Evaluating TBK1 as a Therapeutic Target in Cancers with Activated IRF3


Abstract

TBK1 (TANK-binding kinase 1) is a noncanonical IKK protein kinase that phosphorylates and activates downstream targets such as IRF3 and c-Rel and, mediates NF-κB activation in cancer. Previous reports demonstrated synthetic lethality of TBK1 with mutant KRAS in non–small cell lung cancer (NSCLC); thus, TBK1 could be a novel target for treatment of KRAS-mutant NSCLC. Here, the effect of TBK1 on proliferation in a panel of cancer cells by both generic and pharmacologic approaches was evaluated. In KRAS-mutant cancer cells, reduction of TBK1 activity by knockdown or treatment with TBK1 inhibitors did not correlate with reduced proliferation in a two-dimensional viability assay. Verification of target engagement via reduced phosphorylation of S386 of IRF3 (pIRF3S386) was difficult to assess in NSCLC cells due to low protein expression. However, several cell lines were identified with high pIRF3S386 levels after screening a large panel of cell lines, many of which also harbor KRAS mutations. Specifically, a large subset of KRAS-mutant pancreatic cancer cell lines was uncovered with high constitutive pIRF3S386 levels, which correlated with high levels of phosphorylated S172 of TBK1 (pTBK1S172). Finally, TBK1 inhibitors dose-dependently inhibited pIRF3S386 in these cell lines, but this did not correlate with inhibition of cell growth. Taken together, these data demonstrate that the regulation of pathways important for cell proliferation in some NSCLC, pancreatic, and colorectal cell lines is not solely dependent on TBK1 activity.

Implications: TBK1 has therapeutic potential under certain contexts and phosphorylation of its downstream target IRF3 is a biomarker of TBK1 activity.

Visual Overview: http://mcr.aacrjournals.org/content/12/7/1055/F1.large.jpg.

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Introduction

TBK1 (TANK-binding kinase 1) is a noncanonical IKK kinase, which shares 65% similarity to IKKe and is highly expressed in lung, breast, pancreatic, and colon cancers (1). Besides its key role in innate immune response, increasing evidence indicates that the activation of TBK1 and its close homolog IKKe is associated with the development of human cancers (2–10). TBK1 is an 84 kDa, 729 amino acid–long protein containing an N-terminal kinase domain, a ubiquitin-like domain and two C-terminal coiled-coil domains. TBK1 is an NF-κB–activating kinase, which is constitutively expressed in many normal tissues, including immune cells, brain, lungs, gastrointestinal tract, and reproductive organs (11, 12). TBK1-deficient mice exhibit embryonic lethality due to widespread hepatic apoptosis (13, 14).

TBK1 is regulated through activation by Toll-like receptors (TLR) or cytoplasmic RIG-1–like receptors, and it stimulates type I IFN production via direct phosphorylation of IFN regulatory transcription factor 3 (IRF3) and 7 (IRF7; refs. 15–22). IRF3 phosphorylation at residues Ser-385 and Ser-386 has previously been shown to control its dimerization (23–25) upon which IRF3 translocates to the nucleus (26), and controls transcriptional regulation of cell survival and proliferation (2, 11). In addition to IRF3, several other substrates, i.e., namely cRel, Akt, RalB, SIKE, etc., have been identified that attribute to TBK1 function (7–9, 27–30). TBK1 requires phosphorylation at Ser-172 within the kinase domain activation loop to be activated. Phosphorylation at Ser-172 has been shown previously to induce reorganization of the activation loop allowing the active site in the kinase domain to bind to the substrate (31). TBK1 inhibitors of different chemical structures have been developed by multiple groups and it was shown previously that a PDK1/TBK1 inhibitor, BX795, enhanced phosphorylation of TBK1 while inhibiting...
its activity, suggesting a feedback control mechanism that stimulates phosphorylation of TBK1 (10, 32, 33).

Although the role of TBK1 in cancer still remains unclear, recent studies suggested its role in regulation of cell growth and proliferation, and oncogenic transformation (2, 4, 9). A number of studies have shown that inhibition of TBK1 activity is associated with a decrease in cell proliferation and transformation in breast cancer (34–36). Shen and Hahn (1) first demonstrated the role of both TBK1 and IKKζ in cell transformation. IKKζ, a breast cancer oncogene that is amplified in 30% of breast cancers, mediates activation of NF-κB signaling and is required for transformation in breast cancer (34–36). TBK1 was identified through a functional genomics screen, which showed that a subset of KRAS-mutant non–small-cell lung cancer (NSCLC) cell lines was dependent on TBK1 and that TBK1 was essential for KRAS mutant cancer cell lines (6). This generated an interest in small molecule inhibitors of TBK1 that may provide a novel therapeutic strategy for KRAS-mutant tumors. However, subsequent studies were not able to confirm the proposed synthetic lethal relationship of oncogenic KRAS and TBK1 (5, 8). In these studies, knockdown of TBK1 did not consistently result in a significant alteration of growth, and the sensitivity to TBK1 inhibition by either siRNA or small-molecule inhibitors did not correlate with KRAS status in lung, pancreatic, or colorectal cancer cell lines (5).

Our study aimed to further investigate the role of TBK1 in cancer by studying TBK1 knockdown using RNAi technology as well as small molecule inhibitors. We identified a subset of pancreatic ductal adenocarcinoma cell lines (PDAC) that have high constitutive pIRF3S836 levels and showed that TBK1 inhibitors dose-dependently inhibited pIRF3S836. In addition, shRNA knockdown of TBK1 reduced pIRF3S836 in pancreatic cancer cell lines, and, thus, further supported pIRF3S836 as a target engagement marker for TBK1 activity in these cancer cell lines. However, no robust inhibition of growth was achieved in the two-dimensional proliferation assay in these PDAC cell lines as well as in the majority of NSCLC cell lines either by genetic knockdown or by using TBK1 inhibitors. Our data demonstrate that reduction of TBK1 activity and the subsequent elimination of pIRF3S836 are not sufficient on their own to regulate cell proliferation in the NSCLC and PDAC cell lines that were tested.

Materials and Methods

Cell lines and tool inhibitor treatment conditions

Cell lines (see Supplementary Data) were obtained from various commercial vendors (ATCC, DSMZ, JCRB, and ECACC). The cell line authentication details are listed in the Supplementary Data. All cell lines were grown in recommended media by the ATCC, supplemented with 10% fetal bovine serum (Atlanta Biologicals Inc.). All cultures were maintained at 37°C in a humidified 5% CO2 atmosphere and tested periodically to ensure the absence of mycoplasma. Generally, cells were passaged upon reaching approximately 75% confluence.

Tool inhibitor stocks and dilutions were made in dimethyl sulfoxide solvent. Cell proliferation experiments were carried out in a 96-well format (6 replicates), the cells were plated at a density of 2,000 to 5,000 cells per well. At 24 hours following cell seeding, the cells were treated with the tool inhibitor titrations for 4 days at 37°C and then assayed by using the ATP CellTiter-Glo luminescent cell viability assay (Promega; G7570).

For Incucyte analysis (Essen BioScience, Inc.), cells were plated as described above in 96-well plates, and image-based cell confluence data were collected every 2 hours during live growth.

Plasmid constructs

pLKO.1–shRNAs [targeting TBK1 Coding Sequence (CDS) regions] were purchased from the Broad Institute (Cambridge, MA). Of note, 3′ untranslated region (UTR)–targeting shRNAs and control shRNAs for GFP and KRAS sequences were specifically designed for this study (see Supplementary Data). Human full-length TBK1 cDNA was purchased from Origene (SC11251). shRNA-resistant TBK1 was made by PCR cloning of TBK-WT without the 3′UTR region using newly designed primers, and then subcloned into the pLenti-CMV-Hygro-DEST (w117–1) vector (Addgene) using Gateway cloning. All shRNAs and cDNAs were confirmed by sequencing.

Lentiviral shRNA transduction and proliferation assay

Lentiviral shRNAs were prepared via transfection of the corresponding plasmid DNAs into 293 T cells using Lipofectamine 2000 (Invitrogen; 52887) in OptiMEM ( Gibco; 31985–070) media. The lentiviruses were packaged using the pA8.9 and VSVG packaging vectors in early passage 293 T cells.

Both NSCLC and PDAC cancer cell lines were plated at 150 to 250 K cells per well density in 6-well plates on day 0, and the lentiviral transductions were performed on day 1 using polybrene (8 μg/mL; EMD Millipore # TR-1003-G). The cells were selected in 2 μg/mL puromycin for 3 days and on day 4 cells were plated in 1 μg/mL puromycin (Life Technologies; # A1113803) into 96-well assay plates. Cell viability was assessed on day 8 after viral transductions using the ATP CellTiter-Glo luminescent cell viability assay (Promega; G7570). The growth profiles of cell lines with and without shRNA knockdown were also recorded and analyzed by the Incucyte Imaging System (Essen BioScience, Inc.). The level of knockdown of TBK1 protein was determined by Western blot analysis using the samples collected on day 8.

Protein detection

Cell lysates for Western blotting were collected using RIPA lysis buffer (Boston Bioproducts; BP-115) supplemented with 1× Halt Protease inhibitor (VWR International; VWR # P178415), 5 mMol/L NaF (Sigma-Aldrich Co.) and 10 mMol/L β-glycerophosphate (Sigma-Aldrich Co.). Protein (10–15 μg) was separated on 4% to 15% Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories; 456-1086), and on 4% to 15% Criterion TGX Precast Gel (Bio-Rad Laboratories; 3567085). The proteins were transferred onto a polyvinylidene difluoride membrane using the TransBlot Turbo Transfer System (Bio-Rad Laboratories; 170–4155).

Protein levels from Western blots were quantified by densitometry using the ImageJ software and this was
normalized to the loading control for each sample. The band signal intensities were from the raw chemiluminescent images on films. For IC_{50} calculations, these data were fitted with a standard three-parameter dose–response nonlinear regression fit using GraphPad Prism software.

**Antibodies**

Western blots were probed with antibodies against TBK1 (Cell Signaling Technology; D1B4, CST 3504), phospho-TBK1 S172 (Abgent; AP3627a), IRF3 (Cell Signaling Technology; D83B9, CST 4302), phospho-IRF3 S386 (Epitomics; Clone ID EPR2346, 2562-1), KRAS (Santa Cruz Biotechnology Inc., F234, sc-30) GAPDH (Cell Signaling Technology; horseradish peroxidase-conjugate, 14C10, CST 3683).

**Assays used for initial validation of tool inhibitors**

The TBK1 tool inhibitors were initially screened using the full-length TBK1 protein in a biochemical assay (Ulight Table 1. List of cancer cell lines tested with multiple shRNAs targeting TBK1 and those that resulted in ≥90% knockdown are listed

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue origin</th>
<th>KRAS mutation status</th>
<th>pIRF3^{S386}</th>
<th>IRF3</th>
<th>pTBK1^{S172}</th>
<th>TBK1</th>
<th>shRNAs tested (which had TBK1 KD ≥ 90% by WB)</th>
<th>Growth inhibition (%; normalized to shGFP; average ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H23</td>
<td>Lung</td>
<td>G12C</td>
<td>(−)</td>
<td>NA</td>
<td>(−)</td>
<td>(++)</td>
<td>shT15</td>
<td>shT15: −1.26 ± 1.90 shT17: 82.74 ± 1.41 sh3182: 51.74 ± 1.96 sh3183: −35.02 ± 3.10 sh3185: 48.84 ± 0.95 sh3186: 46.53 ± 0.77 shT15: −3.17 ± 1.33 shT17: 77.34 ± 0.64 sh3182: 36.94 ± 2.23 sh3183: 17.44 ± 1.95 sh3185: 26.63 ± 0.49 sh3186: 34.88 ± 1.99 shT15: −10.06 ± 6.35</td>
</tr>
<tr>
<td>H28</td>
<td>Lung</td>
<td>WT</td>
<td>(−)</td>
<td>NA</td>
<td>(−)</td>
<td>(++)</td>
<td>shT15</td>
<td>shT15: 35.85 ± 9.41 shT15: 35.52 ± 9.44 sh3182: 42.06 ± 9.21 shT15: −0.39 ± 6.65 sh3182: −19.10 ± 6.45 shT15: −6.00 ± 4.99 sh17: 47.00 ± 1.44 sh3182: 48.00 ± 2.99 shT15: 13.68 ± 2.39 sh3182: 31.00 ± 2.61</td>
</tr>
<tr>
<td>SUIT2</td>
<td>PDAC</td>
<td>G12D</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(+)</td>
<td>shT15</td>
<td>shT15: 35.85 ± 9.41 shT15: 35.52 ± 9.44 sh3182: 42.06 ± 9.21 shT15: −0.39 ± 6.65 sh3182: −19.10 ± 6.45 shT15: −6.00 ± 4.99 sh17: 47.00 ± 1.44 sh3182: 48.00 ± 2.99 shT15: 13.68 ± 2.39 sh3182: 31.00 ± 2.61</td>
</tr>
<tr>
<td>WiDr</td>
<td>Colon</td>
<td>WT</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>shT15</td>
<td>shT15: 35.85 ± 9.41 shT15: 35.52 ± 9.44 sh3182: 42.06 ± 9.21 shT15: −0.39 ± 6.65 sh3182: −19.10 ± 6.45 shT15: −6.00 ± 4.99 sh17: 47.00 ± 1.44 sh3182: 48.00 ± 2.99 shT15: 13.68 ± 2.39 sh3182: 31.00 ± 2.61</td>
</tr>
</tbody>
</table>

**NOTE:** The corresponding effects in the two-dimensional cell proliferation assay obtained from ATP CellTiter-Glo (CTG) luminescent cell viability assay using NSCLC, colorectal, and PDAC cancer cell lines is reported as cell growth inhibition as the percentage of shGFP control.

**a**Basal protein levels at 10% fetal bovine serum.
kinase assay; LANCE Ultra ULight, Perkin Elmer) in which two lead series were identified. Six tool inhibitors (Table 3) were selected and these underwent profiling studies in a list of KRAS–wild-type (wt) and -mutant NSCLC cell lines (as described above and in Table 3). In addition, these tool inhibitors were further characterized for their ability to inhibit IRF3 nuclear translocation using a high-content immunofluorescence imaging assay. This assay quantified the amount of IRF3 protein which translocated into the nucleus of the HeLa cell line, a surrogate cell line for tool inhibitor screening; The HeLa cells were stimulated with Poly I:C (Invivogen; tlrl-pic) following treatment with TBK1 tool inhibitors; (Table 3). The assay uses an anti-IRF3 mAb (Epitomics; cat.# 2241-1), and a secondary Ab labeled with a red fluorophore (goat anti-rabbit IgG (H+L), DyLight 549 Conjugated; Thermo Scientific cat. no. 35558). Immunofluorescence image data were analyzed using a Columbus script (Opera/Acapella/Columbus platform for high-content screening—PerkinElmer) that quantified mean intensity of IRF3 signal in the nucleus after image segmentation.

**Kinase panel screening of tool inhibitors**

The kinase inhibitory profiles of inhibitors were evaluated by using 101, 266, and 305 kinase panels at Invitrogen.

**RAS pathway signature analysis**

Gene transcription signature of the RAS pathway was defined as described by Loboda and colleagues (see Supplementary Data; ref. 37).

### Results

**TBK1 dependency did not correlate with the KRAS mutation status**

TBK1 has been proposed as a potential therapeutic target for KRAS-dependent cancer (6). We evaluated the role of TBK1 in KRAS-mutant NSCLC cell lines further using both genetic and pharmacologic approaches. Six shRNA constructs distributed throughout both the coding and the 3’UTR regions were observed to reduce TBK1 protein by ≥90% and reduced viability up to approximately 90% when measured by total cellular ATP levels (Fig. 1). All six shRNAs tested gave a robust knockdown of TBK1 protein in two KRAS-mutant NSCLC cell lines, H23 and A549 (Fig. 1B, top). However, the degree of protein knockdown did not correlate with decreased viability (Fig. 1B, bottom). Specifically, shTBK1-T15 (3’UTR), which showed a very strong level of TBK1 knockdown, had no impact on cell growth in both of these NSCLC cell lines as well as in 7 cell lines tested from PDAC and colorectal cancer cell line panels, including both KRAS-mutant (G12A, G12C, G12D, G12S, and Q61H) and KRAS-wt genotypic profiles (Table 1). Among the six shRNAs tested, only shTBK1-T15 (3’UTR) and shTBK1-T182 (CD5) repeatedly gave ≥90% knockdown in multiple cell lines. On the other hand, shTBK1-T17 (3’UTR) with a partial knockdown of TBK1 protein had potent antiproliferative activity in both H23 and A549 cell lines. The lack of growth inhibition with shTBK1-T182 and shTBK1-T15 in most of the tested PDAC and colorectal cancer cell lines suggested that in these cell lines specifically, antiproliferative effects were not dependent on TBK1.

### Table 2. List of PDACs used for endogenous protein expression profiling for pTBK15172, TBK1, IRF3, pIRF35386, and GAPDH for Western blotting (as presented in Fig. 2A)

<table>
<thead>
<tr>
<th>Cell line ID</th>
<th>Source</th>
<th>Pancreatic cell lines</th>
<th>Kras status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CRL-1420</td>
<td>ATCC</td>
<td>MiaPaca-2</td>
<td>G12V</td>
</tr>
<tr>
<td>2 CRL-2553</td>
<td>ATCC</td>
<td>Panc02.03</td>
<td>G12D</td>
</tr>
<tr>
<td>3 P09</td>
<td>Beller</td>
<td>HuPi4</td>
<td>G12V</td>
</tr>
<tr>
<td>4 CRL-1687</td>
<td>ATCC</td>
<td>Bxp3-3</td>
<td>WT</td>
</tr>
<tr>
<td>5 HTB-134</td>
<td>ATCC</td>
<td>hs766T</td>
<td>Q61H</td>
</tr>
<tr>
<td>6 CRL-1469</td>
<td>ATCC</td>
<td>Panc-1</td>
<td>G12D</td>
</tr>
<tr>
<td>7 CRL-2558</td>
<td>ATCC</td>
<td>PL-45</td>
<td>G12D</td>
</tr>
<tr>
<td>8 CRL-1918</td>
<td>ATCC</td>
<td>Cifp-1</td>
<td>G12V</td>
</tr>
<tr>
<td>9 P15</td>
<td>Beller</td>
<td>Dang</td>
<td>G12V</td>
</tr>
<tr>
<td>10 CRL-1997</td>
<td>ATCC</td>
<td>Hpafl-2</td>
<td>G12D</td>
</tr>
<tr>
<td>11 CRL-1682</td>
<td>ATCC</td>
<td>Aspc-1</td>
<td>G12D</td>
</tr>
<tr>
<td>12 JCRB0182</td>
<td>JCRB</td>
<td>KP-4</td>
<td>G12D</td>
</tr>
<tr>
<td>13 JCRB0177.1</td>
<td>JCRB</td>
<td>KP-1NL</td>
<td>G12D</td>
</tr>
<tr>
<td>14 P21</td>
<td>Beller</td>
<td>Patu 8902</td>
<td>G12V</td>
</tr>
<tr>
<td>15 JCRB1094</td>
<td>JCRB</td>
<td>Suit-2</td>
<td>Not determined</td>
</tr>
<tr>
<td>16 CRL-2554</td>
<td>ATCC</td>
<td>Panc02.13</td>
<td>Q61R</td>
</tr>
<tr>
<td>17 CRL-2557</td>
<td>ATCC</td>
<td>Panc05.04</td>
<td>G12D</td>
</tr>
<tr>
<td>18 CRL-2380</td>
<td>ATCC</td>
<td>Mpanc96</td>
<td>G12V</td>
</tr>
<tr>
<td>19 JCRB0178.1</td>
<td>JCRB</td>
<td>KP-3L</td>
<td>Not determined</td>
</tr>
<tr>
<td>20 JCRB0178.0</td>
<td>JCRB</td>
<td>KP-3</td>
<td>G12V</td>
</tr>
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</table>
pIRF3S386 is a target engagement marker for TBK1 in cancer cell lines

To identify a target engagement biomarker for TBK1 activity, we screened six different cancer cell line panels (a total of 79 cancer cell lines; see Supplementary Data), consisting of 16 NSCLC cell lines, 20 PDAC, 10 colorectal cancer, 16 ovarian cancer, six lymphoma, and 11 glioblastoma multiforme (GBM), for expression of the TBK1 pathway proteins (i.e., IRF3, pIRF3S386, cRel, and RalB) by Western blot analysis (data not shown). From this profiling analysis, PDAC cell lines had the highest basal pIRF3S386 levels that correlated well with high basal pTBK1S172 levels (Fig. 2A and Table 2). However, many of the NSCLC cell lines had very low or undetectable pIRF3S386 levels even following poly I:C stimulation (data not shown). This limited our interpretation of the shRNA knockdown experiments in NSCLC cell lines due to the absence of a viable target engagement marker for the assessment of the TBK1 activity.

Thus, we used the two KRAS-mutant PDAC cell lines, Panc 02.13 and Panc 05.04, which had high basal pIRF3S386 levels, to validate pIRF3S386 as a target engagement marker for TBK1 activity. We performed TBK1 knockdown experiments using four different shRNAs (shT15, sh3182, sh3185, and sh3186) in these two PDAC cell lines. Upon shRNA knockdown of TBK1 in Panc 02.13 and Panc 05.04, the level of TBK1 protein was significantly reduced and this correlated with a concomitant reduction in pIRF3S386 levels, indicating that pIRF3S386 could be used as a target engagement marker for TBK1 activity in cells (Fig. 2B). However, the knockdown of TBK1 did not result in a prominent inhibition of cell proliferation in these two KRAS-dependent PDAC cell lines that had high pIRF3S386 levels (Fig. 2C).

Evaluation of TBK1 tool inhibitors in cancer cell lines with high endogenous pIRF3S386

Complimentary data were obtained by evaluating the impact of a set of potent ATP-competitive TBK1 inhibitors (Table 3). A total of 6 potent TBK1 inhibitors, which were from three different structural series (32, 38, 39), have been synthesized. These inhibitors were characterized by both biochemical as well as cell-based assays (Table 3). The IC50

<table>
<thead>
<tr>
<th>Table 3. TBK1 tool inhibitors: The biochemical and biologic activities and chemical selectivity profiles</th>
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<tr>
<td></td>
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<tr>
<td>-------------------------------------</td>
</tr>
<tr>
<td>Ulight @ 5 μmol/L ATP I50 (nmol/L)</td>
</tr>
<tr>
<td>Ulight @ 250 μmol/L ATP I50 (nmol/L)</td>
</tr>
<tr>
<td>IKK, I50 (nmol/L)</td>
</tr>
<tr>
<td>pIRF3 I50 (Panc05.04 nmol/L)</td>
</tr>
<tr>
<td>IRF3 Translocation I50 (nmol/L)</td>
</tr>
<tr>
<td>CTG H23 I50 (nmol/L)</td>
</tr>
<tr>
<td>CTG H28 I50 (nmol/L)</td>
</tr>
<tr>
<td>CTG A549 I50 (nmol/L)</td>
</tr>
<tr>
<td>CTG H441 I50 (nmol/L)</td>
</tr>
<tr>
<td>Invitrogen (~100×, %)</td>
</tr>
</tbody>
</table>

NOTE: Top row section, First and second rows represent the IC50 values of TBK1 inhibitors obtained from Ulight kinase assay at 5 and 250 μmol/L ATP concentration for inhibition of TBK1 biochemical function; and the third row represents the IC50 values of inhibitors in the same type of assay for inhibition of IKKε biochemical function (at 10 μmol/L ATP). Middle row section, pIRF3 IC50 values calculated from Western blotting quantification of pIRF3S386 levels, IRF3 I50 values obtained from IRF3 nuclear translocation assay, and the cellular IC50 values obtained from ATP CellTiter-Glo (CTG) luminescent cell viability assay in H23, H28, A549 and H441 cell lines, are represented. Bottom row section, Invitrogen kinase panel screening summary representing the percentage of kinases tested with each TBK1 inhibitor that had an IC50 >100× of the IC50 for TBK1 biochemical function in the same screening assay. ([The synthesis of these six tool compounds were reported previously, i.e., Compounds #1–4 (32), compound #5 (38), and compound #6 (39)]. Abbreviation: ND, not determined.)

Phosphorylated IRF3 Is a Biomarker of TBK1 Activity


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values of these inhibitors ranged between 2.3 to 32 nmol/L in the biochemical assay and they were active in the cellular IRF3 translocation assay (Table 3). The compounds were used to treat Panc02.13 cells for 4 hours and displayed near full inhibition of pIRF3S386 signal by Western blotting (Fig. 3A). Interestingly, a compensatory increase in pTBK1S172 levels was observed, suggesting a feedback reactivation response of the TBK1 pathway. All three inhibitors modulated the phosphorylation of pTBK1S172 to varying degrees, but not the total TBK1 levels, which remained unchanged (Fig. 3A).

Multiple PDAC cell lines showed potent inhibition of pIRF3S386 by TBK1 inhibitors (Fig. 3B) and this further supported pIRF3S386 as a valid target engagement marker for measurement of TBK1 cellular activity. Similar data were obtained in multiple colorectal cancer cell lines (data not shown) by these TBK1 inhibitors that substantiated pIRF3S386 as the target engagement marker for TBK1 activity. For several cell lines, we quantified this reduction in pIRF3S386 and then fit the data to a three-parameter hyperbolic–binding function to determine the apparent potency on each cell line based on inhibition of pIRF3S386 (Fig. 3B). The IC_{50} values of these three TBK1 tool inhibitors were between 10 and 850 nmol/L and this was consistent with the expected potency based on an IRF3 nuclear translocation assay (Table 3). These data indicated that the inhibitors were indeed getting into the cells and inhibiting the activity of TBK1.

These inhibitors were then used to evaluate their impact on proliferation in a panel of NSCLC, PDAC, colorectal cancer, ovarian cancer, GBM, and lymphoma cell lines (Fig. 4A; Supplementary Data). A typical dose–response curve is shown for a TBK1 inhibitor, compound #1, and the control MEK inhibitor (PD-0325901) using Panc 02.13 cells (Fig. 4A). The IC_{50} of compound #1 in the cell proliferation assay (IC_{50}: \approx 5 \mu mol/L; Fig. 4A) was significantly higher than its IC_{50} in the cell-based target engagement assay, (IC_{50} pIRF3S386 = 35 nmol/L; Fig. 3B). Among the panel of 18 cancer cell lines screened using these TBK1 inhibitors, a submicromolar IC_{50} was rarely observed (Fig. 4B; Supplementary Data). Therefore, we concluded that the inhibition of the TBK1 pathway, as measured by pIRF3S386, was not sufficient to inhibit the growth of these cancer cell lines.

Using the cell line screening data for compound #1 from a panel of 21 KRAS-wt and -mutant NSCLC cell lines (see Supplementary Data), a RAS gene expression analysis was carried out to assess whether the sensitivity of these NSCLC cell lines to the TBK1 inhibitor (compound #1) correlated...
with the RAS signatures. This approach could potentially uncover a TBK1 KRAS dependency that requires $>95\%$ inhibition of its activity, which was not achieved in our knockdown experiments. This could also explain the large shift in the IC$_{50}$ values of inhibitors that were obtained in the cell growth assays. It was previously shown by Loboda and colleagues (37) that the quantification of RAS-dependent gene expression would provide a better measure of RAS activity in cancer cells than mutations alone. For this purpose, the RAS pathway signatures for this NSCLC cell line panel were calculated using the methods described by Loboda and colleagues (37). The RAS score analysis used expression profiling data from the Broad Cancer Cell Line Encyclopedia (CCLE) database for these cell lines (see Supplementary Data). The IC$_{50}$ values of compound #1 and PD0325901 for the 21 NSCLC cell lines are listed in the Supplementary Data. Our analysis indicated no correlation between the cell potency of the compound #1, and the RAS scores in this panel of 21 NSCLC cell lines (Fig. 4C). These data provided additional evidence that these RAS-driven NSCLC cell lines did not depend on TBK1 alone. In contrast, there was a good correlation between the potency of the positive control MEK inhibitor (PD0325901) and the RAS scores of the NSCLC cell lines that were tested.

**Molecular rescue with shRNA-resistant TBK1 also restores pIRF3$^{S386}$ levels**

Since we validated pIRF3$^{S386}$ as a biomarker for TBK1 activity, we set out to determine whether we could rescue the
The effects of TBK1 shRNA inhibition with shRNA-resistant TBK1 (Fig. 5). The H23 NSCLC cell line was chosen because this was the only cell line that had robust growth inhibition upon TBK1 knockdown with multiple shRNAs (Fig. 1B). As shown earlier, the shTBK1-T17 (targeting the 3’UTR region) effectively knocked down TBK1 in the H23 cell line (≥90%), which resulted in inhibition of cell proliferation (≥80%). The KRAS knockdown by shRNA was used as a positive control (Fig. 1B). In an engineered H23 cell line, which stably overexpressed TBK1-WT cDNA (missing the 3’UTR region), the knockdown of endogenous TBK1 using shTBK1-T17 still inhibited the proliferation of the H23 cell line (Fig. 5A and 5B, bottom) even though shRNA-resistant TBK1 levels were not reduced (by Western blotting), as expected (Fig. 5A and 5B, top). This suggested that the antiproliferative effect, which was originally detected in the H23 cell line after knockdown of TBK1, was not due to on-target effects of TBK1 knockdown. Most importantly, cells expressing shRNA-resistant TBK1-WT cDNA retained full pIRF3S386 in the presence of shTBK1-T17, demonstrating rescue of both the TBK1 protein and its pathway activity, which further supports the value of pIRF3S386 as a validated biomarker for TBK1.

**Discussion**

Advances in targeted therapies for cancer have accelerated in the past 5 years because they often have improved outcomes for selected patient populations. Identifying specific biomarkers for patient selection and for effective pathway inhibition is a key for the development of targeted therapeutics. With this in mind, we evaluated the role of TBK1, a noncanonical IKK, which has been reported to be an important kinase in cancer (4). TBK1 inhibition in a panel
of 79 cancer cell lines (35 KRAS-mutant and 18 KRAS-wt, confirmed by CCLE) was achieved using small-molecule inhibitors as well as shRNAs to knockdown TBK1; however, we did not observe consistent growth reduction in vitro. To evaluate the corresponding pathway effects in cells, we validated pIRF3S386, which is a direct substrate of TBK1, as a target engagement biomarker for TBK1 activity. We also demonstrated good correlation between activated TBK1 (pTBK1S172) and pIRF3S386 in multiple cancer cell lines. Although TBK1 activity was effectively reduced by TBK1 inhibitors and by some short hairpins targeting TBK1, there was no clear evidence of TBK1 being essential in regulation of cell growth and proliferation in these cancer cell lines.

The role of TBK1 in activation of the NF-κB pathway in immune cells via phosphorylation of IkB and IRF3/IRF7 has been extensively documented (6–9); however, the mechanisms that activate proteins and pathways downstream of TBK1 in cancer still remain incompletely understood. Some studies have placed TBK1 downstream of RAS signaling, increasing the potential for TBK1 therapeutics in cancer (6, 8). Upon activation of RAS signaling, RalB, a target of TBK1, was reported to be downregulated and this was shown to control the expression of other downstream targets, which were important in regulation of apoptosis and cell survival (i.e., Bcl-XL and Akt). This further suggested that TBK1 was related to proteins downstream of RAS, which were involved in regulation of cancer cell survival (3, 9). Another study showed that shRNA knockdown of TBK1 inhibited phosphorylation of AKT at Ser-473 and, thus, mediated its prosurvival role in U2OS and HBEC cell lines (8). In our preliminary studies using Western blot analysis to assess the changes in pAKTS473 and pAKTT308 levels in cancer cell lines, no changes were observed either following shRNA knockdown of TBK1 or treating the cell lines with TBK1 inhibitors. Recently, Marion and colleagues (27) also identified SIKE as a TBK1 substrate, and have also shown that TBK1 inhibition resulted in inhibition of TBK1-mediated IRF3 phosphorylation. Furthermore, a systematic RNAi screening study (6) revealed that TBK1 was specifically required for survival of lung cancer cells harboring oncogenic KRAS mutations and also provided additional evidence, suggesting that Bcl-XL was important for TBK1-sensitive lung cancer survival.

Figure 4. Multiple TBK1 kinase inhibitors showed only modest activity in three different cancer cell line panels. A, example growth inhibition curves from Panc 02.13 cells after treating with the titrations of the indicated TBK1 inhibitors and the MEK inhibitor PD-0325901 at day 4 are shown. The viability was assessed using the same method as described in Fig. 1. B, IC50 values from the selected PDAC, colorectal cancer, and NSCLC cancer cell lines using TBK1 inhibitor compound #1. C, correlation analysis of the RAS score versus sensitivity to the TBK1 inhibitor compound #1 in a panel of 26 NSCLC cell lines is shown.

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Phosphorylated IRF3 Is a Biomarker of TBK1 Activity
Figure 5. Rescue experiment using an engineered H23-TBK1-WT overexpression cell line and the 3’UTR-targeting shRNA-T17. A, confirmation of the efficient knockdown of TBK1 using shT17 in the H23 cell line (by Western blotting, top), and the growth inhibition phenotype in a two-dimensional cell viability assay (bottom). B, an engineered cell line stably expressing TBK1-WT cDNA (H23-TBK1-WT) was used to attempt the rescue of the shRNA-T17 antiproliferative effect. Top, the change in expression levels of TBK1, pTBK1 (S172), and pIRF3 (S386) and IRF3 by Western blotting, and bottom is showing the change in growth profiles upon shRNA knockdown with the indicated shRNAs. shRNA lentiviral transductions were done in parallel in both H23 native and the H23-TBK1-WT overexpression cell lines, and the cell growth profiles were monitored using the viability assay at day 8 as described in Fig. 1. This experiment was repeated two times.

Our results did not demonstrate a dependency of TBK1 in KRAS-mutant NSCLC cell lines, consistent with some other recent reports, even though some shRNAs and inhibitors can reduce cell proliferation (5, 8). In two prior TBK1 shRNA knockdown studies, there was no overlap in NSCLC cell lines tested, and the reported growth inhibition used different shRNAs (6, 8). Furthermore, in prior reports, the rescue of the growth inhibition phenotype using shRNA-resistant TBK1 was not performed. Here, we used a larger overlapping set of shRNAs, including two novel 3’UTR-targeting shRNAs. We also included two NSCLC cell lines previously shown to be sensitive to TBK1 knockdown (A549 and H23) as well as many other KRAS-mutant cancer cell lines. Collectively, these knockdown studies argue that the role of TBK1 in cell proliferation and growth mechanisms still remain to be elucidated. Identifying these determinants and codependencies, especially in the context of RAS mutations, could still have significant implications for targeted therapy of KRAS-mutant cancers (40).

Using small-molecule kinase inhibitors of TBK1 is a complementary approach to a genetic knockdown strategy for clarifying the role of TBK1 in cancer. Recently, small-molecule inhibitors of TBK1 tested in lung, pancreas, and colon cancer cell lines revealed a small subset of cell lines sensitive to TBK1 inhibition but this sensitivity did not correlate with KRAS dependence (5, 8). Here, we used TBK1 inhibitors of three different structural classes and found only weak growth reduction and this activity did not correlate with reduction of pIRF3S386 levels. In addition, the activity observed in a large panel of cell lines did not correlate with the RAS signatures of those cell lines (37). Therefore, TBK1 inhibition alone is not sufficient to drive a robust growth inhibition phenotype in these cell lines. It is possible that by combining inhibitors of other KRAS-activated pathways would be a more effective treatment paradigm (40).

TBK1 integrates a variety of upstream signals via stimulation of TLRs and directly modulates the function of numerous downstream targets. Indeed, it is unclear what TBK1 substrates are important for cancer survival, and we have provided evidence that pIRF3S386 is a direct substrate of TBK1 for some NSCLC, PDAC, and colorectal cancer cell lines. Previous studies have demonstrated the potential for TBK1 to autophosphorylate when overexpressed in 293 T cells (3). However, it is unclear what other endogenous kinases can activate TBK1, though it is known that ubiquitination regulated by its binding partners TRAM, TRIF, TRAF3, and RalB, controls its activation. In the presence of TBK1 inhibitors, TBK1 remains phosphorylated and in fact, TBK1 phosphorylation seems to increase, suggesting a feedback mechanism that stimulates the endogenous activating kinase (10). It also is likely that depending on what upstream signals are activating TBK1 (i.e., TLR4, TGFβ, etc.) different downstream targets might be involved in regulating the pathway. Recent advances in the availability and specificity of TBK1 inhibitors coupled with validated biomarkers such as pIRF3S386 should provide improved tools to help address these issues and hopefully provide better insights into our understating of the complex network of TBK1 regulatory pathways in cancer. Continued research on these emerging pathways will hopefully yield key insights into TBK1 biology and establish important directions into treating diseases targeted through TBK1.

Disclosure of Potential Conflicts of Interest
Q. Pan is a senior principal scientist at Boehringer Ingelheim. P. Strack is a director at Merck & Co. E.J. Morris is a Scientist at Merck. No potential conflicts of interest were disclosed by the other authors.

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