Development of a High-Throughput Assay for Identifying Inhibitors of TBK1 and IKKε

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Abstract
IKKε and TBK1 are noncanonical IKK family members which regulate inflammatory signaling pathways and also play important roles in oncogenesis. However, few inhibitors of these kinases have been identified. While the substrate specificity of IKKε has recently been described, the substrate specificity of TBK1 is unknown, hindering the development of high-throughput screening technologies for inhibitor identification. Here, we describe the optimal substrate phosphorylation motif for TBK1, and show that it is identical to the phosphorylation motif previously described for IKKε. This information enabled the design of an optimal TBK1/IKKε: substrate peptide amenable to high-throughput screening and we assayed a 6,006 compound library that included 4,727 kinase-focused compounds to discover in vitro inhibitors of TBK1 and IKKε. 227 compounds in this library inhibited TBK1 at a concentration of 10 μM, while 57 compounds inhibited IKKε. Together, these data describe a new high-throughput screening assay which will facilitate the discovery of small molecule TBK1/IKKε: inhibitors possessing therapeutic potential for both inflammatory diseases and cancer.

Introduction
The IKK family of kinases consists of four family members, the canonical IKKα and IKKβ, as well as two noncanonical family members, IKKε and TBK1. Together, this family of kinases regulates a myriad of critical cellular processes including inflammation, survival, proliferation, senescence, and autophagy [1–4]. Consistent with these numerous functions, aberrant IKK signaling can result in susceptibility to diseases such as inflammatory disorders and cancer [1,3,5,6]. The canonical IKK complex, which consists of IKKα, IKKβ, and a regulatory subunit, NEMO, is a point of convergence for a variety of stimuli. Upon activation, the canonical IKKs, primarily IKKβ, phosphorylate IkBα, the inhibitor of NF-kB, which promotes the ubiquitination and degradation of IkBα [3,7,8]. The transcription factor NF-kB is then freed to accumulate in the nucleus and activate the transcription of a number of target genes involved in inflammatory and stress responses [3,7,8].

In contrast to the canonical IKKs, IKKε and TBK1 are activated by a smaller subset of inflammatory stimuli, and are especially critical for antiviral responses [6,7,9]. These kinases phosphorylate and activate the transcription factors IRF3, IRF7, and STAT1, promoting a Type 1 interferon response [10–14]. These kinases also activate NF-kB, but the mechanism by which this occurs in unclear since they do not phosphorylate both of the serines on IkBα which are required for IkBα degradation [15,16]. IKKε and TBK1 can also promote oncogenesis. For example, IKKε is overexpressed in some breast and ovarian cancers, and TBK1 was recently shown to be important for Ras-induced cell transformation [17–20]. In spite of the important role these kinases play in both inflammatory and oncogenic signaling, few inhibitors have been identified. BX-795, a small molecule inhibitor of 3-phosphoinositide-dependent protein kinase 1 (PDK1), inhibits both IKKε and TBK1 at low nanomolar concentrations in vitro (IC50 at 41 nM and 6 nM, respectively) [21,22]. However, BX-795 lacks selectivity as 16 out of 76 tested kinases were inhibited by BX-795 in the nM range [21]. It was also recently shown that a series of azabenimidazole derivatives inhibits these kinases in the low nM range, but 6 of 79 kinases tested using one of these compounds were inhibited in a range within 10-fold of TBK [23]. These results suggest that IKKε and TBK1 are suitable targets for small molecule inhibitor development, but the need for the development of selective inhibitors of IKKε and TBK1 remains.

The development of high throughput assays to identify inhibitors of TBK1 and IKKε was hindered until recently by the absence of information regarding the substrate specificities of these enzymes. Peptide substrates for IKKε and TBK1 are frequently based on the IKKβ phosphorylation sites in IkBα, even...
though there is no evidence that all IKK family members phosphorylate the same substrate repertoires. In fact, the recently published phosphorylation motifs for IKK\(\alpha\), IKK\(\beta\) and IKKe suggest that these kinases do have overlapping, but quite different, optimal peptide substrates, although a detailed comparison of the ability of IKK family members to phosphorylate these different peptide substrates has not been performed [24–26]. The phosphorylation motif for TBK1 has not been previously reported.

Here, a positional scanning peptide library (PSPL) technology was used to determine the optimal phosphorylation motif for TBK1. We demonstrate that the substrate specificity of TBK1 is identical to that of IKKe, but differs from the phosphorylation motif of IKK\(\beta\) at key positions. Importantly, we also demonstrate that, like IKKe, TBK1 phosphorylates its predicted optimal peptide (TBK1-Tide) more efficiently than an optimal peptide for IKK\(\beta\) or a peptide containing the IKKe phosphorylation sites present in IxB\(\alpha\). We then used this information to develop and validate an IKKe/TBK1 peptide substrate appropriate for high-throughput chemical screening and executed a high-throughput screen (HTS) against both TBK1 and IKKe.

**Results**

While it is clear that misregulation of TBK1 activity can promote inflammatory disorders and may play a role in oncogenesis, the role of TBK1 in these signaling pathways is poorly understood. Determining the substrate specificity of TBK1, therefore, would facilitate both the prediction of novel TBK1 substrates and the development of high-throughput assays to identify effective TBK1 inhibitors. To this end, we utilized PSPL technology to determine the optimal TBK1 phosphorylation motif using GST-TBK1 purified from HEK293T cells [27,28]. This technology employs 198 biotinylated peptide libraries, which are used as substrates in individual solution-based kinase assays. Each peptide library has a mixture of serine and threonine at a fixed central position and also has one other position fixed to one of the 20 naturally-occurring amino acids. Phosphothreonine and phosphotyrosine were also included at the fixed positions to allow the identification of primed phosphorylation events. All other positions contain a degenerate mixture of amino acids. Following a kinase reaction, the biotinylated peptides are separated in a capillary channel etched in a quartz microfluidic device 

**2. microfluidic capillary electrophoresis (MCE). MCE operates on the principle that small fluorescently-labeled peptide substrates from ~100 nanoliter sized aliquots of reaction samples are separable in a capillary channel etched in a quartz microfluidic chip ratio when a current is applied [29,30]. This technique has been widely adopted as a gold standard assay in the profiling of small molecule inhibitors of kinases and can be tested in a high-throughput fashion [31,32]. TBK1-Tide was synthesized with an N-terminal 5’-Carboxyfluorescein (5-FAM) dye for use as a substrate. The TBK1-Tide synthesized for HTS (5-FAM-Ala-ADADYASLDWDA-NH2) retained all of the residues critical for phosphorylation by TBK1 and IKKe, but had the -1 and -4 Asp...
residues changed to Ala in order to decrease the likelihood of Asp isomerization. TBK1 and IKKe were characterized for their behavior with this substrate and found to have Km values for ATP of 7.5 μM and 4.7 μM respectively (Figure 4A-B). Because of the MCE system's limits of detection, TBK1-Tide was fixed at a concentration of 1 μM and Km values for substrate were not determined. The enzyme concentrations were then titrated and fixed to 120 nM for TBK1 and 81 nM for IKKe. These values were chosen to give 30% conversion of substrate to product after 2 hours of incubation (data not shown).

Compounds were screened at a final concentration of 10 μM in 0.1% DMSO. Two libraries of compounds were tested. The Library of Pharmacetically Active Compounds (LOPAC) consists of 1280 known bioactive small molecules, including 300 FDA approved drugs including antibiotics, and compounds targeting gene regulation and expression, multi-drug resistance, apoptosis, ion channels, neurotransmission, calcium signaling, lipid signaling and phosphorylation regulation. This library was tested in duplicate to establish the reproducibility of the screen (Figure S2). A second kinase-focused library consisting of 4,727 unique and “rule of five” compliant compounds was also tested (Figures 5A and B) [33].

Results from the HTS were found to follow a quasi-normal distribution (Figures 5A and B) [34]. Compounds that showed inhibition values greater than three standard deviations from the mean value (50% inhibition for TBK1 and 30% for IKKe) were considered active. Active compounds from each target were clustered based on structural similarity using Pipeline Pilot software [35] and filtered for drug-like properties using the REOS filter (Table 1) [36]. Active compounds from the kinase cassette that showed selectivity for either enzyme were re-tested in 10 point dose response curves to establish potency values. This screen demonstrated that 227 compounds in the library inhibited TBK1 at a concentration of 10 μM and 57 compounds inhibited IKKe at a concentration of 10 μM (Figure 6). The structures of the five most active compounds are shown in Figure 7, including 4 TBK1-specific inhibitors and 1 dual TBK1/IKKe inhibitor. All of these compounds inhibited these kinases at concentrations of less than 1 μM.

As IKKe and IKKa are closely related to TBK1 and IKKe, it was important to determine the specificity of any candidate inhibitors. A secondary screen was therefore performed to evaluate the ability of the top-scoring TBK1 and IKKe inhibitors to inhibit the canonical IKKes. It was determined that IKKβ phosphorylated TBK1-Tide efficiently enough to perform this secondary screen, though for IKKa the commercially available Caliper FL-1 peptide was more efficiently phosphorylated than TBK1-Tide (data not shown). The ATP Km of IKKβ for TBK1-Tide was 2 μM and the ATP Km of IKKe for FL-1 was 134 μM (Figure S3). Enzyme concentrations were titrated and fixed to 11.5 μM for IKKa and 1.1 μM for IKKβ and the screen was performed as described above for TBK1 and IKKe. Importantly, few of the compounds which inhibited TBK1 or IKKe also inhibited IKKa or IKKβ.
and none of the most effective TBK1/IKKe inhibitors were effective against IKKa and IKKB (Figures 6, 7 and Table S1). Together, these data confirm that the inhibitors identified in this screen are specific for TBK1 or TBK1/IKKe among the IKK family members.

**Discussion**

The development of effective small-molecule screening technologies for kinases is dependent on appropriately measuring changes in enzyme activity. While phosphorylation of a known protein substrate can be measured as a reporter for kinase activity,
a peptide substrate is usually superior, as it is easier to generate large, consistent quantities, and is more amenable to the development of non-radioactive assays. However, the generation of an optimal peptide substrate requires a thorough understanding of kinase substrate specificity, and this information is only available for a small fraction of the >500 protein kinases in the human genome. The substrate specificities of three IKK family members, IKKa, IKKB and IKKe, have recently been described [24–26].

Like IKKe, TBK1 is a noncanonical IKK family member which regulates Type I interferon signaling and may play a role in oncogenesis. Here, a positional scanning peptide library technology was utilized to identify the optimal phosphorylation motif for TBK1 (Figure 1) [27]. The substrate specificity of TBK1 is

![Figure 4. ATP Km determination for TBK1 and IKKe.](image)

Enzymatic reactions of A) TBK1 or B) IKKe were incubated at room temperature with 10 ATP concentrations varying from 333 μM to 0.017 μM in three fold dilutions. Reactions were sampled on the Caliper EZReader system at 9.35 minute intervals over a 3 hour period. Percent conversions were calculated from relative heights of product and substrate peaks and used to calculate velocity and ATP Km in Graph Pad Prism.

doi:10.1371/journal.pone.0041494.g004

![Figure 5. Distribution of compound activity. A–B) The kinase library was screened at 10 μM in a single concentration format against enzymatic reactions of A) TBK1 and B) IKKe. The distribution of activity is shown as a frequency histogram based on the number of compounds active at each level (% Inhibition). The data follow a normal distribution.](image)

doi:10.1371/journal.pone.0041494.g005
identical to that of related kinase IKKα. Interestingly, the substrate specificities of the noncanonical IKKs share overlapping characteristics with the substrate specificity of the canonical IKKs, but the optimal peptide substrates for these kinases are quite different (Figure 2). These data allowed the generation of a peptide substrate for TBK1 and IKKα (TBK1-Tide) which is amenable to high-throughput screening. This technology was then used to screen the LOPAC and Kinase libraries, and the number of chemical clusters and singleton hits as described in the text.

table1

<table>
<thead>
<tr>
<th>TBK1</th>
<th>IKKα</th>
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<tr>
<td>N_unfiltered hits = 227 (@ inh.&gt;50%)</td>
<td>N_unfiltered hits = 57 (@ inh.&gt;30%)</td>
</tr>
<tr>
<td>N_filtered drug-like = 184</td>
<td>N_filtered drug-like = 33</td>
</tr>
<tr>
<td>N_LOPAC = 18</td>
<td>N_LOPAC = 12</td>
</tr>
<tr>
<td>N_Kinase lib = 166</td>
<td>N_Kinase lib = 21</td>
</tr>
<tr>
<td>N_clusters = 11 (N_min_cluster ≥ 3)</td>
<td>N_clusters = 0 (N_min_cluster ≥ 3)</td>
</tr>
<tr>
<td>N_singletons = 105</td>
<td>N_singletons = 27</td>
</tr>
</tbody>
</table>

Number of active compounds (N) detected in each screen for total number detected (unfiltered), number after drug-like filtering (filtered.drug-like), hits from the LOPAC and Kinase libraries, and the number of chemical clusters and singleton hits as described in the text.

doi:10.1371/journal.pone.0041494.t001

Figure 6. Relative activity of active compounds. Activity of selected compounds tested in the potency screen are shown as a heat map scaled from <1 μM (purple) to >10 μM (red) against IKKα, IKKβ, IKKε, and TBK1.

doi:10.1371/journal.pone.0041494.g006

Identification of TBK1 and IKKε Inhibitors

Materials and Methods

Antibodies, Plasmids, and Reagents

GST-TBK1 was created by PCR cloning into the BamHI site of the pEBG vector. GST-TBK1 K38A was created using a modification of the QuickChange Site-directed mutagenesis protocol (Stratagene). GST-IKKα, GST-IKKβ, and GST-IKKε for in vitro kinase assays were generated and used as previously reported [24–26]. TBK1-Tide (ADDDYDSLDWDAKKK), TBK1-Y5A (ADDDADSLDWDAKKK), and TBK1-L8A (ADDDYDSADWDAKKK) used in Figure 1 were created and HPLC purified under contract by the Tufts University Core Facility. The TBK1 peptide used in Figures 2 and 3 (ADADYASLDWDAKK) was generated to decrease the likelihood of Asp isomerization observed

Figure 6. Relative activity of active compounds. Activity of selected compounds tested in the potency screen are shown as a heat map scaled from <1 μM (purple) to >10 μM (red) against IKKα, IKKβ, IKKε, and TBK1.

doi:10.1371/journal.pone.0041494.g006
with the original TBK1-Tide. This peptide, as well as IKK\(\beta\)-Tide-pT (ADpTRYESIDEEAKKK) and IKK\(\beta\)-Tide-A (ADARYESIDEDEEAKKK) were also generated and HPLC purified by the Tufts University Core Facility. The IkBa (IKK substrate) peptide was obtained from Upstate. The FL-1 peptide (5-FAM-AKRRRLSSLRA-COOH) was obtained from Caliper Life Sciences. For all library screening, recombinant IKK\(\alpha\), IKK\(\beta\), IKKe and TBK1 were purchased from Life Technologies (Invitrogen).

Cell Culture, Transfection, Immunoprecipitations, and Western Blotting

HEK-293T cells were obtained from ATCC and were grown in DMEM containing 10% FBS. Transfection was performed by polyethylenimine. For preparation of recombinant kinases, cells were lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM \(\beta\)-glycerophosphate, 1 mM PMSF, 1 mM sodium orthovanadate, 1 \(\mu\)g/mL leupeptin, 1 \(\mu\)g/mL pepstatin, and 10 nM Calyculin A.

Design of 4,727 Member Kinase-focused Library

More than 100K compounds were initially reviewed in the form of SD files from Life Chemicals, ChemDiv, Asinex and Enamine. These kinase-focused libraries were designed by their respective vendors using one or more of the following approaches: 1) searching virtual and physical general purpose libraries for compounds similar to known kinase inhibitors, 2) selecting or synthesizing compounds having a hinge-binding motif, e.g. heterocycles with a high likelihood to bind the kinase hinge motif conserved in nearly every kinase-small molecule X-ray structure, and 3) structure- or ligand-based virtual screening on representative kinase structures. Following an analysis of each vendor’s library, the UNC CICBDD acquired 4,727 compounds (from all four vendors) that all were unique and “rule of five” compliant [33].

High-throughput Screen for TBK1, IKKe, IKK\(\alpha\) and IKK\(\beta\) Inhibitors

5-FAM-labeled TBK1-Tide for library screening was generated in the High-Throughput Peptide Synthesis and Arrays Core Facility at University of North Carolina at Chapel Hill. The 1,280 compound Library of Pharmaceutically Active Compounds (Sigma) and the UNC CICBDD 4,727 compound kinase-focused library were evaluated for their ability to inhibit phosphorylation of 5-FAM-TBK1-Tide (TBK1, IKKe, IKK\(\beta\)) or FL-1 (IKK\(\alpha\)) using the MCE Ezreader system from PerkinElmer.

In vitro Kinase Assays

Kinase buffer contained 50 mM Tris (pH 7.5), 12 mM MgCl\(_2\), 1 mM \(\beta\)-glycerophosphate, 100 \(\mu\)M ATP, and 10 \(\mu\)Ci \(\gamma\)-\(^{32}\)P-ATP/reaction. Reactions were incubated at 30°C for 1h. Recombinant GST-TBK1 and GST-TBK1 K38A used in the positional scanning peptide library assay were generated as described above and the assay was performed as described previously [27,28]. MCE assay buffer contained 50 mM Heps (pH 7.4), 0.01% Triton X-100, 10 mM MgCl\(_2\), 1 mM DTT, 0.01% BSA and ATP at the determined Km (4.7 \(\mu\)M for IKKe, 7.5 \(\mu\)M for TBK1, 134 \(\mu\)M for IKK\(\alpha\), and 2 \(\mu\)M for IKK\(\beta\)). IKKe reactions were incubated for 2 hours at room temperature and TBK1, IKK\(\alpha\), and IKK\(\beta\) reactions were incubated for 6 hours at room temperature.

Supporting Information

Figure S1 Selectivity values for TBK1. Following the PSPL assay, relative affinities for each amino acid at each position relative to the phosphorylation site were calculated.

Figure S2 Comparison of duplicate compound assay values. A–B) The LOPAC library was screened in duplicate at 10 \(\mu\)M in a single concentration format against enzymatic reactions of A) TBK1 and B) IKKe. Results from the first determination are shown on the horizontal axis (Result 1) and the second determination is shown on the vertical axis (Result 2).

Figure S3 ATP Km determination for IKK\(\alpha\) and IKK\(\beta\). Enzymatic reactions of A) IKK\(\alpha\) and B) IKK\(\beta\) were incubated at room temperature with 10 ATP concentrations varying from...
333 μM to 0.017 μM in three fold dilutions. Reactions were sampled on the Caliper EZReader system at 9.35 minute intervals over a 3 hour period. Percent conversions were calculated from relative heights of product and substrate peaks and used to calculate velocity and ATP Km in Graph Pad Prism.

Table S1  Most active compounds from the LOPAC set. Values represent percent inhibition of the listed kinase isof orm when treated with the indicated inhibitor at a concentration of 10 μM after 2 hours (at completion of the assay as described in the text).

References

Acknowledgments
We thank members of the Baldwin lab and CICBDD for helpful discussions.

Author Contributions
Conceived and designed the experiments: JEH ASB WPJ. Performed the experiments: JEH MAP JGC. Analyzed the data: JEH WPJ DK XW. Contributed reagents/materials/analysis tools: LCC. Wrote the paper: JEH ASB WPJ.

PLoS ONE | www.plosone.org 8 July 2012 | Volume 7 | Issue 7 | e41494