SBI-0640756 Attenuates the Growth of Clinically Unresponsive Melanomas by Disrupting the eIF4F Translation Initiation Complex

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Abstract

Disrupting the eukaryotic translation initiation factor 4F (eIF4F) complex offers an appealing strategy to potentiate the effectiveness of existing cancer therapies and to overcome resistance to drugs such as BRAF inhibitors (BRAFi). Here, we identified and characterized the small molecule SBI-0640756 (SBI-756), a first-in-class inhibitor that targets eIF4G1 and disrupts the eIF4F complex. SBI-756 impaired the eIF4F complex assembly independently of mTOR and attenuated growth of BRAF-resistant and BRAF-independent melanomas. SBI-756 also suppressed AKT and NF-κB signaling, but small-molecule derivatives were identified that only marginally affected these pathways while still inhibiting eIF4F complex formation and melanoma growth, illustrating the potential for further structural and functional manipulation of SBI-756 as a drug lead. In the gene expression signature patterns elicited by SBI-756, DNA damage, and cell-cycle regulatory factors were prominent, with mutations in melanoma cells affecting these pathways conferring drug resistance. SBI-756 inhibited the growth of NRAS, BRAF, and NF1-mutant melanomas in vitro and delayed the onset and reduced the incidence of Nras/Ink4a melanomas in vivo. Furthermore, combining SBI-756 and a BRAFi attenuated the formation of BRAF-resistant human tumors. Taken together, our findings show how SBI-756 abrogates the growth of BRAF-independent and BRAF-resistant melanomas, offering a preclinical rationale to evaluate its antitumor effects in other cancers.

Introduction

The emergence of effective inhibitors for BRAF-mutant melanoma has had major impact on the clinical management of melanoma (1). However, the initial success of such treatments has been limited due to the propensity of melanomas to develop resistance (2). In most cases, mechanisms underlying BRAF inhibitor (BRAFi) resistance include activation of genetic or epigenetic pathways that circumvent targeted BRAF and restore MAPK and related signaling to levels sufficient to fuel tumorigenesis (2). This outcome has led to development of combination therapies targeting both BRAF and associated pathways, such as MEK and PI3K (3), albeit, with limited success. Furthermore, 50% of melanomas, such as those harboring NRAS and NF1 mutations, lack BRAF mutations, and are thus not amenable to BRAFi therapy (4). Thus, tumor chemoresistance and the lack of therapies for BRAF wild-type (WT) tumors remains a major clinical challenge.

We identified BI-69A11, which inhibits both AKT and NF-κB signaling (5) and attenuates melanoma development and progression in both human xenografts and mouse genetic models (6–8). In efforts to improve the biophysical properties of BI-69A11, we identified SBI-0640756 (SBI-756), which retained the biologic effects of the original compound while possessing superior pharmacokinetics. Extended characterization of SBI-756 identified eIF4G1 as its direct target. eIF4G1 is a large scaffolding protein that is a key component of the eukaryotic translation initiation factor 4F (eIF4F) complex (9). Small translational repressors, eIF4E-binding proteins (4E-BP), associate with eIF4E, and impair its binding to eIF4G and the eIF4F complex assembly (10). mTORC1-mediated phosphorylation of 4E-BPs leads to their dissociation from eIF4F, enabling eIF4E interaction with eIF4G and the formation of the eIF4F complex (10). Although required for cap-dependent translation of all nuclear-encoded mRNAs, increased eIF4F levels stimulate translation of mRNAs encoding cancer-promoting proteins while having only a marginal effect on translation of house-keeping mRNAs (11). Correspondingly, elevated eIF4F activity has been linked to resistance to BRAF- and MEK-targeted therapies (12). SBI-756 targeting of the eIF4G1 disrupts the eIF4F complex assembly, even in BRAF-resistant melanomas.
melanoma. Correspondingly, SBI-756 attenuates resistance to BRAFi and inhibits NRAS- and NFI-mutant melanomas.

**Materials and Methods**

**Western blot analysis and antibodies**

Cells were rinsed with PBS and lysed as previously described (8). Protein concentration was determined using Coomassie Plus Protein Assay Reagent (Thermo Scientific). Equal amounts of cell lysate proteins (50 μg) were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences). Membranes were blocked (5% BSA/TBST, 1 hour) and incubated with primary antibodies (1 hour at room temperature or overnight at 4°C), with shaking. Following three TBST washes, membranes were incubated for 1 hour at room temperature secondary antibodies (1:10,000). Detection and quantifications were made using Odyssey Infrared Imaging System (LiCor Biosciences), or by exposing them to X-ray film. Antibodies against p-AKT, p-PRAS40, p-IKK, p-eIF4E, p-mTOR, p-p70S6K, p-pS6, p-4E-BP1, p-SGK3, AKT, PRAS40, and tubulin were obtained from Santa Cruz Biotechnology. Secondary antibodies were goat anti-rabbit Alexa-680 F(ab′)2 (Molecular Probes) and goat anti-mouse IRDye 800 F(ab′)2 (Rockland Immunocchemicals). All antibodies were used according to the suppliers’ recommendations.

**Cell culture**

Melanoma lines were obtained from the Wistar Institute, Yale University, TGen, NCI, and ATCC and maintained in high-glucose Dulbecco modified Eagle medium (HyClone) with 5% FBS and 1% penicillin–streptomycin at 37°C in 5% CO2. E1A/RAS transformed WT and 4E-BP double knockout (DKO) mouse embryonic fibroblasts (MEF) were described previously (1). For cell line authentication, short tandem repeat (STR) analysis was performed on isolated genomic DNA with the GenePrint 10 System from Promega, and peaks were analyzed using GeneMarker HID from Softgenetics. Allele calls were searched against STR databases maintained by ATCC (www.atcc.org), DSMZ (www.dsmz.de), Texas Tech University Children’s Oncology Group (cogcell.org), and the Wistar Institute Melanoma Cell STR Profiles (http://www.wistar.org/lab/meenhard-herlyn-dvm-dsc/page/melanoma-cell-str-profiles). Authentication was last performed on August 24, 2015.

**m7GTP pull-down assay**

As previously described (13), cells growing in 100 mm plates were washed (cold PBS), collected, and lysed in 50 mM/L MOPS/KOH (7.4), 100 mM/L NaCl, 50 mM/L NaF, 2 mM/L EDTA, 2 mM/L EGTA, 1% NP40, 1% Na-DOC, 7 mM/L β-mercaptoethanol, protease inhibitors, and phosphate inhibitor cocktail (Roche). Lysates were incubated with m7GDP-agarose beads (Jena Bioscience; 20 minutes), washed (4 times) with 50 mM/L MOPS/KOH (7.4), 100 mM/L NaCl, 50 mM/L NaF, 0.5 mM/L EDTA, 0.5 mM/L EGTA, 7 mM/L β-mercaptoethanol, 0.5 mM/L/PMSE, 1 mM/L NaVO4 and 0.1 mM/L/GTP. Bound proteins were eluted by boiling the beads in loading buffer. m7GDP-agarose pulled down material was analyzed by Western blot analysis.

**Results and Discussion**

To improve the biophysical properties of BI-69A11, we designed and synthesized over 60 BI-69A11 analogues (Fig. 1A). Following iterative structure–activity relationship (SAR), we selected four analogues with improved pharmacokinetics (Supplementary Table S1; Fig. 1A). Of those, SBI-756 and SBI-726 exhibited superior properties (60× improved aqueous solubility and up to 100× improved permeability), and a favorable pharmacokinetic profile, while not exerting toxicity (Supplementary Tables S1 and S2; Supplementary Fig. S1A and S1B).

Examination of four human melanoma lines revealed that SBI-756 and SBI-726 were comparable with BI-69A11 in their anti-proliferative effects (Fig. 1B), and inhibition of Akt and NFκB activity (Fig. 1C). SBI-756 elicited comparable toxicity in melanoma cells and melanocytes, but was less toxic against nontransformed fibroblasts (Supplementary Fig. S1C). SBI-756 and SBI-726 were more effective than BI-69A11 in attenuating colony formation by BRAFi- and NRAS-mutant melanoma cells (Fig. 1D).

On the basis of its overall properties (in vivo and in vitro), we selected to further characterize SBI-756.

To identify proteins that interact and may serve as direct targets for SBI-756 we performed gas chromatography/lipid mass spectrometry (GC/MS-MS) using biotinylated BI-69A11. Of the 74 proteins that bound specifically (outcompeted using 10× excess of soluble BI-69A11) was eIF4G1 (Supplementary Table S3). We thus set to determine whether the eIF4F complex is indeed disrupted by SBI-756, and further, whether the effect of SBI-756 on AKT, NFκB, and mTOR is dispensable for its effect on eIF4F as well as for suppression of melanoma growth.

We next determine whether SBI-756 dissociates eIF4G1 from the eIF4F complex using m7GTP-agarose pull-down, which captures the eIF4F complex. SBI-756 effectively dissociated eIF4G1 from the eIF4E in a dose-dependent manner, which was accompanied by a concomitant increase in 4E-BP1:eIF4E binding (Fig. 2A), reflective of impaired eIF4F complex formation. Inhibition of the eIF4F complex was also confirmed for the parent compound BI-69A11, although SBI-756 was more potent (Supplementary Fig. S2A).

SBI-756 also inhibits the AKT/mTORC1 signaling and mTORC1 inhibition disrupts the eIF4F complex via activation of 4E-BPs (10). To determine whether SBI-756 impedes eIF4F assembly directly or via mTORC1, we employed 4E-BP1/2 DKO MEFs, wherein mTOR inhibition does not impair the eIF4F assembly (13). Whereas torin1 induced dissociation of eIF4G1 from eIF4E in WT but not in 4E-BP2 DKO MEFs, SBI-756 reduced eIF4G1:eIF4E association in both WT and 4E-BP DKO MEFs (Fig. 2B). Likewise, SBI-756, but not torin1, attenuated the proliferation of E1A/RAS-transformed 4E-BP DKO MEFs (Fig. 2C). These results substantiate that the eIF4F complex on the eIF4F complex assembly is largely mTOR-independent.

As levels of the eIF4F complex inversely correlate with the effectiveness of various cancer therapies (12, 14), we assessed the integrity of the eIF4F complex after treating melanoma cells with a combination of BRAFi vemurafenib (aka PLX4032) and SBI-756. Comparing A375 melanoma cultures that are sensitive to BRAFi vemurafenib (PLX4032) and resistant derivatives (A375R), BRAFi slightly reduced eIF4G1 association with eIF4E, whereas SBI-756 had a more robust effect (Fig. 2D).

Significantly, SBI-756, but not BRAFi, promoted a dose-dependent dissociation of eIF4G1 from eIF4E in A375R (Fig. 2D), Lu1205R and WM7593R cells (Supplementary Fig. S2B). Further-
Figure 1.
Development and characterization of SBI-756. A, more than 60 analogues were synthesized targeting numerous BI-69A11 regions including the linker (red), aryl groups (blue and green), and benzimidazole ring (gray). Top analogues are shown. B, mutant BRAF (Lu1205) or mutant NRAS (WM1346) melanoma lines were plated (triplicates 384-well plates; 1,500 cells per well) and cell viability was assessed 48 hours after treatment with indicated compounds. Growth inhibition is calculated as percentage of DMSO-treated controls and is plotted against the log drug concentration. C, UACC903 cells were treated with DMSO or indicated concentrations of BI-69A11 analogues for 24 hours and whole-cell lysates were immunoblotted with indicated antibodies. D, indicated cultures were plated at low density (500 cells/well in 6-well plates) and grown in medium containing indicated compounds. The number of colonies formed after 10 days in culture was determined by crystal violet staining.
more, a SBI-756/BRAFi combination decreased the amount of eIF4G1 bound to eIF4E, which was not seen following BRAFi treatment alone (Supplementary Fig. S2C and S2D). While consistent with a recent study reporting that compounds that target eIF4E (such as 4EGI-1) or eIF4A (such as rocaglate derivatives) synergize with BRAFi to inhibit proliferation of BRAFi-resistant cells (12), our findings demonstrate the effectiveness of SBI-756 in disrupting the eIF4F complex by targeting the eIF4G1.

To identify signaling pathways affected by SBI-756, we performed reverse-phase protein array analysis of BRAF- and NRAS-mutant melanomas (Supplementary Table S4). Unsupervised clustering of proteins that significantly ($P < 0.05$) changed expression/phosphorylation levels with SBI-756 treatment for 24 hours demonstrated a consistent inhibitory effect in both cell lines on mTOR signaling, and multiple translation initiation regulators, reflected by markedly decreased phosphorylation of S6, mTOR,
P70S6K as well as TSC2 (Supplementary Fig. S3A; Supplementary Table S4). Of interest, SBI-756 did not affect MAPK or JAK–STAT signaling pathways. Western blot analysis confirmed the dose-dependent inhibitory effects of SBI-756 on these proteins and a decrease in 4E-BP1 phosphorylation in UACC903 and A375 cell lines (Supplementary Fig. S3B).

The effect of SBI-756 on mTOR, AKT, and NF-kB, led us to determine whether modification of SBI-756 could reduce its effect on these signaling pathways while retaining its effect on the eIF4F complex. Among SBI-756 derivatives, at least one (SBI-755199) was found to be as effective in inducing melanoma cell death (Fig. 3A) while exhibiting reduced inhibition of AKT, TSC2, PRS6, and NFkB activity (Fig. 3B). Notably, SBI-755199 retained its effective inhibition of mRNA translation (Fig. 3C). The latter was performed using a bicistronic construct, which enables measuring eIF4F complex underpin its biologic activity thereby providing translation. These data substantiate that the effects of SBI-756 on the eIF4F complex underpin its biologic activity thereby providing the basis for the SAR-based screen of SBI-756 analogues.

Dose–response analysis of SBI-756 in 21 melanoma lines identified two groups representing respective SBI-756–sensitive and -resistant lines (>2-fold expression difference in IC_{50} between groups; Fig. 3D). Evaluation of gene expression data from these cell lines enabled mapping differentially expressed genes (DEG) for each group. In total, we detected 1,533 significant DEGs between sensitive and the more resistant cells (P < 0.05; fold change > 1.5). Analysis of gene enrichment within canonical pathways (Supplementary Table S5) identified higher expression levels of genes involved in DNA damage repair (Supplementary Fig. S5B; Supplementary Table S6). Among these genes exhibiting lower expression in resistant lines were key cell-cycle regulatory proteins, including, CDKN1A, CDKN2A, and RB1 (P < 3.0 × 10^{-14}).

As genes conferring drug resistance can often reveal pathways that underlie the drug response, we established multiple SBI-756–resistant clones from UACC903 and UACC3629 cultures. Exome sequencing of individual clones identified a total of 587 protein-coding gene variants in 550 genes that were not detected in the SBI-756–sensitive parental cultures (Supplementary Fig. S3C; Supplementary Table S6). Of these somatic mutations, 81.1% (n = 517) were missense or nonsense single nucleotide variants, with the remaining 11.9% (n = 70) consisting of splicing or frameshift mutations that alter reading frames. IPA analysis indicated enrichment of numerous proteins (n = 174) implicated in melanoma (P = 3.8 × 10^{-13}). Among genes with mutations identified in multiple SBI-756–resistant clone, are those implicated in fatty-acid beta oxidation (P = 0.0008) and DNA damage response (P = 0.004) and cell-cycle checkpoint control (P = 0.01; Supplementary Table S7). These pathways were previously described to be affected upon inhibition of the eIF4E pathway at the level of translation (15–17). Consistent with these observations, a clear G2/M transition was identified following SBI-756 treatment in parental (sensitive) but not in the SBI-756–resistant melanoma cells (Supplementary Fig. S3D).

To confirm the effect of SBI-756 on the eIF4F complex in BRAFi-resistant cultures on neoplastic growth, we assessed SBI-756 effectiveness on their two-dimensional (2D) growth in vitro (with BRAFi–PLX4032) and on tumorigenesis in vivo (with BRAFi–PLX4720). Growth in 2D and colony-forming efficiency (CFE) were effectively attenuated in both parental (sensitive) and resistant cultures, with NF1-mutant melanoma lines exhibiting equal or greater sensitivity (Fig. 4A and Supplementary Fig. S4A and S4B). Effectiveness of SBI-756 in NF1-mutant melanoma was further confirmed in primary cultures (Supplementary Fig. S4C).

In vivo we first evaluated SBI-756 using an inducible NraG_{Q61K}/\text{Ink}^{b/\text{d}} genetic model in which melanoma tumors emerge within 16 to 20 weeks (Fig. 4B). Administration of SBI-756 only, starting 11 weeks after genetic inactivation of \text{Ink}^{b/\text{d}} and induction of NraG_{Q61K} (about 10–14 days prior to tumor appearance), delayed tumor onset (from 20–26 weeks), and reduced tumor incidence, by 50%, compared with the control nontreated group (Fig. 4B). No signs of toxicity were identified during and after administration of SBI-756 (21-week period), consistent with earlier studies with BI-69A11 (17). Given its effectiveness as a single agent, one would expect that combination with MAPK inhibitors could offer novel therapeutic modalities for Nras-mutant tumors.

In vivo, we monitored growth of A375 tumors in immunodeficient mice subjected to either BRAFi alone or BRAFi combined with SBI-756. Notably, SBI-756 did not elicit toxicity in mice, which was monitored by liver function and body weight (Supplementary Table S2, Supplementary Fig. S1B). Growth of established tumors (~250 mm^{3}) was largely inhibited by treatment with either BRAFi alone or a combination of BRAFi plus SBI-756 (Fig. 4C). However, as seen in human melanoma tumors, in the BRAFi-treated group (3/5) resumed growth whereas no tumors were seen in mice (0/4) treated with the drug combination (Fig. 4C), suggesting that combining SBI-756 with BRAFi antagonizes BRAFi-resistant melanoma in vivo. When we allowed tumors to reach 500 mm^{3} before initiating treatment, 5 of 7 (75%) mice subjected to BRAFi treatment alone relapsed as drug-resistant tumors (4/5 within 4–6 weeks), whereas only 3 of 6 (50%) mice subjected to combination treatment developed resistance, albeit more slowly (2/3 after 8 weeks; Supplementary Fig. S4D). Notably, assessing SBI-756 effect on the eIF4F complex in vivo revealed a time-dependent disruption of the eIF4F complex in melanoma tumors grown in animals that were subjected to treatment with both BRAFi and SBI-756 (up to 8 hours, consistent with the half-life of SBI-756; Fig. 4D). These results demonstrate the disruption of the eIF4F complex in vivo, consistent with the effectiveness of SBI-756 in overcoming BRAFi-resistant phenotype.
Figure 3.
Characterization of SBI-756 analogues. A–C, SBI-756 and indicated analogues were assessed for their effect on growth of UACC903 melanoma cells 24 hours after their addition (A); AKT, TSC2, PRS6, and NFkB signaling pathway 4 hours after their addition (B); inhibition of translation in vitro using the bicistronic Renilla/HCV-IRES firefly luciferase constructs (C). D, twenty-one melanoma lines were treated with serial 2-fold dilutions of SBI-756, yielding final drug concentration ranges of 100 μmol/L to 0.2 nmol/L, and cell viability was assessed using CellTiter Glo after 72 hours. Cells were subgrouped according to IC50 values that identified seven most resistant and seven most sensitive cell lines.
in the resistant melanoma, while SBI-756 retains its effectiveness, underlies the novelty and importance of eIF4G1 targeting. Significantly, the low dose and the lack of in vivo toxicity observed for SBI-756 makes it an even more attractive for further evaluation.

Genetic support for the significance of the eIF4F complex in cancer was provided through the characterization of the haploinsufficient elf4e mice, which were found to be more resistant to tumor development (18). Correspondingly, efficacy of a plethora of inhibitors, including PI3K, mTOR, HER2, and MAPK, is limited
in cells that exhibit high eif4E–4E-BP ratio and correlates with their inability to disrupt the eIF4F complex (14). Moreover, recent findings show that the efficacy of 4EGI-1, which is thought to directly target eIF4E:eIF4G association is likely to be predeter-


data (provided animals, acquired and managed patients, Development of methodology: 


cined by the eIF4E-4E-BP ratio. As SBI-756 decreases eIF4F levels independently of mTOR and cellular eIF4E–4E-BP ratio, its combination with currently available inhibitors is an appealing ther-


terapy that allows targeting the eIF4F complex inde-


dependently of mTOR, eIF4E, or 4E-BP status in the cell.

Disclosure of Potential Conflicts of Interest

M.A. Davies reports receiving commercial research grants from Astrazeneca, GSK, Merck, Oncotheryne, Roche/Genentech, and Sanofi-Aventis. He is also a consultant/advisory board member for GSK, Novartis, Roche/Genentech, Sanofi-Aventis, and Vaccinex. No potential conflicts of interest were disclosed by the other authors.

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