a*^{-/-} melanoma model. Administration of BI-69A11 at an early stage after induction abolished melanoma development and remarkably, tumor development was still inhibited by 36% when BI-69A11 treatment was initiated at late stage when melanomas had started to appear. These data suggest that BI-69A11 might be a candidate for further development and clinical assessment.

Figure 1. BI-69A11 administration inhibits mutant *BRAF* and mutant *NRAS* melanoma cell growth in vitro, in xenografts tumor models, and is associated with altered gene expression of interferon- and cell death-related functional networks

(A) Mutant *BRAF* (Lu1205 and WM793) or mutant *NRAS* (WM1346 and WM1366) melanoma cells were plated in 384-well plate at a density of 1,500 cells per well and allowed to grow overnight. DMSO (starting point) or BI-69A11 then was added at the indicated concentrations (0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, or 10 μ M). The cell viability was assessed using ATPlite after 48 h. The cell viability was calculated as a percentage of DMSO-treated controls and plotted against the log of the drug concentration using GraphPad Prism. Experiments were performed in triplicates. (B) Venn diagram displaying genes with >1.5 fold-change in expression induced by BI-69A11 treatment in responsive mutant *BRAF* (Lu1205/WM793, blue circle) compared to mutant *NRAS* (WM1346/WM1366, red circle) melanoma cell lines. Two hundred and twenty-three genes exhibited altered expression uniquely in mutant *BRAF* cell lines, whereas 208 genes exhibited altered expression in mutant *NRAS* cell lines. Ninety-five genes exhibited overlapping altered expression between the 2 groups. Of these 95 genes, Ingenuity analysis (IPA, Networks analysis) identified inflammation/interferon-related and cell death clusters, consisting of 18 and 26 genes, respectively, as those that were most significantly modified. The specific altered genes, fold changes, up/down-regulation and p-values of these 95 genes are presented in Table S1. (C) Human UACC903 melanoma xenografts were implanted subcutaneously into the right flank of nude mice. When tumors reached the size of 50 mm³,

vehicle (V) or BI-69A11 (2.2 mg/kg; D) was administered by IV (tail vein) twice per week. Numbers indicate mouse number. Tumor size was measured at the indicated time points.

Figure 2. IP administration of BI-69A11 inhibits melanoma development in the *Nras*^(Q61K)::*Ink4a*^{-/-} genetic model mice

Mice that harbor the *Nras*^(Q61K)::*Ink4a*^{-/-} genotype were given vehicle or BI-69A11 at a dose of 0.5mg/kg by IP injection, two times per week. Tumor development was monitored twice per week. Following their harvest, tumors were subjected to pathological assessment and IHC staining for S100 and DCT to confirm their classification as melanoma (Fig S2). (A) Time curve indicates the incidence of melanoma development in the *Nras*^(Q61K)::*Ink4a*^{-/-} animals subjected to treatment 10 weeks after induction of mutant *Nras* expression. (B) The latency of melanoma development in mice that were treated with vehicle or BI-69A11 at 24 weeks after the activation of mutant *Nras* expression.

Figure 3. The effects of BI-69A11 administration on the levels of CD45-, Ki67-, and TUNEL-staining, and quantitation of its effects by gene expression microarray analysis
 (A) Representative images of tumors from vehicle-(left) or BI-69A11-treated (center) *Nras^(Q61K)::Ink4a^{-/-}* mice that were immunofluorescently stained for CD45 (red) and Dapi (blue), 20x magnification. Scale bar = 100 μ m. The vehicle- and BI-69A11-treated tumor pictures are representative of approximately 70 and 85% of total images evaluated, respectively. The graph (right) represents the mean percentage of CD45-positive cells per field. $N = 3$ mice per treatment group. $P=0.0018$. (B) Ki67 analysis of tumor sections from vehicle (blue)- and BI-69A11 (red)-treated mice. Ki67 staining were quantitated by ImageJ software and represented as Ki67-positive nuclei. $N = 9$ vehicle-treated mice, and $N= 6$ BI-69A11-treated mice. $P=0.0106$. (C) TUNEL analysis of tumor sections from vehicle (blue)- and BI-69A11 (red)-treated mice. TUNEL staining were quantitated by Image J software and represented as TUNEL positive nuclei. $N = 8$ vehicle-treated mice, and $N= 6$ BI-69A11-treated mice. $P=0.8645$. In B & C), quantitations were performed on fields of cells with equivalent numbers of cells (DAPI-positive nuclei, data not shown.). (D) The 3 most up- and 3 most downregulated functional clusters identified within the top 10% of altered genes (>2 -fold change, $p>0.05$) in the BI-69A11-non-responsive tumors in the *Nras^(Q61K)::Ink4a^{-/-}* mice.