Published in final edited form as:

Pigment Cell Melanoma Res. 2011 August ; 24(4): 703–713. doi:10.1111/j.1755-148X.2011.00867.x.

Effective inhibition of melanoma by BI-69A11 is mediated by dual targeting of the AKT and NF- κ B pathways

Yongmei Feng¹, Elisa Barile¹, Surya K. De¹, John L. Stebbins¹, Apple Cortez¹, Pedro Aza-Blanc¹, Jessie Villanueva², Meenhard Heryln², Stan Krajewski¹, Maurizio Pellecchia¹, Ze'ev A. Ronai¹, and Gary G. Chiang¹

¹ Sanford-Burnham Medical Research Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037

² The Wistar Institute, Molecular and Cellular Oncogenesis Program, Philadelphia, PA 19104

Summary

In melanoma, the activation of pro-survival signaling pathways, such as the AKT and NF- κ B pathways, are critical for tumor growth. We have recently reported that the AKT inhibitor BI-69A11 causes efficient inhibition of melanoma growth. Here, we show that in addition to its AKT inhibitory activity, BI-69A11 also targets the NF- κ B pathway. In melanoma cell lines, BI-69A11 inhibited TNF- α -stimulated IKK α / β and I κ B phosphorylation as well as NF- κ B reporter gene expression. Furthermore, the effective inhibition of melanoma growth by BI-69A11 was attenuated upon NF- κ B activation. Mechanistically, reduced NF- κ B signaling by BI-69A11 is mediated by the inhibition of sphingosine kinase 1, identified in a screen of 315 kinases. Significantly, we demonstrate that BI-69A11 is well-tolerated and orally active against UACC 903 and SW1 melanoma xenografts. Our results demonstrate that BI-69A11 inhibits both the AKT and NF- κ B pathways and that the dual targeting of these pathways may be efficacious as a therapeutic strategy in melanoma.

Significance—Although B-RAF and MEK inhibitors have shown promise in the clinic against melanoma, the development of resistance to these singly targeted agents inevitably results. These observations underscore the plasticity of melanoma to chemotherapeutic agents and further emphasize the need to apply combinatorial targeting of signaling pathways as a strategy to maximize therapeutic response. The PI3K/AKT and NF- κ B signaling pathways are altered in melanoma, presenting additional opportunities for target inhibition. Our studies demonstrate that the AKT inhibitor, BI-69A11, also inhibits the NF- κ B pathway and that dual inhibition of both pathways is responsible for the anti-tumor efficacy of this molecule.

Keywords

melanoma; AKT; NF- κ B; targeted therapy

Introduction

Targeted therapy for melanoma has primarily focused on the RAS/MEK/ERK pathway, due to the prevalence of B-RAF and N-RAS mutations, which are found in over 50% of melanomas for B-RAF and 15–30% for N-Ras (Sekulic et al., 2008; Solit and Rosen, 2010). Recently, a phase I study with PLX4032, an inhibitor of mutant BRAF, showed impressive results in patients with metastatic melanoma harboring the V600E BRAF mutation (Bollag et al., 2010; Flaherty et al., 2010). However, as observed with molecularly-targeted agents in other malignancies, tumors initially responsive to PLX4032 developed secondary resistance.

Furthermore, intrinsic resistance of V600E BRAF-bearing tumors to PLX4032 was also observed in some patients as well as in V600E BRAF-bearing melanoma cell lines (Bollag et al., 2010; Flaherty et al., 2010; Lee et al., 2010). These observations underscore the plasticity of melanoma in acquiring resistance to chemotherapeutic agents and highlight the importance of targeting multiple signaling pathways in order to achieve a sustainable therapeutic response.

Two additional signaling pathways known to be dysregulated in melanoma are the AKT and NF- κ B pathways. These pathways have been intensively studied over the past two decades for target discovery and small molecule inhibitor development (Madhunapantula and Robertson, 2009). The protein kinase AKT is activated downstream of class I phosphatidylinositol-3-kinase (PI3K), which generates the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP₃) from its precursor, phosphatidylinositol 4,5-bisphosphate (PIP₂). Activation of PI3K by growth factor receptor tyrosine kinases or heterotrimeric G-protein coupled receptors, results in the generation of PIP₃, which subsequently recruits AKT to the plasma membrane and results in the dual phosphorylation of AKT at T308 and S473, by the protein kinases PDK1 and mTOR, respectively (Alessi et al., 1997; Sarbassov et al., 2005). Following these activating phosphorylation events, AKT in turn phosphorylates a large number of downstream substrates involved in apoptosis, cell-cycle regulation, protein translation and metabolism (Bhaskar and Hay, 2007; Plas and Thompson, 2005). AKT activation is antagonized by the tumor suppressor PTEN, a lipid phosphatase that dephosphorylates PIP₃ to PIP₂. In melanoma, activation of AKT occurs in approximately 50% of human melanomas, with inactivation of PTEN by mutation or epigenetic silencing observed in approximately 60% of these cases (Dhawan et al., 2002; Robertson, 2005).

The NF- κ B family of transcription factors is involved in a diverse spectrum of cellular processes, including inflammation, cell cycle regulation, cell migration/invasion, and apoptosis (Ghosh and Karin, 2002). In the canonical NF- κ B pathway, the transcriptional activity of NF- κ B is tightly regulated by I κ B binding, which sequesters NF- κ B in the cytoplasm. Activation of the canonical NF- κ B pathway by ligands such as TNF- α leads to the activation of the I κ B kinase (IKK) complex, which consists of the kinase subunits IKK α , IKK β , and the regulatory subunit IKK γ (NEMO). The activated IKK complex phosphorylates I κ B, triggering its proteasomal degradation and allowing for the nuclear translocation of NF- κ B and transcription of target genes such as IAP, FLIP, Bcl-XL, and cyclin D (Deveraux et al., 1998; Guttridge et al., 1999; Micheau et al., 2001; Ravi et al., 2001). In melanoma, constitutive activation of the NF- κ B pathway is frequently observed due to upregulation of IKK activity, either by increased expression of IKK activating kinases or by increased expression of cytokines and chemokines arising from B-RAF or N-RAS mutation (Dhawan and Richmond, 2002; Norris and Baldwin, 1999; Ueda and Richmond, 2006; Yang and Richmond, 2001). Furthermore, the requirement for IKK activity in melanoma is underscored by a recent publication whereby deletion of IKK β abrogates H-Ras driven melanoma development in *Ink4a/Arf*^{-/-} mice (Yang et al., 2010).

We have been studying BI-69A11, a small molecule inhibitor of AKT originally identified through an in silico approach (Forino et al., 2005). BI-69A11 binds to the AKT catalytic site and inhibits the kinase activity in an ATP-competitive manner with an IC₅₀ of 2.3 μ M in vitro (Forino et al., 2005). In cell-based assays using melanoma, prostate, and breast cell lines, BI-69A11 reduced AKT S473 phosphorylation and inhibited cell proliferation via increased cell death. In particular, cells that exhibited elevated AKT activity, such as UACC 903 cells harboring both B-RAF mutation and PTEN inactivation, were more sensitive to cell killing by BI-69A11 (Gaitonde et al., 2009). Furthermore, BI-69A11 effectively inhibited melanoma growth as tumor xenografts in vivo (Gaitonde et al., 2009).

In this study, we further characterize the AKT inhibitor BI-69A11 and find that in addition to its AKT inhibitory activity, BI-69A11 also targets the NF- κ B pathway through a mechanism that is consistent with sphingosine-1-kinase inhibition. Importantly, the dual targeting of both the AKT and NF- κ B pathways is essential for the inhibition of melanoma growth by BI-69A11. Furthermore, we demonstrate that oral administration of BI-69A11 is well-tolerated and effective towards inhibiting melanoma growth in UACC903 xenograft and SW1 syngeneic tumor models.

Results

BI-69A11 inhibits the NF- κ B pathway

Previous studies with BI-69A11 had shown high efficacy in inhibiting melanoma growth in xenograft models using concentrations as low as 0.5 mg/kg, which was somewhat unexpected given the results from in vitro and cell-based assays (Gaitonde et al., 2009). One explanation for these discordant results is that BI-69A11 may affect other signaling pathways in addition to AKT. To address this possibility, a screen of 100 kinases was performed to identify additional protein kinases inhibited by BI-69A11 using the Invitrogen Select Screen kinase profiling service. Among the kinases inhibited by BI-69A11 in vitro were IKK α , IKK β , and CHK2, which play key roles in NF- κ B signaling and DNA damage signaling, respectively (Ghosh and Karin, 2002; Reinhardt and Yaffe, 2009). We first tested whether BI-69A11 inhibited the NF- κ B pathway in cells. Stimulation of UACC 903 melanoma cells, harboring B-RAF and PTEN mutation, with TNF- α led to a time-dependent increase in phosphorylation of IKK α/β and I κ B and, subsequently, degradation of I κ B (Fig. 1A). However, pre-treatment of UACC 903 cells with 10 μ M BI-69A11 abrogated TNF- α stimulated IKK α/β and I κ B phosphorylation and increased the stability of I κ B. We also tested the effect of BI-69A11 on two additional melanoma cell lines: MeWo, a human cell line which does not harbor B-RAF or N-Ras mutation but are p53 mutant, and SW1, a mouse melanoma cell line which harbors N-Ras mutation, (Supplemental Table 1 and (Qi et al., 2008)). In both cell lines, we found a dose-dependent inhibition of IKK α/β and I κ B phosphorylation and increased stability of I κ B by BI-69A11 (Fig. 1B), again demonstrating that BI-69A11 inhibits the NF- κ B pathway. Additionally, both MeWo and SW1 cells also showed decreased viability and AKT activity in the presence of BI-69A11 (Fig. S1). We next examined whether the inhibition of IKK α/β and I κ B phosphorylation and subsequent I κ B stabilization affected NF- κ B transcriptional activity using a luciferase reporter assay. Stimulation of NF- κ B luciferase-transfected MeWo cells with TNF- α led to a 16-fold increase in luciferase activity (Fig. 1C). Incubation of reporter transfected-MeWo cells with BI-69A11 prior to TNF- α stimulation abrogated NF- κ B-dependent luciferase induction in a dose-dependent manner, confirming that BI-69A11 inhibits NF- κ B signaling. We also tested the effect of BI-69A11 on the other hit revealed by our in vitro kinase screen, CHK2. As mentioned previously, the ATM/CHK2 axis plays an important role in DNA damage signaling in cells (Reinhardt and Yaffe, 2009). Following γ -irradiation, which induces the DNA damage response, both ATM and CHK2 were activated in the absence of BI-69A11, as evidenced by phosphorylation of ATM at S1981 and Chk2 at T68. However, treatment of cells with BI-69A11 prior to γ -irradiation did not inhibit ATM or CHK2 phosphorylation or the phosphorylation of the CHK2 substrate, Cdc25C (Fig. S2), demonstrating that BI-69A11 does not inhibit CHK2 in cells. Taken together, our data demonstrate that BI-69A11 inhibits the cytokine-induced NF- κ B pathway in addition to inhibiting AKT.

Dual inhibition of AKT and NF- κ B is required for inhibiting melanoma growth

In order to test the relative contribution of inhibiting the AKT and NF- κ B pathways in the BI-69A11-mediated reduction of melanoma growth, we utilized the AKT inhibitor GSK690693. GSK690693 is a potent AKT inhibitor (IC₅₀ 2 nM in vitro), and has shown

variable anti-proliferative activity depending on the tumor cell tested (Rhodes et al., 2008). As expected, treatment of UACC 903 cells with GSK690693 inhibited phosphorylation of AKT and its downstream substrate PRAS40 in a dose dependent manner (Fig. 2A). In contrast to our observations with BI-69A11, GSK690693 did not inhibit TNF- α induced I κ B phosphorylation and degradation (Fig. 2B). Furthermore, GSK690693 did not inhibit UACC 903 cell proliferation to the same extent as BI-69A11 (Fig. 2C). This suggests that AKT inhibition alone is insufficient to inhibit melanoma cell growth and that both AKT and NF- κ B inhibition are required to efficiently block growth. To test this hypothesis, we examined whether activation of NF- κ B signaling could abrogate the effect of BI-69A11 on melanoma cell viability. We first tested this in WM35 melanoma cells, where NF- κ B was activated via siRNA-mediated knockdown of I κ B. In WM35 cells transfected with non-targeting siRNA, BI-69A11 reduced cell viability by 70% (Fig. 2D). In contrast, knockdown of I κ B increased cell viability in the presence of BI-69A11 by approximately two-fold, demonstrating that activation of NF- κ B signaling confers resistance to BI-69A11. We confirmed our hypothesis by examining the effect of BI-69A11 on SK-MEL-5 and SK-MEL-28 melanoma cell lines, both of which exhibit constitutive NF- κ B activity (Yang and Richmond, 2001). As predicted, both cell lines exhibited increased resistance to BI-69A11, with EC₅₀ values 4- to 10-fold higher than that obtained with UACC 903 melanoma cells (Fig. 2E), which corresponded to a reduced effect of BI-69A11 on IKK α / β phosphorylation and I κ B stabilization in SK-MEL-5 cells (Fig. 2F). SK-MEL-5 cells also exhibited lower levels of AKT signaling compared to UACC 903 cells, which was unaffected by BI-69A11 (Fig. S3), suggesting that a lack of dependence on AKT signaling in SK-MEL-5 cells also contributes to the relative resistance of these cells to BI-69A11. Taken together, our results demonstrate that both AKT and NF- κ B inhibition by BI-69A11 are required for efficient blockade of melanoma growth.

Inhibition of the NF- κ B pathway by BI-69A11 is mediated by SPHK1

The observation that BI-69A11 additionally reduced IKK α / β phosphorylation in addition to I κ B phosphorylation suggested that BI-69A11 could affect NF- κ B signaling at, or above, the level of IKK activation. As IKK activity is also regulated by phosphorylation, we carried out an expanded Invitrogen SelectScreen for kinases potentially inhibited by BI-69A11, increasing the total number of kinases screened to 315. From this screen, we identified sphingosine kinase 1 and 2, Aurora B, and Fes as potential cellular targets of BI-69A11; no known IKK activating kinases were affected by BI-69A11. We initially focused our efforts on sphingosine kinase 1 (SPHK1), which catalyzes the formation of sphingosine-1-phosphate (S1P) and is required for NF- κ B activation (Alvarez et al., 2010). The activity of SPHK1 was inhibited by BI-69A11 in vitro, with an IC₅₀ value of 1.4 μ M (Fig. 3A), which is similar to the IC₅₀ value determined for BI-69A11 inhibition of AKT (Forino et al., 2005). Because the balance between the levels of S1P and its precursors (sphingosine and ceramide) is important for mediating cell growth versus apoptosis (French et al., 2006), we assessed whether increasing S1P levels attenuated the effects of BI-69A11 on cell viability. Indeed, addition of exogenous S1P to UACC 903 cells increased the viability of cells treated with 10 μ M BI-69A11 three- to four-fold over control cells which lacked added S1P (Fig. 3B). As SPHK1 is required for TNF- α -induced NF- κ B activation, we next tested whether adding S1P could rescue the BI-69A11 mediated inhibition of NF- κ B transcriptional activity. Similar to our previous results, BI-69A11 (2 μ M) reduced TNF- α induced NF- κ B activity by over two-fold (Fig. 3C), and this inhibition could be rescued by the addition of exogenous S1P. Therefore, our data suggest that the underlying mechanism for reduced NF- κ B signaling in BI-69A11-treated cells additionally involves the inhibition of SPHK1.

B-RAF inhibitor-resistant melanoma lines are still sensitive to BI-69A11

Although initially effective, chronic B-RAF inhibitor treatment eventually leads to the emergence of resistance through a variety of mechanisms, including receptor tyrosine kinase upregulation (e.g. PDGF β , IGF-1R) or B-RAF-independent reactivation of MAPK signaling (e.g. N-Ras mutation, MAP3K8), but not through secondary mutations in B-RAF which abrogate inhibitor binding (Johannessen et al., 2010; Nazarian et al., 2010; Villanueva et al., 2010). In particular, studies with melanoma cells that acquired resistance to the B-RAF inhibitor SB-590885 showed elevated ERK and IGF-1R/PI3K/AKT activation and combined treatment of these resistant cells with MEK and PI3K inhibitors induced cell death (Villanueva et al., 2010), suggesting that therapies targeting these pathways are viable alternatives. We examined the effect of BI-69A11 on the 451Lu cell line, which are sensitive to B-RAF inhibition, and its B-RAF inhibitor-resistant derivative, 451Lu-R3 (Villanueva et al., 2010). Despite their differential sensitivity to the B-RAF inhibitor, SB-590885 (451Lu: EC₅₀, 670 nM; 451Lu-R3: EC₅₀, 5.97 μ M) (Fig. 4A), both 451Lu and 451Lu-R3 cell lines were sensitive to BI-69A11, with EC₅₀ values of 1.73 μ M and 1.38 μ M, respectively, (Fig. 4B) similar to our results observed with other BI-69A11-sensitive melanoma lines. In addition, combining BI-69A11 with SB-590885 led to additive inhibition of melanoma viability in 451Lu cells, but not 451Lu-R3 cells (Fig. 4C, D). Therefore, our results indicate that the BI-69A11 sensitivity of melanoma cells is unaffected by mechanisms leading to B-RAF inhibitor resistance and suggest that the dual AKT and NF- κ B inhibition achieved by BI-69A11 may be a viable therapeutic strategy in melanoma refractory to B-RAF inhibitors.

Oral administration of BI-69A11 effectively inhibits melanoma xenograft growth

Our previous study had shown that intraperitoneal administration of BI-69A11 inhibited melanoma xenograft growth (Gaitonde et al., 2009). In order to extend these findings, we determined whether BI-69A11 was also active when administered orally. Initial MTD assays showed some evidence of toxicity at 200 mg/kg (data not shown). However, oral administration of BI-69A11 up to 100 mg/kg was well tolerated, with no significant alterations in body weight or blood chemistry compared to PBS or vehicle controls (Supplemental table 2 and Fig. S4). Importantly, liver enzyme function was not compromised, and analysis of liver sections by H & E staining revealed no pathology (Fig. S4). Thus, we next tested the effect of oral administration on the growth of human UACC 903 and SK-MEL-5 melanoma cells as xenografts in nude mice and mouse melanoma SW1 cells in an immune-competent syngeneic tumor model (Bhoulmick et al., 2004). For both UACC 903 and SW1 cell lines, BI-69A11 administration at a dose of 100 mg/kg reduced tumor size by over 2-fold compared to vehicle-treated animals (Fig. 5A). In contrast, the growth of SK-MEL-5 xenografts was unaffected by BI-69A11 (Fig. S5), consistent with the resistance of these cells observed in our cell culture experiments. Using TUNEL staining, UACC 903 and SW1 xenografts from treated animals exhibited higher levels of apoptosis compared to vehicle-treated animals, suggesting that reduced tumor growth in treated animals is due, at least in part, to increased apoptosis (Fig. 5B). In addition, the UACC 903 and SW1 xenografts from BI-69A11-treated animals exhibited higher I κ B expression than vehicle-treated controls, consistent with our observations with NF- κ B pathway inhibition in cultured cells. Therefore, oral administration of BI-69A11 is well tolerated in animals and effectively inhibits melanoma growth in tumor xenografts by causing increased apoptosis, consistent with inhibition of NF- κ B signaling.

Discussion

An emerging recognition in cancer therapy is the need to combine treatment against multiple targets in order to achieve efficient therapeutic index. In melanoma, extensive efforts for

treatment with B-RAF and MEK inhibitors have resulted in initial positive clinical effects (Bollag et al., 2010; Flaherty et al., 2010; Paraiso et al., 2010). Nevertheless, due to the reappearance of resistant tumors under single-agent regimens, it is well recognized that combination with other cellular targets must be accomplished in order to prevent tumor recurrence. Among key signaling pathways that are modified in melanoma, in addition to the MAPK signaling axis are PI3K and NF- κ B (Govindarajan et al., 2007; Robertson, 2005; Ueda and Richmond, 2006). It is estimated that the percentage of melanomas bearing activated AKT is equal, if not greater, to those that harbor activated ERK signaling (Robertson, 2005). Similarly, active NF- κ B signaling is often seen in melanoma and provides an alternative pathway to the AKT signaling axis by which these tumors achieve resistance against apoptosis (Ivanov et al., 2001; Ueda and Richmond, 2006). Furthermore, the importance of NF- κ B signaling in melanoma was recently demonstrated in IKK β knockout mice, which fail to develop melanoma when crossed to the *TetOn-Hras^{v12}*, *Ink4a/Arf^{-/-}* mouse model (Yang et al., 2010). Thus, extensive studies devoted towards the identification of inhibitors to the AKT and NF- κ B pathways represent additional means by which to target melanoma.

Although BI-69A11 was originally selected on the basis of its AKT-inhibitory activity against purified enzyme in vitro and in cell-based assays (Forino et al., 2005; Gaitonde et al., 2009), its ability to elicit a potent anti-tumor response in melanoma xenografts raised the notion that BI-69A11 may have other cellular targets. Indeed, kinase profiling experiments identified IKKs as a target of BI-69A11, prompting an examination of the effects of BI-69A11 on NF- κ B signaling. Importantly, we found that BI-69A11 blocked TNF- α stimulated I κ B phosphorylation/degradation, and NF- κ B transcriptional activity, consistent with IKK inhibition. However, BI-69A11 also inhibited activating phosphorylation of IKK α/β , which implicated components of the pathway upstream of IKKs.

The PI3K/AKT pathway has been shown to activate the NF- κ B pathway, although the underlying mechanism(s) are controversial (Delhase et al., 2000; Dhawan et al., 2002; Ozes et al., 1999). This raised the possibility that the NF- κ B inhibition by BI-69A11 was linked to the previously reported effect of this compound on AKT. However, during TNF- α stimulated NF- κ B activation, neither AKT nor PRAS40 phosphorylation was affected by BI-69A11 (Fig. S6). Furthermore, AKT inhibition by the well-characterized inhibitor GSK690693 did not affect TNF- α stimulated NF- κ B activation in UACC 903 cells. Taken together, our data suggest that BI-69A11-mediated inhibition of the NF- κ B pathway is independent of its effects on AKT and involve other upstream components.

Additional kinase profiling experiments revealed several other kinases (SPHK1, SPHK2, FES, and AURKB) as potential targets of BI-69A11. Among these kinases, we focused our efforts on SPHK1 (sphingosine kinase), due to its requirement for NF- κ B activation (Alvarez et al., 2010), which is consistent with our observations. SPHK1 is an emerging drug target in a wide variety of cancers, and SPHK1 inhibitors have shown efficacy in pre-clinical models (French et al., 2006; Kapitonov et al., 2009; Paugh et al., 2008; Shida et al., 2008). At the time, we chose not to pursue FES, as its expression was reported to be downregulated in melanoma cell lines (Easty and Bennett, 2000). Aurora B (AURKB) also was not pursued because of published data showing low nanomolar sensitivity of SK-MEL-5 and SK-MEL-28 cells to the Aurora B/C inhibitor GSK1070916 (Hardwicke et al., 2009) and our observed resistance of these cell lines to BI-69A11 despite the similar IC₅₀ values of BI-69A11 towards AURKB (1.73 μ M), AKT, and SPHK1. In contrast, addition of exogenous S1P, which we posited would bypass the requirement for SPHK1, was able to rescue the inhibition of NF- κ B transcriptional activity elicited by BI-69A11 and partially abrogated the effect of BI-69A11 on cell viability. Taken together, our data indicate that SPHK1 is a relevant target of BI-69A11 in cells.

Significantly, our results suggest that the anti-tumor efficacy of BI-69A11 is derived from both the inhibition of the NF- κ B pathway and the previously demonstrated inhibition of AKT. Inhibition of AKT alone, as achieved with GSK690693, is insufficient to affect UACC 903 growth in culture. Similarly, restoration of NF- κ B activity, either by constitutive IKK activation or by I κ B knockdown, partially abrogates the effect of BI-69A11. Therefore, targeting these two pathways in melanoma possessing the appropriate cellular context may be an effective strategy to inhibit melanoma growth. Interestingly, we demonstrate that BI-69A11 still retains efficacy in melanoma cells that have acquired resistance to B-RAF inhibitors, suggesting that these alterations do not change sensitivity to AKT or NF- κ B pathway inhibition.

Finally, we extended our previous findings by examining the oral administration of BI-69A11 on the growth of melanoma xenografts. In contrast to intraperitoneal administration of BI-69A11, which showed dose-limiting toxicity at 5 mg/kg (Gaitonde et al., 2009), oral administration of BI-69A11 was well tolerated up to 100 mg/kg, with effective inhibition of UACC 903 melanoma growth in a xenograft model and SW1 melanoma growth in an immune-competent syngeneic model over the treatment period. Consistent with the increased resistance of SK-MEL-5 to BI-69A11 observed in vitro, SK-MEL-5 xenograft growth was not significantly affected by BI-69A11, which we attribute to the activated NF- κ B signaling present in this cell line. Importantly, in the cell lines sensitive to BI-69A11, we observed increased apoptosis in the tumors from treated animals concomitant with increased I κ B levels, both of which are consistent with NF- κ B inhibition. Therefore, oral administration of BI-69A11 appears to be a viable route of delivery that retains anti-tumor efficacy.

Several AKT inhibitors are currently in clinical trials for a variety of tumor types and encompass ATP competitive inhibitors, allosteric inhibitors, and anti-sense oligonucleotide inhibitors (Clinicaltrials.gov). Two orally available ATP-competitive inhibitors, GSK2141795 and GSK2110183 (GlaxoSmithKline), are currently in Phase I and II clinical trials for solid tumors and hematological malignancies. GDC-0068 (Array Biopharma/Genentech) is another orally available ATP-competitive inhibitor currently in Phase I trials for solid tumors. MK-2206 (Merck) is an orally available, allosteric inhibitor that has shown synergism when combined with other chemotherapies in pre-clinical models (Hirai et al., 2010) and is currently in Phase I and II trials for advanced solid tumors and hematologic malignancies. Perifosine/KRX-0401 (KERYX Biopharmaceuticals) is another allosteric inhibitor targeting the AKT PH domain, although Phase II clinical trials in solid tumors with perifosine as a single agent have shown little efficacy (Gills and Dennis, 2009). Finally, RX-0201 (Rexahn), an anti-sense oligonucleotide inhibitor of AKT1, is currently in Phase II trials for metastatic pancreatic cancer in combination with gemcitabine. We are actively pursuing the discovery of analogs of BI-69A11 with improved potency and pharmacological properties towards the goal of identifying potential clinical candidates.

Overall, our results here demonstrate that targeting both the AKT and NF- κ B pathways, as accomplished by BI-69A11, represents an efficient strategy for inhibiting melanoma growth. Importantly, we show that BI-69A11 retains efficacy against melanoma that have acquired resistance to B-RAF inhibitors. Future studies will determine the efficacy of BI-69A11 or its second-generation analogs as an alternative to, or in combination with, B-RAF and MEK inhibitors in melanoma.

Materials and methods

Cell culture

UACC 903, MeWo, WM35, and mouse SW1 melanoma cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Mediatech, Manassas, VA) with 10% fetal bovine serum and maintained at 37°C in 5% CO₂. SK-MEL-5 and SK-MEL-28 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad CA) with 10% fetal bovine serum and maintained at 37°C in 5% CO₂.

Antibodies, reagents, and siRNAs

Antibodies against phospho-IKK α / β (S180/S181), IKK, phospho-I κ B (S32), I κ B, phospho-AKT (S473), ATM, and phospho-Cdc25C (S216) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against I κ B used for IHC were purchased from Biolegend (San Diego, CA). Antibodies against phospho-PRAS40 (T246), PRAS40, phospho-ATM (S1981), and phospho-CHK2 (T68) were obtained from Millipore (Billerica, MA). The AKT antibody was from Invitrogen (Carlsbad, CA). Compound BI-69A11 was synthesized and purified as described (Gaitonde et al., 2009). Human TNF- α was purchased from Cell Signaling Technology (Danvers, MA). GSK690693 and SB-590885 were obtained from SYN|thesis med chem (San Diego, CA). Sphingosine-1-phosphate was purchased from Cayman Chemical (Ann Arbor, MI). siRNAs against I κ B (AGUCAGAGUUCACGGAGUU, GCUGAUGUCAACAGAGUUA) were obtained from Dharmacon/ThermoFisher (Lafayette, CO).

Cell treatment and immunoblotting

To determine the effect of compounds on phospho-AKT, cells were plated at 75% confluency and grown overnight on 60 mm plates. Compounds were added to the plate, incubated for 4 h and harvested for immunoblot analysis. For phospho-IKK/phospho-I κ B experiments, cells were plated as described above and grown overnight. Compounds were added to cells for 1 h prior to stimulation with 20 ng/ml TNF- α and harvested for immunoblotting. For harvest, cells were rinsed with PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 10 μ g/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 μ M microcystin-LR, 2.5 mM sodium orthovanadate). The protein concentration was determined using Bio-Rad protein assay solution. Equal amounts of cell lysate (50 μ g) were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences, Waltham, MA). Membranes were blocked with 3% BSA/TBST for 1 h and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C with shaking. Following three times wash with TBST, membranes were incubated with second antibody for 1 h at room temperature. Bound antibodies were detected with horseradish peroxidase-conjugated protein A (GE Biosciences, Piscataway, NJ) for polyclonal antibodies or horseradish peroxidase-conjugated anti-mouse antibody (GE Biosciences, Piscataway, NJ) for monoclonal antibodies followed by enhanced chemiluminescence using Western Lightning reagent (PerkinElmer Life Sciences, Waltham, MA). For γ -irradiation experiments, cells were exposed to 10 Gy using a Gammacell 40 Exactor research irradiator (MDS Nordion, Ottawa, ON, Canada).

Cell proliferation assays

UACC 903 cells were seeded in 96-well plates at 5,000 cells per well and allowed to attach overnight. Compounds were added to the cells and cell viability was determined with the CellTiter-Blue kit (Promega, Madison WI) or ATPlite kit (PerkinElmer Life Sciences,

Waltham, MA) according to the manufacturer's protocol. EC₅₀ values were determined using Prism (Graphpad Software, San Diego, CA).

Luciferase reporter assay

Ninety percent confluent MeWo cells were transiently transfected with the NF- κ B-dependent firefly luciferase reporter plasmid NF- κ B-Luc and Renilla luciferase plasmid pRL-TK (Kosco et al., 2008) using Lipofectamine2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). After 48 h, cells were plated at 1×10^5 cells/well in 24-well plate and allowed to attach overnight. Cells were then treated with different concentration of BI-69A11 for 1h followed by addition of 20 ng/ml TNF- α . Luciferase assay was performed using the dual luciferase kit (Promega, Madison, WI) according to the manufacturer's protocol after 4 h. Luciferase activity was normalized to Renilla luciferase activity as a control for transfection efficiency.

Tumor studies

All animal studies were conducted in the Animal Facility at Sanford-Burnham Medical Research Institute in accordance with the Institutional Animal Care and Use Committee guidelines. For the UACC 903 and SK-MEL-5 xenograft studies, six to seven-week old female nu/nu mice were purchased from Harlan Laboratories (Indianapolis, IN) and allowed to acclimatize for 1 week. UACC 903 cells (1×10^6 , suspended in 200 μ l sterile PBS) or SK-MEL-5 (1×10^6 , suspended in 200 μ l sterile PBS) were injected into the subcutaneous tissue of the flank. For the SW1 syngeneic tumor studies, six to seven-week old female C3H/HeJ mice were purchased from JAX (Bar Harbor, ME) (Bhoulmick et al., 2004). Mouse SW1 melanoma cells (1×10^6 , suspended in 200 μ l sterile PBS) were injected into the subcutaneous tissue of the flank.

When xenograft size reached a volume of between 10–20 mm³, the mice were sorted into 8 mice per group with the average tumor volumes distributed equally between the groups. Vehicle control (Hot Rod Chemistry Formulation #1, Pharmatek, San Diego, CA), or BI-69A11 at 30 and 100 mg/kg was administered by oral gavage three times a week. The mice were maintained in a pathogen-free environment with free access to food. Body weight and tumor volume were measured twice a week. Tumor size was measured with linear calipers and calculated using the formula: $[(\text{length in millimeters} \times (\text{width in millimeters})^2) / 2]$. The mice were sacrificed after 4 weeks and the tumors were fixed in Z-Fix (Anatech, Battle Creek, MI), and embedded in paraffin for immunohistochemical analysis.

Immunohistochemistry

Immunohistochemical studies were performed on paraffin-embedded tissue sections of tumor extracted at the time of sacrifice. Serial sections (5-mm thick) of paraffin-embedded tissues were fixed on silane-coated glass slides, deparaffinized, and subjected to antigen retrieval (Target Retrieval Solution, Dako, Carpinteria, CA). Following blocking, slides were incubated with the indicated antibodies overnight, washed in PBS, and incubated with the appropriate HRP-conjugated secondary antibody (anti-rabbit polymer-HRP, Dako, Carpinteria, CA). Bound antibodies were visualized with DAB+ Chromogen solution (Dako, Carpinteria, CA) and counterstained with hematoxylin. For the TUNEL assay, tumor sections were stained using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Slides were scanned with the ScanScope XT system (Aperio Technologies, Vista, CA) and quantitation was carried out using ImageScope software (Aperio Technologies, Vista, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Gavin Robertson for providing us with the melanoma cell lines used in this study. We also thank the SBMRI animal facility for technical assistance with the xenograft studies. This study was supported by NIH grants CA114046 (M.H.) and CA128814 (Z.R.).

References

- Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balph. *Curr Biol*. 1997; 7:261–9. [PubMed: 9094314]
- Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY, Maceyka M, Jiang H, Luo C, Kordula T, et al. Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature*. 2010; 465:1084–8. [PubMed: 20577214]
- Bhaskar PT, Hay N. The two TORCs and Akt. *Dev Cell*. 2007; 12:487–502. [PubMed: 17419990]
- Bhoumik A, Gangi L, Ronai Z. Inhibition of melanoma growth and metastasis by ATF2-derived peptides. *Cancer Res*. 2004; 64:8222–30. [PubMed: 15548688]
- Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, Cho H, Spevak W, Zhang C, Zhang Y, Habets G, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature*. 2010; 467:596–9. [PubMed: 20823850]
- Delhase M, Li N, Karin M. Kinase regulation in inflammatory response. *Nature*. 2000; 406:367–8. [PubMed: 10935625]
- Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J*. 1998; 17:2215–23. [PubMed: 9545235]
- Dhawan P, Richmond A. A novel NF-kappa B-inducing kinase-MAPK signaling pathway up-regulates NF-kappa B activity in melanoma cells. *J Biol Chem*. 2002; 277:7920–8. [PubMed: 11773061]
- Dhawan P, Singh AB, Ellis DL, Richmond A. Constitutive activation of Akt/protein kinase B in melanoma leads to up-regulation of nuclear factor-kappaB and tumor progression. *Cancer Res*. 2002; 62:7335–42. [PubMed: 12499277]
- Easty DJ, Bennett DC. Protein tyrosine kinases in malignant melanoma. *Melanoma Res*. 2000; 10:401–11. [PubMed: 11095400]
- Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, O'dwyer PJ, Lee RJ, Grippo JF, Nolop K, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010; 363:809–19. [PubMed: 20818844]
- Forino M, Jung D, Easton JB, Houghton PJ, Pellecchia M. Virtual docking approaches to protein kinase B inhibition. *J Med Chem*. 2005; 48:2278–81. [PubMed: 15801821]
- French KJ, Upson JJ, Keller SN, Zhuang Y, Yun JK, Smith CD. Antitumor activity of sphingosine kinase inhibitors. *J Pharmacol Exp Ther*. 2006; 318:596–603. [PubMed: 16632640]
- Gaitonde S, De SK, Tcherpakov M, Dewing A, Yuan H, Riel-Mehan M, Krajewski S, Robertson G, Pellecchia M, Ronai Z. BI-69A11-mediated inhibition of AKT leads to effective regression of xenograft melanoma. *Pigment Cell Melanoma Res*. 2009; 22:187–95. [PubMed: 19175524]
- Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell*. 2002; 109(Suppl):S81–96. [PubMed: 11983155]
- Gills JJ, Dennis PA. Perifosine: update on a novel Akt inhibitor. *Curr Oncol Rep*. 2009; 11:102–10. [PubMed: 19216841]
- Govindarajan B, Sligh JE, Vincent BJ, Li M, Canter JA, Nickoloff BJ, Rodenburg RJ, Smeitink JA, Oberley L, Zhang Y, et al. Overexpression of Akt converts radial growth melanoma to vertical growth melanoma. *J Clin Invest*. 2007; 117:719–29. [PubMed: 17318262]

- Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS Jr. NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol.* 1999; 19:5785–99. [PubMed: 10409765]
- Hardwicke MA, Oleykowski CA, Plant R, Wang J, Liao Q, Moss K, Newlander K, Adams JL, Dhanak D, Yang J, et al. GSK1070916, a potent Aurora B/C kinase inhibitor with broad antitumor activity in tissue culture cells and human tumor xenograft models. *Mol Cancer Ther.* 2009; 8:1808–17. [PubMed: 19567821]
- Hirai H, Sootome H, Nakatsuru Y, Miyama K, Taguchi S, Tsujioka K, Ueno Y, Hatch H, Majumder PK, Pan BS, et al. MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. *Mol Cancer Ther.* 2010; 9:1956–67. [PubMed: 20571069]
- Ivanov VN, Fodstad O, Ronai Z. Expression of ring finger-deleted TRAF2 sensitizes metastatic melanoma cells to apoptosis via up-regulation of p38, TNFalpha and suppression of NF-kappaB activities. *Oncogene.* 2001; 20:2243–53. [PubMed: 11402319]
- Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA, Emery CM, Stransky N, Cogdill AP, Barretina J, et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature.* 2010; 468:968–72. [PubMed: 21107320]
- Kapitonov D, Allegood JC, Mitchell C, Hait NC, Almenara JA, Adams JK, Zipkin RE, Dent P, Kordula T, Milstien S, et al. Targeting sphingosine kinase 1 inhibits Akt signaling, induces apoptosis, and suppresses growth of human glioblastoma cells and xenografts. *Cancer Res.* 2009; 69:6915–23. [PubMed: 19723667]
- Kosco KA, Cerignoli F, Williams S, Abraham RT, Mustelin T. SKAP55 modulates T cell antigen receptor-induced activation of the Ras-Erk-AP1 pathway by binding RasGRP1. *Mol Immunol.* 2008; 45:510–22. [PubMed: 17658605]
- Lee JT, Li L, Brafford PA, Van Den Eijnden M, Halloran MB, Sproesser K, Haass NK, Smalley KS, Tsai J, Bollag G, et al. PLX4032, a potent inhibitor of the B-Raf V600E oncogene, selectively inhibits V600E-positive melanomas. *Pigment Cell Melanoma Res.* 2010; 23:820–7. [PubMed: 20973932]
- Madhunapantula SV, Robertson GP. The PTEN-AKT3 signaling cascade as a therapeutic target in melanoma. *Pigment Cell Melanoma Res.* 2009; 22:400–19. [PubMed: 19493313]
- Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J. NF-kappaB signals induce the expression of c-FLIP. *Mol Cell Biol.* 2001; 21:5299–305. [PubMed: 11463813]
- Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, Chen Z, Lee MK, Attar N, Sazegar H, et al. Melanomas acquire resistance to B-Raf(V600E) inhibition by RTK or N-RAS upregulation. *Nature.* 2010; 468:973–7. [PubMed: 21107323]
- Norris JL, Baldwin AS Jr. Oncogenic Ras enhances NF-kappaB transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways. *J Biol Chem.* 1999; 274:13841–6. [PubMed: 10318790]
- Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature.* 1999; 401:82–5. [PubMed: 10485710]
- Paraiso KH, Fedorenko IV, Cantini LP, Munko AC, Hall M, Sondak VK, Messina JL, Flaherty KT, Smalley KS. Recovery of phospho-ERK activity allows melanoma cells to escape from BRAF inhibitor therapy. *Br J Cancer.* 2010; 102:1724–30. [PubMed: 20531415]
- Paugh SW, Paugh BS, Rahmani M, Kapitonov D, Almenara JA, Kordula T, Milstien S, Adams JK, Zipkin RE, Grant S, et al. A selective sphingosine kinase 1 inhibitor integrates multiple molecular therapeutic targets in human leukemia. *Blood.* 2008; 112:1382–91. [PubMed: 18511810]
- Plas DR, Thompson CB. Akt-dependent transformation: there is more to growth than just surviving. *Oncogene.* 2005; 24:7435–42. [PubMed: 16288290]
- Qi J, Nakayama K, Gaitonde S, Goydos JS, Krajewski S, Eroshkin A, Bar-Sagi D, Bowtell D, Ronai Z. The ubiquitin ligase Siah2 regulates tumorigenesis and metastasis by HIF-dependent and -independent pathways. *Proc Natl Acad Sci U S A.* 2008; 105:16713–8. [PubMed: 18946040]

- Ravi R, Bedi GC, Engstrom LW, Zeng Q, Mookerjee B, Gelinas C, Fuchs EJ, Bedi A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-kappaB. *Nat Cell Biol.* 2001; 3:409–16. [PubMed: 11283615]
- Reinhardt HC, Yaffe MB. Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2. *Curr Opin Cell Biol.* 2009; 21:245–55. [PubMed: 19230643]
- Rhodes N, Heerding DA, Duckett DR, Eberwein DJ, Knick VB, Lansing TJ, McConnell RT, Gilmer TM, Zhang SY, Robell K, et al. Characterization of an Akt kinase inhibitor with potent pharmacodynamic and antitumor activity. *Cancer Res.* 2008; 68:2366–74. [PubMed: 18381444]
- Robertson GP. Functional and therapeutic significance of Akt deregulation in malignant melanoma. *Cancer Metastasis Rev.* 2005; 24:273–85. [PubMed: 15986137]
- Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science.* 2005; 307:1098–101. [PubMed: 15718470]
- Sekulic A, Haluska P Jr, Miller AJ, Genebriera De Lamo J, Ejadi S, Pulido JS, Salomao DR, Thorland EC, Vile RG, Swanson DL, et al. Malignant melanoma in the 21st century: the emerging molecular landscape. *Mayo Clin Proc.* 2008; 83:825–46. [PubMed: 18613999]
- Shida D, Takabe K, Kapitonov D, Milstien S, Spiegel S. Targeting SphK1 as a new strategy against cancer. *Curr Drug Targets.* 2008; 9:662–73. [PubMed: 18691013]
- Solit D, Rosen N. Oncogenic RAF: a brief history of time. *Pigment Cell Melanoma Res.* 2010; 23:760–2. [PubMed: 20973931]
- Ueda Y, Richmond A. NF-kappaB activation in melanoma. *Pigment Cell Res.* 2006; 19:112–24. [PubMed: 16524427]
- Villanueva J, Vultur A, Lee JT, Somasundaram R, Fukunaga-Kalabis M, Cipolla AK, Wubbenhorst B, Xu X, Gimotty PA, Kee D, et al. Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell.* 2010; 18:683–95. [PubMed: 21156289]
- Yang J, Richmond A. Constitutive IkappaB kinase activity correlates with nuclear factor-kappaB activation in human melanoma cells. *Cancer Res.* 2001; 61:4901–9. [PubMed: 11406569]
- Yang J, Splittgerber R, Yull FE, Kantrow S, Ayers GD, Karin M, Richmond A. Conditional ablation of Ikkb inhibits melanoma tumor development in mice. *J Clin Invest.* 2010; 120:2563–74. [PubMed: 20530876]

Fig. 1. Inhibition of the NF- κ B pathway by BI-69A11

A, UACC 903 cells were incubated with either vehicle (DMSO) or BI-69A11 (10 μ M) for 1 h prior to TNF- α stimulation. Cells were harvested at the indicated time points and whole cell lysates were immunoblotted with the indicated antibodies. **B**, UACC 903, MeWo, or SW1 cells were incubated with either vehicle (–, DMSO) or the indicated BI-69A11 concentrations for 1 h prior to TNF- α stimulation. Cells were harvested at 5 minutes following stimulation and whole cell lysates were immunoblotted with the indicated antibodies. **C**, MeWo cells were transfected with a NF- κ B firefly luciferase reporter construct along with Renilla luciferase. 72 h post-transfection, cells were treated with the indicated concentrations of BI-69A11 for 1 h, stimulated with TNF- α for 4 h as indicated, and assayed for luciferase activity. Bars represent average normalized fold induction $\pm$ S.D.

Fig. 2. Both AKT and NF- κ B inhibition are required for growth inhibition by BI-69A11

A, UACC 903 cells were treated with vehicle (–, DMSO) or the indicated concentrations of GSK690693 for 4 h. Whole cell lysates were immunoblotted with the indicated antibodies. **B**, UACC 903 cells were treated with vehicle (–, DMSO) or the indicated concentrations of GSK690693 for 1 h, prior to TNF- α stimulation for 5 min. Whole cell lysates were immunoblotted with the indicated antibodies. **C**, UACC 903 cells were incubated for 72 h with the indicated concentrations of BI-69A11 (open circles) or GSK690693 (filled squares) and cell viability was assessed by CellTiter Blue assay. EC₅₀ values for BI-69A11 and GSK690693 were 1.8 μM and 83 μM , respectively, as calculated from the non-linear curve fit (Prism). Error bars, S.D. **D**, WM35 cells were transfected with non-targeting or I κ B-targeting siRNAs in triplicate. 48 h post-transfection, cells were treated with vehicle or 10 μM BI-69A11 and cell viability was assessed by CellTiter Blue 24 h post-treatment. Error bars, S.D.; t-test: *, $p < 0.05$; ***, $p < 0.001$. **E**, UACC 903, SK-MEL-5, and SK-MEL-28 cells were incubated for 72 h with the indicated concentrations of BI-69A11 and cell viability was assessed by CellTiter Blue assay. EC₅₀ values for BI-69A11 in UACC 903, SK-MEL-5, SK-MEL-28 cells were 1.1 μM , 5 μM , and 11.6 μM , respectively, as calculated from the non-linear curve fit (Prism). Error bars, S.D. **F**, SK-MEL-5 or UACC 903 cells

were incubated with either vehicle (–, DMSO) or the indicated BI-69A11 concentrations for 1 h prior to TNF- α stimulation. Cells were harvested at 5 minutes following stimulation and whole cell lysates were immunoblotted with the indicated antibodies

Fig. 3. BI-69A11 inhibits SPHK1

A, In vitro inhibition data from Invitrogen SelectScreen for BI-69A11 towards SPHK1. Purified SPHK1 was incubated with the indicated concentrations of BI-69A11 and assayed for kinase activity. Error bars, S.D. **B**, UACC 903 cells incubated in DMEM + 0.5% FBS were treated with the indicated concentrations of sphingosine-1-phosphate (S1P) for 1 h prior to addition of the indicated concentrations of BI-69A11. Cell viability was assessed by CellTiter Blue at 4 h. **C**, MeWo cells were transfected with a NF- κ B firefly luciferase reporter construct along with Renilla luciferase. 72 h post-transfection, cells were treated with vehicle (DMSO), 2 μ M BI-69A11 or 5 μ M S1P with 2 μ M BI-69A11, stimulated with TNF- α for 4 h as indicated, and assayed for luciferase activity. Bars represent average normalized fold induction $\pm$ S.D.

Fig. 4. Melanoma cells resistant to the B-RAF inhibitor SB-590885 are sensitive to BI-69A11
A, 451Lu (filled squares, B-RAF inhibitor sensitive) and 451Lu-R3 (open circles, B-RAF inhibitor resistant) were incubated for 72 h with the indicated concentrations of SB-590885 and cell viability was assessed by ATPlite assay. EC₅₀ values for 451Lu and 451Lu-R3 were 670 nM and 5.97 μM, respectively, as calculated from the non-linear curve fit (Prism). **B**, 451Lu (filled squares) and 451Lu-R3 (open circles) were incubated for 72 h with the indicated concentrations of BI-69A11 and cell viability was assessed by ATPlite assay. EC₅₀ values for 451Lu and 451Lu-R3 were 1.7 μM and 1.4 μM, respectively, as calculated from the non-linear curve fit (Prism). **C**, 451Lu cells were incubated for 72 h with the indicated BI-69A11 concentrations alone (filled squares), combined with 0.1 μM SB-590885 (open circles), or combined with 1.0 μM SB-590885 (filled triangles) and cell viability was assessed by ATPlite assay. EC₅₀ values for BI-69A11 were 1.7 μM (alone), 1.1 μM (+ 0.1 μM SB-590885), and 990 nM (+ 1.0 μM SB-590885), respectively. **D**, 451Lu-R3 cells were incubated for 72 h with the indicated BI-69A11 concentrations alone (filled squares), combined with 0.1 μM SB-590885 (open circles), or combined with 1.0 μM SB-590885 (filled triangles) and cell viability was assessed by ATPlite assay. EC₅₀ values for BI-69A11 were 1.4 μM (alone), 1.4 μM (+ 0.1 μM SB-590885), and 1.2 μM (+ 1.0 μM SB-590885), respectively. Error bars, S.D. for all panels.

Fig. 5. Oral administration of BI-69A11 inhibits melanoma tumor growth

A, human UACC 903 melanoma xenografts were implanted subcutaneously into the flank of nude mice or mouse SW1 melanoma cells were implanted into the syngeneic host C3H/HeJ. Starting at a tumor size of 10 mm³, vehicle or the indicated concentrations of BI-69A11 were administered by oral gavage 3 times per week. Tumor size was measured by calipers at the indicated time points. Error bars, S.E.M. **B**, TUNEL and IκB staining of tumor sections from vehicle and BI-69A11-treated UACC903 or SW1 xenograft mice after 28 d. Representative pictures for TUNEL (10x) and IκB staining (20x) are shown. TUNEL and IκB staining were quantitated by ImageScope software and represented as TUNEL positive nuclei/area and IκB immunoscore/area. Error bars, S.E.M.