IL2 Inducible T-cell Kinase, a Novel Therapeutic Target in Melanoma


Abstract

Purpose: IL2 inducible T-cell kinase (ITK) promoter CpG sites are demethylated in melanomas compared with nevi. The expression of ITK in melanomas, however, has not been established and requires elucidation.

Experimental Design: An ITK-specific monoclonal antibody was used to probe sections from deidentified, formalin-fixed paraffin-embedded tumor blocks or cell line arrays and ITK was visualized by IHC. Levels of ITK protein differed among melanoma cell lines and representative lines were transduced with four different lentiviral constructs that each contained an shRNA designed to knockdown ITK mRNA levels. The effects of the selective ITK inhibitor BI 10N on cell lines and mouse models were also determined.

Results: ITK protein expression increased with nevus to metastatic melanoma progression. In melanoma cell lines, genetic or pharmacologic inhibition of ITK decreased proliferation and migration and increased the percentage of cells in the G0–G1 phase. Treatment of melanoma-bearing mice with BI 10N reduced growth of ITK-expressing xenografts with BI 10N reduced growth of ITK-expressing xenografts.

Conclusions: We conclude that ITK, formerly considered an immune cell–specific protein, is aberrantly expressed in melanoma and promotes tumor development and progression. Our finding that ITK is aberrantly expressed in metastatic melanomas suggests that inhibitors of ITK may be efficacious for melanoma treatment. The efficacy of a small-molecule ITK inhibitor in the Tyr-Cre/Ptennull/BrafV600E mouse melanoma model supports this possibility.

Introduction

Advances in understanding the genetic aberrations associated with melanoma initiation and progression have led to the recent U.S. Food and Drug Administration approval of three small-molecule inhibitors against components in the BRAF–MEK pathway (1–3). However, these systemic therapies rarely lead to melanoma cures, even if used in combination (4). Next-generation sequencing analyses of melanoma specimens identified a handful of genetic aberrations that may have “driver” roles in melanoma development and progression but some distinct melanoma subtypes bear none of the identified “driver” genetic aberrations (5). Current immunotherapies have admittedly led to cures, albeit in a small group of patients (6–8). Although early clinical trials testing combinations of antibodies against immune checkpoint proteins have shown great promise, the toxicities and long-term efficacy are unknown and under investigation (9).

Using DNA-methylation profiling to discriminate primary cutaneous melanomas from benign moles, we determined that the promoter for IL2 inducible T-cell kinase (ITK) was significantly hypomethylated in primary melanomas compared with nevi (10). This finding was unexpected because the expression of ITK, a member of the TEC family of tyrosine kinases that includes the Bruton’s tyrosine kinase (BTK), is normally restricted to distinct immune cell subsets. In T cells, ITK functions downstream of the T-cell receptor and is important for T-cell activation, development, differentiation, and production of many proinflammatory cytokines (11). Given previous reports about the potential for lineage reprogramming of melanoma cells with acquisition of neural or angiogenic properties (12, 13), we hypothesized that...
Tissue procurement

Human tissues analyzed (Supplementary Table S1) were obtained as deidentified, formalin-fixed paraffin-embedded tumor blocks from the University of North Carolina (UNC; Chapel Hill, NC) Health Care archives under Institutional Review Board (IRB)-approved protocols. Tissue sections and tissue microarray construction

Four micron-thick whole tissue sections (WTS) of nevi (n = 30) and primary invasive melanomas (n = 20) were used for tumor tissue analyses. Tissue microarrays (TMA) of metastatic melanomas (n = 82) containing triplicate cores (0.6 mm) from each specimen were also constructed. A pathologist (P.A. Groben) reviewed hematoxylin and eosin (H&E; hematoxylin 7211, eosin 7111, Richard-Allan)-stained tissue sections to confirm the specimens’ diagnoses and marked representative tumor areas on the H&E slides for cores to be included in TMAs. TMA blocks were cut into 4-μm thick sections. A pathologist examined H&E slides of the TMAs to confirm tumor presence.

Cell culture and Western blot analyses

Cells were grown in 10% FBS (S12450, Atlanta Biologicals) in base media without antibiotic unless noted. Human melanoma cell lines A375, VMM 39, SKMEL 103, SKMEL 153, SKMEL 147, RPMI-8322, and PMWK were used for the cell biology experiments. A375, VMM 39, SKMEL 103, and SKMEL 147 were passaged in DMEM (Gibco). SKMEL 131, SKMEL 153, and RPMI-8322 were grown in RPMI-1640 media. PMWK cells were passaged in Alpha Minimum Essential Medium (MEM) media (Gibco) with 0.5× minimal essential amino acids. The sources and base media for the cell lines have been reported (15) except for SKMEL 235 and WM 1232 (obtained from Wistar, M. Herlyn) and grown in RPMI-1640 (11875-093, Gibco) and TII 2% [80% MCDB 153 (M-7403, Sigma), 20% L-15 media (41300-070, Gibco)], 2% FBS (S12450, Atlanta Biologicals), 5 μg/mL bovine insulin (I-5500, Sigma), 1.68 mmol/L Calcium Chloride (C-5670, Sigma). Cell lines have been tested and authenticated and verified as mycoplasma free. Normal human melanocytes (NHM; Clonetech) were cultured and Western blot analyses were performed as described previously (16).

Melanocyte-melanoma cell line array construction

Cell line arrays (CLA) were constructed from 38 melanoma cell line and three NHM pellets that were fixed in 10% buffered formalin (SF398-Fisher) for 16 to 24 hours, washed twice in 70% ethanol, clotted in 2% low-melting agarose (BP165-25, Fisher), processed, and embedded in paraffin wax (23-021-400, Fisher). Cell pellet blocks were sectioned and H&E-stained to verify the quality of the cell clots and guide CLA construction. Three 1-mm diameter cores were removed from each cell pellet block and randomly embedded into recipient CLA blocks, which were cut into 5-μm thick sections.

Specimen pathology review

Melanomas were reviewed by a dermatopathologist. Demographic characteristics were extracted from the clinical record.

Antibodies, tissue staining protocols, imaging, and analysis

ITK (rabbit monoclonal Y401; Abcam ab32039) was utilized to probe human ITK. GAPDH (Abcam ab9484 mouse monoclonal), cyclin D2 (Santa Cruz Biotechnology sc-563056), and LC3 (Novus Biologicals NB 100-2220) were used for Western blot analyses. Other antibodies and tissue staining protocols for the IHC experiments are summarized in Supplementary Table S2. Single or dual IF on CLAs, melanoma TMAs, and WTS of nevi and melanomas were performed in the Bond fully-automated slide staining system as previously described (17).

H&E stained WTS, CLA, and melanoma TMA slides were digitally imaged at ×20 magnification using the Aperio ScanScope XT (Aperio Technologies). High-resolution acquisition (×20 objective) of fluorescently stained slides in the DAPI, Cy3, and Cy5 channels was performed in the Aperio-FL (Aperio Technologies) and fluorescently stained CLAs in PM2000 (HistoRx). Fluorescently stained WTS and TMAs (ITK-S100) were submitted for analysis through Spectrum using HistoRx automated quantitative analysis (AQUA) software version 2.2. Expression of ITK protein labeled by Cy5 (red) was measured in an S100-specific tumor mask labeled by Alexa555 (green; ref. 6). ITK expression in CLAs was measured in the autofluorescent (Cy3) mask. Same specimen array cores were averaged. AQUA scores were normalized across the TMAs and WTS using cell line standards run in every batch. The Aperio Area Quantification FL algorithm was used to quantify ITK (Cy5) colocalization with CD3, CD19, CD56, CD57, CD68, MPO, and MCT markers (Cy3) in three primary melanomas and two melanomas metastatic to lymph nodes from 5 different patients.
Lenti-shRNA Core Facility. Lentivirus was produced according to library TRC-Hs1.0 (Human) were supplied by the UNC-CH’s TRCN0000010023 (ITK7) from the Thermo Scientific TRCN0000010021 (ITK5), TRCN0000010022 (ITK6), and BRAFV600E.

Melanoma mutational status

The BRAF (exons 11 and 15) and NRAS (exons 2 and 3) mutational status of primary melanomas and cell lines was determined as reported (19). Metastatic melanoma TMA cores were stained with BRAF VE1 antibody (20) and scored for determination as reported (19). Metastatic melanoma TMA cores were stained with BRAF VE1 antibody (20) and scored for determination as reported (19). Metastatic melanoma TMA cores were stained with BRAF VE1 antibody (20) and scored for determination as reported (19). Metastatic melanoma TMA cores were stained with BRAF VE1 antibody (20) and scored for determination as reported (19).

shRNA lentivirus production and use

Lentiviral shRNA constructs TRCN0000010020 (ITK4), TRCN0000010021 (ITK5), TRCN0000010022 (ITK6), and TRCN0000010023 (ITK7) from the Thermo Scientific TRC shRNA library TRC-Hs1.0 (Human) were supplied by the UNC-CH’s Lenti-shRNA Core Facility. Lentivirus was produced according to the ViraPower Lentiviral Packaging Mix instructions (44-2050, Invitrogen). Approximately \(1 \times 10^7\) lentiviral particles were added to transduce approximately 50% of the cells in a 7.5 cm dish. On day 2, media were removed and fresh complete media added then on day 3 media were replaced with fresh complete media containing puromycin (final concentration 10 \(\mu\)g/mL). Cells were allowed to grow for 4 days before use.

BI 10N, a small-molecule ITK inhibitor

BI 10N (21) was from Changchun Discovery Sciences Ltd. Aliquots of a 1,000 \(\times\) stock solution in DMSO were prepared and stored at \(-20^\circ\)C. Carma Biosciences performed selectivity assays.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed using the Immobiline DryStrip 7 cm, pH 6 to 11 gel system according to the manufacturer’s specifications (17-6001-94, GE Healthcare). Gels were then transferred and Western blot analyses were performed using the Y401 antibody.

Proliferation and migration assays

Human melanoma cell lines were added to 10 cm\(^2\) 6-well dishes at a density of 50,000 cells per well. BI 10N was added in DMEM; DMSO was used as a drug vehicle control. Cells were harvested using Trypsin (0.025%) in PBS solution (R-001-100, Gibco) containing 0.01% EDTA for approximately 5 minutes. Cells were counted using the Countess Automated Cell Counter (C10227, Life Technologies). Graphs were generated using GraphPad Prism version 5 (GraphPad Software).

Single-cell tracking was performed to calculate the average motility rate, as described previously (22). Cells were incubated for 24 hours with BI 10N before tracking. At least 50 cells were tracked at each BI 10N concentration.

EdU – FxCycle violet staining of melanoma cells

Melanoma cells were grown to approximately 60% confluence in T25 tissue culture flasks (Coming Product #430639). Cells were labeled with Click-iT EdU Alexa Fluor 488 (C10425, Invitrogen) followed by detection using FxCycle Violet (F-10347, Invitrogen), as per manufacturer’s recommendations. Data acquisition was accomplished using CyanADP from Beckman Coulter, and cell-cycle analysis accomplished using Summit (version 4.3) software (DAKO).

Caspase glo 3/7 assay

The Promega Caspase-GLO 3/7 Assay Kit (G8090, Promega) was utilized as per the manufacture’s protocols. Ten thousand melanoma cells were plated in 96-well dishes in quadruplicate with the indicated drug concentrations. Of note, 100 nmol/L staurosporine was used as the positive control.

Reverse Phase Protein Array

PMWs and RPMI-8322 cells were treated with increasing BI 10 concentrations, lysates were produced, and the MD Anderson core facility performed Reverse Phase Protein Array (RPPA) analyses (23).

In vivo studies

All mice were housed and followed in the UNC LCCC mouse phase I unit (MP1U) under UNC-CH Institute for Animal Care and Use Committee approved protocols. Ten-week-old male nude athymic mice (Jackson Labs 000819) were subcutaneously injected into the flank with 500,000 cells, which were previously suspended in a 50:50 mixture of Matrigel Basement Membrane Matrix (356234, BD Biosciences) and Hank’s Solution with 2% FBS. The Tyr-Cre/Ptennull/BrafV600E GEMM was induced as described (24). Treatment began when tumors reached an approximate size of 60 mm\(^3\). BI 10N was administered orally via medicated diet (25) at 15 mg/kg/d. Body mass and body condition score (26) were monitored weekly via caliper measurements for the duration of the experiment as a marker for toxicity. Tumor volume was calculated using the formula: \((\text{width}^2 \times \text{length})/2\).

Statistical analysis

Statistical analyses of IF data were accomplished using R software (The R project for Statistical Computing). Mann–Whitney and Kruskal–Wallis tests were used to compare ITK expression with clinical attributes in subsets of nevi, primary melanoma, and metastatic melanoma samples. Mann–Whitney tests were used to compare ITK expression with mutational status in melanoma metastases (mutant vs. wild type) and genetic alterations (negative versus positive) in cell lines.

Linear regression analyses were performed for shRNA cell proliferation data using logarithmically transformed baseline normalized cell counts (\(y\)) against days (\(x\)). Similar regression analyses were performed for mouse tumor growth data using the square root-transformed tumor volume (\(y\)) against weeks (\(x\)). Likelihood ratio tests were conducted to assess whether slopes between control and treatment groups were statistically different from each other. Differences in melanoma cell motility among different treatment groups were assessed using Mann–Whitney \(U\) tests. \(P\) values were Bonferroni corrected to account for multiple comparisons of different treatment groups for each cell line.
For RPPA data, a linear regression line was fitted using protein expression as the dependent variable and BI 10N concentration as the independent variable to analyze RPPA data. t tests were used to assess whether the estimated slopes significantly differed from zero. The P values of the t tests for slope were adjusted using the FDR control to account for multiple testings. Data were analyzed through the use of QIAGEN’s Ingenuity Pathway Analysis (IPA).

Microarray analysis
Analysis of microarray data (Gene Expression Omnibus accession number GSE54623) was performed using Biometric Research Branch (BRB, National Cancer Institute, Bethesda, MD) array tools software (27). The quantitative trait analysis (QTA) tool used the Spearman correlation coefficient to identify genes whose expression was correlated with ITK expression in melanoma cell lines. The correlated (P<0.005) genes were investigated to determine whether they were overrepresented in Biologic Biochemical Image Database (28), Kyoto Encyclopedia of Genes and Genomes (29), BioCarta (30), Panther (31), or Reactome (30) pathways.

Study approval
Human tissue studies were IRB approved under UNC protocols 07-0450 and 09-0737. Animal experiments were approved by the UNC Institutional Animal Care and Use Committee.

Results
ITK protein expression in melanocytic tissues
Sections of normal skin, benign nevus, primary melanoma, and metastatic melanomas were dually probed with antibodies against ITK (red) and S100 (green; a marker for melanocytic lineage cells) and visualized using immunofluorescence (IF; example shown in Fig. 1A). ITK in melanocytic lineage cells of benign nevi (n=30), primary melanomas (n=20), and metastatic melanomas (n=70) was quantified in the S100-positive cells using AQUA (refs. 32, 33; Fig. 1B and Supplementary Table S1). Mean ITK expression was higher in primary melanomas than benign nevi, and even higher in metastatic melanomas (P<0.001). The mean ITK protein levels of nevi and melanomas were each not significantly associated (all P>0.05) with demographic or tumor characteristics, and the melanomas’ ITK protein levels were not associated with BRAFV600E status (Supplementary Table S1).

ITK protein expression in immune cell subsets
Because ITK has been shown to have a role in Th2-mediated responses, which have been previously associated with ineffective antitumor responses (34), we investigated whether ITK was expressed in immune subsets infiltrating the tumors. To accomplish this, we performed dual color IF analysis of ITK and markers of immune cell subsets on three primary melanomas and two
melanomas metastatic to lymph nodes from 5 patients. ITK expression was observed in a few small mononuclear cells in the melanoma microenvironment, but did not colocalize with CD3⁺, CD19⁺, CD68⁺, myeloperoxidase (MPO)⁺, mast cell tryptase (MCT)⁺, CD56⁺, or CD57⁺ markers for T cells, B cells, macrophages, neutrophilic granulocytes, mast cells, and subsets of natural killer cells (Supplementary Fig. S1A). We therefore could not confirm ITK expression in the most abundant tumor-infiltrating immune cell subsets.

Melanoma cell line characterization and ITK levels
To investigate whether ITK expression is associated with a particular gene expression or mutation signature, we first probed a melanocyte-melanoma CLA with the ITK antibody (Supplementary Table S2). These melanoma cell lines had previously undergone next-generation sequencing (35) and gene expression profiling (16). The three different NHM cultures in the CLA had a mean ITK signal of 38.5 (range 26.7–52.3) in arbitrary IF units, whereas 38 melanoma cell lines had a mean ITK signal of 278 (range 30.4–4,941). To group the melanoma cell lines in the CLA by ITK levels, we used the ITK IF arbitrary units obtained for each cell line to classify them as ITK low (ITK_LO; <38.5, the mean NHM IHC value), intermediate (ITK_INT; 38.5–<77.0, twice the mean NHM IHC ITK signal), or high (ITK_HI; ≥77.0; Supplementary Table S3). In comparison, Western blot analyses of five of these cell lines give an order from lowest to highest ITK expression (PMWK, SKMEL 147, A375, RPMI-8322, to VMM 39) indistinguishable from the IF determined order (Fig. 2A).

There were no significant associations (all P > 0.05) of ITK protein levels with NRAS and BRAF mutational status or CDKN2A, PTEN, or TP53 genetic alterations in the cell lines (data not shown). QTA was performed to correlate ITK expression with gene expression changes identified by the next-generation sequencing and gene expression profiling. However, no (P < 0.05, Bonferroni corrected) gene or pathway alterations were associated with ITK expression (data not shown).

ITK protein depletion using shRNA
To confirm ITK’s presence in melanoma cells and elucidate its role in melanoma, we stably transduced melanoma cell lines with lentiviral particles that contained a single shRNA designed to knockdown ITK mRNA levels (ITK 4, 5, 6, or 7). For negative controls, we transduced the same melanoma cell lines with lentiviral vectors containing a scrambled (SCR) shRNA sequence. As shown in Fig. 2B, Western blot analysis of whole-cell lysates showed a >95% decrease in the ITK protein level of VMM 39 (ITK_HI) cells stably transduced with shRNAs ITK 4, 5, and 7, but ITK 6 and SCR shRNAs appeared inactive. No increase in dead cells or other morphologic changes were noted in the lentivirally transduced melanoma cells. Comparable results were noted with the RPMI-8322 (ITK_HI) cell line after lentiviral transduction (data not shown).
not shown), whereas ITK protein levels in the PMWK (ITKLO) cell line were too low to reliably measure significant changes in ITK protein levels (Fig. 2A).

The shRNAs were utilized to study the effects of decreased ITK protein expression on melanoma cell proliferation, cell-cycle profile, apoptosis, and motility. Melanoma cell lines VMM 39 and RPMI-8322 (both ITKHIJ) transduced with shRNAs ITK 4, 5, or 7 proliferated at a decreased rate (P < 0.05, Bonferroni corrected) compared with those transduced with SCR (Fig. 2C and Supplementary Table S4). shRNAs ITK 5 and 7 had no affect (P > 0.05) on PMWK (ITKLO) proliferation, although ITK 4 slightly decreased the proliferation of PMWK compared with SCR.

To assess whether ITK suppression induced changes in the cell cycle, incorporation of 5-ethynyl-2'-deoxyuridine (EdU) was measured to determine de novo DNA synthesis (S-phase). In addition, each cell line was stained using FxCycle violet to measure DNA content. Inhibition of ITK expression in the VMM 39 (ITKHI) and RPMI-8322 (ITKHI) cell lines increased the proportion of cells in G<sub>0</sub>-G<sub>1</sub> (FxCycle<sub>low</sub> EdU<sub>low</sub>) and decreased the number of cells in S phase (FxCycle<sub>medium</sub>/low-/EdU<sub>high</sub> EdU<sup>low</sup>). There were no significant cell-cycle changes in PMWK (ITKLO) melanoma cells transduced with any of the shRNAs (data not shown).

Because ITK regulates migration in T cells (36, 37), we investigated the effect of ITK expression on melanoma cell line motility quantified by single-cell tracking analysis (38). Migration rates of the ITK 4, 5, or 7- transduced VMM 39 (ITKHI) and RPMI-8322 (ITKHI) cell lines were significantly decreased (P < 0.05, Bonferroni corrected) compared with the corresponding SCR-transduced cell lines (Fig. 2D and Supplementary Table S4). No significant changes in the migration rate of the PMWK (ITKLO) cells were observed following transduction with any of the lentiviral clones.

BI 10N decreases ITK activity in melanoma cells

To assess the effect of pharmacologic inhibition of ITK activity in melanoma cell lines, we used BI 10N, a small-molecule inhibitor of ITK (21, 39). We investigated the potency and selectivity of BI 10N at a concentration of 200 nmol/L against 56 kinases using a cell-free assay (Supplementary Table S5). IC<sub>50</sub>s were measured for the kinases most potently inhibited by BI 10N in the initial screen, and tight binding studies were performed as needed. BI 10N is a highly selective inhibitor of ITK, and this affinity is described by an apparent dissociation constant (K<sub>d</sub>,apparent) of 8.6 pmol/L. Of the 56 protein kinases tested, BI 10N is at least 1,000-fold more selective for ITK than for 51 of the protein kinases and is about 10-fold selective over three members of the neurotrophic tyrosine receptor kinase family (TRKA, TRKB, TRKC) and the SRC family member nonreceptor tyrosine kinase YES. BI 10N is about 1,000-fold more selective for ITK than for the seven other SRC family tyrosine kinases tested, including SRC and FYN.

To assess whether ITK expressed in melanoma cells is active, we probed phosphorylation at Tyr-180 and 511 residues (40) in whole-cell lysates from high ITK-expressing melanoma cell lines with the commercially available antibodies 4F10 and 8D11, but could not identify a correctly migrating band. To quantitate ITK phosphorylation in the melanoma cells, we therefore performed two-dimensional electrophoresis on whole-cell lysates obtained from RPMI-8322 cells followed by Western blot analysis using the ITK Y401 antibody. Lysates profiled included those from untreated cells, phosphatase-treated cells, and from cells treated with different concentrations of BI 10N for 3 days before harvest (Fig. 3A). In untreated RPMI-8322 cells, two differentially charged immune reactive bands that migrated with the appropriate weight for ITK were identified as ITK by Western blot analysis. However, one of the immune reactive bands was absent in extracts treated with lambda phosphatase and from extracts from cells treated
with concentrations of BI 10N \( \geq 25 \) nmol/L. In untreated cells, about 14% of ITK is phosphorylated.

To determine whether the ITK’s catalytic activity stimulates the proliferation and migration of melanoma cell lines, we inhibited ITK using BI 10N. Proliferation assays were conducted on PMWK (ITK\textsubscript{LO}), VMM 39 (ITK\textsubscript{HI}), RPMI-8322 (ITK\textsubscript{LO}), and other melanoma cell lines (Fig. 3B, data not shown, and Supplementary Table S4). In these studies, BI 10N was added at the inception of the assay at the indicated concentrations when the cells were plated. Similar to the equivalent shRNA studies, proliferation of the ITK\textsubscript{HI} cell lines (VMM 39, RPMI-8322, and SKMEL 153) and an ITK\textsubscript{INT} cell line (A375) decreased by about 50% in the presence of 50 nmol/L BI 10N, whereas the effects on the proliferation of PMWK (ITK\textsubscript{LO}) cells were weaker and required 200 nmol/L BI 10N. ITK catalytic activity inhibition by BI 10N reduced ITK\textsubscript{HI} cell proliferation and migration similar to shRNA suppression of ITK expression. Isogenic overexpression of ITK was not performed to confirm these results.

Cell-cycle analysis of VMM 39 (ITK\textsubscript{HI}) and RPMI-8322 (ITK\textsubscript{LO}) cell lines using EdU labeling and FxCycle Violet staining indicated that, similar to the shRNA studies, treatment of cells with BI 10N increased the percentage of cells in the G0–G1 phase and decreased the percentage of cells in the S phase in a dose-dependent manner (Supplementary Fig. S1B). No cell-cycle–specific changes were observed between the treated and untreated PMWK (ITK\textsubscript{LO}) cells. BI 10N did not affect the percentage of cells in pre-G0–G1 in PMWK, RPMI-8322, or VMM 39 cells (data not shown). To further assess the effects of ITK activity on apoptosis, caspase-3/7 activities in PMWK, RPMI, and VMM 39 were assessed; no consistent effects by BI 10N on the caspase activity levels were noted (data not shown).

The effects of BI 10N on melanoma cell migration were measured for PMWK (ITK\textsubscript{LO}), VMM 39 (ITK\textsubscript{HI}), RPMI-8322 (ITK\textsubscript{LO}), and additional melanoma cell lines (Fig. 3C, data not shown, and Supplementary Table S4), by single-cell tracking analysis. Similar to the results with the shRNA clones targeting ITK, the migration by the ITK\textsubscript{HI} cell lines (VMM39, RPMI-8322, and SKMEL 153) decreased about 50% in the presence of 10 to 25 nmol/L BI 10N, whereas an ITK\textsubscript{INT} cell line (A375) and the ITK\textsubscript{LO} cell line PMWK required BI 10N concentrations of 100 nmol/L or greater, to significantly decrease migration.

Effects of ITK activity on specific cellular proteins

Westerns were performed on RPMI-8322 cells treated with BI 10N administered at concentrations as high as 50 nmol/L for 3 days. Inhibition of ITK resulted in cyclin D2 (CCND2) decrease by about one-third but no change in LC3 (MAP13LC3A) level (related to autophagy; Supplementary Fig. S2A). AKT and ERK protein levels and phosphorylation were unchanged by Western blot analyses (data not shown).

To further investigate the effects of ITK activity on cells, RPPAs (282 proteins or phosphorylated species) were performed on triplicate extracts of PMWK and RPMI-8322 cells treated with up to 50 nmol/L BI 10N. The RPPA results confirmed the phosphor Western analyses we performed for AKT and ERK showing no change in the phosphorylation state of those proteins (data not shown). IPA using a cutoff FDR value of 0.15 indicated that the most affected pathway was p53 signaling with cell-cycle arrest being influenced but not apoptosis or cell survival (Supplementary Table S6 and Supplementary. S2B). RPPA-determined levels of protein analyzed in the p53 pathway are plotted in Supplementary Fig. S2C.

Efficacy of BI 10N in murine melanoma models

To investigate whether BI 10N reduces melanoma growth in vivo, we treated mouse melanoma models with medicated chow containing BI 10N (21). Dose-finding studies based on the published literature were performed to establish an effective dose of 15 mg per kg (mpk)/day. No signs of toxicity (e.g., weight loss, lethargy) were noted in immunodeficient or genetically engineered mice at this dose. Human melanoma xenograft mice were established from two melanoma cell lines with high ITK expression (VMM 39 and RPMI-8322) and from a lower ITK-expressing line, A375. Treatment of each xenograft mouse model with BI 10N at 15 mpk significantly slowed tumor growth in the VMM 39 and RPMI-8322 mice, whereas no such effect was seen in the melanoma xenograft mice established from A375 cells (Fig. 4A).

Given ITK’s possible effect on immune system function, we tested BI 10N in immunocompetent mice with autochthonous melanoma. For this purpose, we used the Tyr-CRE\textsuperscript{ER\textsuperscript{TM}}\textsuperscript{B-Raf\textsuperscript{F600E}}\textsuperscript{Pten\textsuperscript{LO}}\textsuperscript{B-Raf\textsuperscript{V600E}}\textsuperscript{B-Raf\textsuperscript{V600E}} genetically engineered murine model (GEMM; ref. 24). In this system, endogenous tumors featuring V600E B-Raf mutation and Pten loss are generated by tissue-specific CRE recombinase activation in melanocytes. Melanomas from this model expressed high levels of ITK in all five excised tumors tested (Fig. 4B). Mice were dosed with BI 10N after the tumors reached a size of about 60 mm\textsuperscript{3}. Treatment of mice with BI 10N at 15 mpk resulted in arrest of tumor growth compared with the untreated mice (Fig. 4C). These results suggest that ITK kinase activity is a driver of tumor growth in a GEMM of melanoma. GEMM mice survive significantly longer when treated with BI 10N (Fig. 4D). Similar to the treated cell lines, cyclin D2 was decreased by at least one-third in BI 10N-treated versus untreated tumors, whereas LC3 showed no change (data not shown).

Discussion

Our work provides several lines of evidence that ITK, a gene whose promoter CpG sites are hypomethylated in primary melanomas compared with benign nevi, is expressed in a majority of metastatic and primary melanoma tumors. Specifically, we show that ITK protein expression increased with tumor progression from nevus to primary melanoma and even more so to metastatic melanoma. Furthermore, suppression of ITK expression or kinase function in melanoma cells reduced cell attributes associated with tumorigenesis, including proliferation and motility. Finally, inhibition of ITK activity using BI 10N reduced tumor growth in both animal models and in addition extended the survival of the Tyr-Cre/Pten\textsuperscript{LO}/B-Raf\textsuperscript{V600E} mice.

To our knowledge, this is the first report that ITK is highly expressed in nonhematopoietic tissues and even more so by a solid tumor malignancy, such as melanoma. One group reported low-level expression of ITK mRNA in colon cancer tumor tissues, but commented that its expression was probably derived from contaminating host cells (41). Previous studies about the putative oncogenic role of ITK have been limited to its increased or aberrant expression in T-cell malignancies (42–44). It has also been reported that an ITK inhibitor had cytotoxic effects on malignant T cells (43). We are not aware of previous studies reporting ITK inhibition of solid tumor malignancies.
Our review of the 226 melanoma samples that have undergone next-generation sequencing and putative copy-number analysis as part of The Cancer Genome Atlas (TCGA) Project (45, 46) using the GISTIC tool revealed no evidence of copy number alterations or somatic mutations that could account for the ITK expression in the cells or clinical samples reported here. The lack of ITK gene copy number alterations in the TCGA melanomas together with our finding that melanomas are hypomethylated in the ITK promoter relative to nevi (10) and express ITK protein suggests that epigenetic rather than genetic mechanisms, at least in part, account for the high ITK expression in melanoma.

Therapeutic targeting of ITK in melanoma should critically consider its bystander effect on the host immune response given the overwhelming evidence that a functioning immune system is fundamental for antitumor responses against this disease. Of note, our investigations failed to demonstrate ITK expression in several tumor-infiltrating immune cell subsets such as T cells, polymorphonuclear cells, mast cells, natural killer cells, and macrophages. Importantly, the antitumor effect of BI 10N remained significant in immunocompetent mice with melanoma, suggesting at least the lack of a significant negative effect of ITK inhibition on the host antitumor response.

In this work, we characterized the kinase inhibitor profile of BI 10N, a small-molecule selective inhibitor of ITK previously used in animal studies (21). BI 10N was highly selective for ITK over a large panel of kinases, but not for three TRK family members and the SFK member YES. Although TRKA and TRKB are highly expressed in melanoma (47), the effects of BI 10N are most likely mediated through the inhibition of ITK as the effects of BI 10N on...
melanoma cells are similar to those achieved with ITK depletion using shRNAs.

A limitation to our work is that the mechanism by which ITK increases cell proliferation and migration remains unknown. BI 10N treatment did not change AKT and ERK phosphorylation levels in RPMI-8322 cells. However, evidence presented indicates that the mechanism by which ITK activity drives the cell cycle may involve changes in the p53 pathway, and in particular modulation of cyclin levels, but further studies are needed to clarify these issues.

In summary, ITK appears to be a driver of melanoma as demonstrated by its expression in most of the metastatic melanomas examined and by the significant effects of BI 10N on the proliferation and migration of melanoma cells and its efficacy in mouse melanoma models. We are currently investigating rational treatment combinations between ITK inhibitors and standard treatments for metastatic melanoma in various preclinical melanoma models that may serve as the basis for future clinical trials in metastatic melanoma. Besides BI 10N, several other ITK inhibitors previously have been developed to target Th2 dominant autoimmune, inflammatory, and infectious diseases (48, 49). Notably, ibrutinib (IMBRUVICA, Pharmacyclics, Inc.), an irreversible inhibitor of BTK and ITK (50), has been granted approval by the U.S. FDA. We conclude that ITK is a novel target for the treatment of melanoma that, at least in animal models, is amenable to pharmacologic intervention.

Disclosure of Potential Conflicts of Interest

S.J. Moschos is a consultant/advisory board member for Merck and Prometheus. D.S. Rubenstein is an employee of Sutro/GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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