Development of an HTS-Compatible Assay for the Discovery of ASK1 Signalosome Inhibitors Using AlphaScreen Technology

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ABSTRACT

Genetic target validation studies have demonstrated that the apoptosis signal-regulating kinase 1 (ASK1) represents an important target for the treatment of rheumatoid arthritis, cardiac diseases, and several neurodegenerative disorders. To identify small-molecule inhibitors of ASK1, we have developed a high-throughput screening-compatible, homogenous, biochemical assay using AlphaScreen technology. This novel assay design utilizes purified stress-activated ASK1 signalosome complex, and it monitors phosphorylation of its full-length native substrate, MKK6. The assay has been optimized in a 384-well format and validated by screening the Sigma LOPAC library. The results presented here demonstrate that the assay is sensitive and robust with a Z' factor value of 0.88 ± 0.04 and a signal-to-background ratio of 11, indicating that this assay can be used to screen large chemical libraries to discover novel inhibitors of ASK1.

INTRODUCTION

The apoptosis signal-regulating kinase 1 (ASK1) is a ubiquitously expressed mitogen-activated protein kinase kinase (MAP3K) that is involved in the modulation of cell differentiation, survival, apoptosis, and senescence.¹ In resting cells, ASK1 is associated with scaffold proteins within high-molecular-mass complexes (1,000–2,000 kDa), designated as the ASK1 signalosome.^{2,3} ASK1 is activated by environmental and biological stressors, including lipopolysaccharides, reactive oxygen species such as H_2O_2 , tumor necrosis factor alpha, endoplasmic reticulum stress, influx of calcium ions, cytokines, Fas ligand, and GPCR agonists.^{4–6} In addition to its central role in responding to cellular stress, ASK1 also mediates physiological processes such as neuronal differentiation, synaptic plasticity, and the innate immune response.^{7–9} The manner in which ASK1 signalosome is regulated and responds to this wide array of conditions is only beginning to be understood. The activity of ASK1 signalosome complex is controlled by a number of mechanisms, including post-translational modification and protein-protein interactions. For example, redox imbalance induces molecular rearrangements within the ASK1 signalosome, and ASK1 autophosphorylation (residue Thr838 located in the activation loop) results

in MKK3/6 and MKK4/7 recruitment and phosphorylation, which, in turn, leads to the activation of p38 and JNK pathways, respectively. Furthermore, mechanisms that either directly modulate the catalytic activity of the kinase or indirectly alter its specificity for a particular substrate, its stability, or its subcellular localization have been identified.^{3,10–14} Thus, ASK1 signaling is tightly regulated by multiple and distinct molecular events.

Excessive ASK1 signalosome activity has been linked to nervous system disorders, cardiac diseases, diabetes, and inflammation. Consequently, ASK1 signalosome has emerged as a possible drug target for the treatment of these pathological conditions. In addition, ASK1 knockout mice exhibit no developmental abnormalities and appear healthy under normal laboratory conditions, suggesting that selective ASK1 inhibitors may be well tolerated therapeutically.¹⁵ Recently, small-molecule inhibitors of ASK1 were identified using *in silico* approaches. However, these inhibitors exhibit low potency (IC_{50} , 3–13 μ M), and further medicinal chemistry attempts failed to improve the activity of these molecules.^{16,17}

In this study, we present the development of a robust and sensitive high-throughput compatible biochemical assay that will enable the discovery of new small-molecule inhibitors of ASK1 signalosome. Since *in vitro* assays using purified kinase domains and synthetic substrates may not be a true reflection of *in vivo* kinase-substrate interactions and phosphorylation, we expressed and purified the stress-activated ASK1 signalosome, and biotinylated full-length MKK6, to enable and develop a high-throughput screening (HTS)-compatible Amplified Luminescent Proximity Homogenous Assay (AlphaScreen®). We validated the assay by screening the Sigma LOPAC library. Importantly, we demonstrate that the ASK1 inhibitor AlphaScreen assay is robust and sensitive with an average Z' factor value of 0.88 ± 0.04 and a signal-to-background (S/B) ratio of 11. Several hits, many of which were known kinase inhibitors, were identified and confirmed, indicating that our assay is suitable for the identification of small molecules which are capable of blocking ASK1-mediated MKK6 phosphorylation. Thus, the assay we describe here can be used to screen large chemical libraries to discover novel inhibitors targeting stress-activated ASK1 signalosome.

MATERIALS AND METHODS

Cell Culture and Reagents

Human embryonic kidney cells (HEK293T) were purchased from American Type Culture Collection (ATCC). HEK293T cells were maintained at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's

modified Eagle's medium (Invitrogen), containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. All of the chemicals including the library of pharmacologically active compounds (Sigma LOPAC1280™) were purchased from Sigma-Aldrich. The LOPAC library was reformatted at a 2.5 mM concentration in dimethyl sulfoxide (DMSO) into 384-well format source plates obtained from Greiner Bio-One. ASK1 full-length protein fused to an N-terminal GST-tag (cat# PV3809) was purchased from Invitrogen. Recombinant human MKK6 protein fused to an N-terminal Mal-E tag (cat# 14-304) was purchased from Millipore. ASK1 (cat# 3762), MKK6 (cat# 9264) and phospho-specific MKK6 antibodies (cat# 9236) were purchased from Cell Signaling Technology. Mouse monoclonal anti-Flag (FLAG M2, cat# F3165) antibody was purchased from Sigma-Aldrich. IRDye-labeled antibodies and streptavidin were purchased from Licor. The AlphaScreen reagents were purchased from PerkinElmer.

Protein Expression, Purification, and Biotinylation

Biotinylation of recombinant human MKK6 protein fused to an N-terminal Mal-E tag was performed using EZ-Link Micro Sulfo-NHS-LC-Biotinylation Kit according to the manufacturer's protocol (cat# 21935; Pierce). Generation of the ASK1 expression construct has been previously described by us.¹² Briefly, for ASK1, HEK293T cells were transiently transfected with an ASK1-expressing construct at 24 h after plating using calcium phosphate precipitation. Sixteen hours after transfection, the medium was replaced with fresh medium and cells were cultured at 37°C in 5% CO₂ for an additional 24 h. For ASK1 signalosome purification, cells were washed with ice-cold phosphate-buffered saline pH 7.0 and extracted in ice-cold M-PER lysis buffer (Thermo Scientific) containing Complete protease inhibitors (Roche Diagnostic) and a phosphatase inhibitor cocktail (Sigma-Aldrich). The lysate was incubated on ice for 10 min and centrifuged at 14,000 *g* for 15 min at 4°C. The protein was purified from the cleared lysate by anti-FLAG M2 affinity chromatography (Sigma-Aldrich). A 1 mL bed volume of FLAG-agarose was equilibrated in buffer A (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). The clarified lysate was diluted in buffer A to a concentration of 1 mg/mL and loaded onto the column at 0.25 mL/min. The column was washed with 20 column volumes of buffer A and eluted using buffer B (0.1 M glycine-HCl, pH 3.5). The eluted protein was dialyzed against buffer A and concentrated by filtration to a final concentration of 0.3 mg/mL. DTT and glycerol were added to the purified sample to a final concentration of 2 mM and 10%, respectively, and stored in aliquots at –80°C.

For purification of the MKK6 substrate, *Escherichia coli* BL21 (AI) cells were co-transformed with the pDEST14-Avi-MKK6-FLAG expression construct and the BirA plasmid (GeneCopoeia). A 10 mL overnight culture was used to inoculate 500 mL LB media containing 50 µg/mL ampicillin and 33 µg/mL chloramphenicol. Cultures were grown at 37°C until the OD₆₀₀ reached ~0.5. The temperature was adjusted to 30°C to enable optimal expression. D-biotin (Supelco) was added at a final concentration of 50 µM, and expression of MKK6 and BirA was induced for 4 h with 0.2% L-arabinose and 0.5 mM IPTG, respectively. Bacteria were collected by centrifugation, and

proteins were extracted with B-PER lysis buffer (Thermo Scientific) containing complete protease inhibitors; incubated on ice for 10 min; and clarified by centrifugation at 4°C (14,000 *g* for 15 min). The protein was purified by anti-FLAG M2 affinity chromatography (Sigma-Aldrich). A 1 mL bed volume of Flag-agarose was equilibrated in buffer A (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). The clarified lysate was diluted in buffer A to a concentration of 1 mg/mL and loaded onto the column at 0.25 mL/min. The column was washed with 20 column volumes of buffer A and eluted using buffer B (0.1 M glycine-HCl, pH 3.5). The eluted protein was equilibrated with wash buffer. DTT, EGTA, and glycerol were added to the protein sample to a final concentration of 2, 0.1 mM, and 10%, respectively.

Immunoblotting

Protein concentration of the lysates was measured using the BCA Protein Assay Reagent (Pierce). Thirty microgram of protein was added to Laemmli sample buffer containing 4% of 2-mercaptoethanol and heated to 90°C for 5 min. The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (4%–12%) and blotted onto nitrocellulose using the iBlot system (Invitrogen). The membranes were blocked with Odyssey blocking buffer (Licor) and probed with the indicated primary antibody (1:333) for 45 min using SNAP i.d. (Millipore) system. After washing with Odyssey blocking buffer, the blots were incubated for 45 min with the appropriate IRDye-labeled secondary antibodies (Licor) and the blots were visualized using Odyssey infrared imaging (Licor) and Licor Image Studio Lite software.

ASK1 Signalosome Inhibitor AlphaScreen Assay

Experiments were performed in low volume, 384-well, white Optiplates (Greiner Bio-One) under green light conditions (100 lx) at room temperature. Detection buffer (DB) was prepared by mixing Protein A Acceptor beads and anti-phospho-MKK6 (1:200) in 25 mM Tris-HCl, 200 mM NaCl, 100 mM EDTA 0.3% BSA, and 0.01% Triton X-100. After a 1 h incubation period at room temperature, donor beads were added (80 µg/mL) and the mix was directly added to the kinase reaction. Kinase assay was performed in kinase buffer (25 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, 0.01% Triton X-100, pH 7.4). The assay was performed under the following conditions: 5 µL of kinase buffer containing stress-activated ASK1 signalosome and adenosine triphosphate (ATP) (60 µM) were mixed with 5 µL of kinase buffer containing the test compound and incubated for 10 min before the addition of 5 µL of kinase buffer containing MKK6. Reactions were stopped by the addition of 5 µL of DB. After 16 h of incubation in the dark at room temperature, the AlphaScreen signal was measured at a wavelength of 520–620 nm using the Envision (PerkinElmer).

Data Analysis

To monitor assay sensitivity and evaluate the robustness of the assay, S/B ratios and Z' factor values were calculated using the equations previously described.¹⁸ IC₅₀ values were calculated by non-linear regression analysis (sigmoidal dose response; variable slope) using Prism software (GraphPad Software, Inc.). Data analysis

nalosome and its full-length substrate, MKK6, it was possible to develop an HTS-compatible assay. The principles of the AlphaScreen assay are outlined schematically in Figure 1.

Expression and Purification of ASK1 and Biotinylated MKK6

We have previously described the expression and purification of stress-activated ASK1 signalosome.¹² In order to generate biotinylated MKK6 substrate, full-length human MKK6 fused at the C-terminus to the FLAG epitope and at the N-terminus to the avidin tag (Avi-MKK6-FLAG) was co-expressed with a biotin ligase (BirA) in *E. coli*. The recombinant biotinylated Avi-MKK6-FLAG protein was purified by affinity chromatography, and the purity was examined by SDS gel electrophoresis. Coomassie staining and Western blot analysis showed that the purified Avi-MKK6-FLAG protein migrated as one major band at ~40 kDa (Fig. 2A, B). In addition, Avi-MKK6-FLAG biotinylation was confirmed using dye-labeled

for the LOPAC¹²⁸⁰ compound library was performed using Symyx Assay Explorer version 3.2 (Symyx Technologies, Inc.).

RESULTS

Assay Principle

The aim of this work was to develop a robust 384-well *in vitro* biochemical assay using AlphaScreen technology and to validate the assay as HTS compatible by pilot screening the Sigma-LOPAC collection. We observed earlier in our studies that the commercially available reagents produced low-emission signals, resulting in poor S/B (≤ 2) even at high kinase and substrate concentrations (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/adt) and, consequently, were not suitable for the development of a robust HTS-compatible assay. We and others previously reported that ASK1 exists within a higher-order protein complex which requires an initial stress for activation. Thus, we evaluated whether using the intact human stress-activated ASK1 sig-

Fig. 2. Characterization of recombinant Avi-MKK6-FLAG. Human Avi-MKK6-FLAG protein was expressed in *Escherichia coli* and purified using an anti-FLAG M2 affinity column. (A) The purity of the Avi-MKK6-FLAG recombinant protein was determined using Coomassie blue-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). A band of the anticipated molecular weight (~40 kDa) was observed. (B) Immunoblots of purified biotinylated Avi-MKK6-FLAG protein were probed with a specific anti-MKK6 antibody, an anti-phospho-MKK6 antibody, or dye-labeled streptavidin. No detectable band was observed using an anti-phospho-MKK6 antibody.

streptavidin. Importantly, the purified Avi-MKK6-FLAG protein was not phosphorylated at serine 207, as revealed by Western blot analysis (Fig. 2B) and by mass spectrometry (data not shown). Thus, the purified Avi-MKK6-FLAG protein provides an ideal full-length substrate for the detection of ASK1-mediated MKK6 phosphorylation *in vitro*.

ASK1 Signalosome Inhibitor AlphaScreen Assay
Optimization and Validation

We previously determined the stress-activated ASK1 signalosome $K_m(ATP)$ value as 18 μM by measuring the incorporation of radioactive phosphate into full-length MKK6 using a 96-well format radiometric

filtration binding assay.¹² To assess whether a chemiluminescent signal can be detected using an AlphaScreen format, a concentration matrix of ASK1 signalosome and MKK6 was tested and incubated with kinase buffer containing 20 μM ATP. After a 30 min incubation period, DB containing the donor and acceptor beads was added to stop the reaction and then, the phosphorylation of Avi-MKK6-FLAG was detected. In the presence of low nanomolar concentration of ASK1 signalosome (3 nM) and MKK6 (30 nM), we observed a five- to seven-fold increase in S/B (Fig. 3A). Time course experiments conducted with 3 nM of ASK1 and 30 nM of MKK6 in the presence of 20 μM ATP were carried out. After non-linear regression analysis

Fig. 3. Optimization of ASK1 signalosome inhibitor AlphaScreen assay. **(A)** Cross-titration of purified ASK1 signalosome versus purified Avi-MKK6-FLAG. Kinase and substrate were incubated in kinase buffer that was supplemented with 20 μM ATP for 20 min. Phosphorylation of MKK6 at serine 207 was detected using AlphaScreen technology. **(B)** Dependence of AlphaScreen signal (signal to background [S/B]) on incubation time. Kinase assays were conducted with 3 nM of ASK1, 30 nM of MKK6, and 20 μM ATP. Kinase reactions were stopped at the indicated time. **(C)** Dependence of AlphaScreen signal (S/B) on incubation time with the detection buffer (DB). After addition of the DB containing the donor and acceptor beads, the signal was measured at the indicated time. **(D)** Dependence of AlphaScreen signal (S/B) on ATP concentration. After 60 min, ASK1 (3 nM)-catalyzed phosphorylation of MKK6 (30 nM) was terminated, and AlphaScreen signal was detected. **(E)** Staurosporine IC_{50} was determined using the ASK1 inhibitor AlphaScreen assay. Thirty nanomolar of MKK6, 3 nM of ASK1, and 20 μM of ATP were incubated in the presence of an increasing concentration of staurosporine. After 60 min, the reactions were terminated and AlphaScreen signal was detected. The experiment was performed in quadruplicate, $S/B=11$, and $Z'=0.79$. Data are average $\pm$ standard deviation (SD).

(one site binding [hyperbola]), AlphaScreen signal intensity demonstrated linear dependence of ~60 min, resulting in S/B of ~11 (Fig. 3B). After a 16 h incubation time with the DB, we observed the highest S/B ratio (Fig. 3C).

As shown in Figure 3D, the K_m for ATP determined with the AlphaScreen assay was in good agreement with the K_m value obtained using radiometric filtration binding assay.¹² Thus, for the ASK1 signalosome inhibitor AlphaScreen assay, we choose to perform the kinase reaction for 60 min in a 15 μ L volume with kinase and a substrate concentration of 3 and 30 nM in the presence of 20 μ M ATP (Table 1).

To further evaluate the HTS compatibility of the ASK1 signalosome inhibitor AlphaScreen assay, we determined Z' factor values in the presence of staurosporine, a well-characterized non-selective kinase inhibitor. The Z' factor value was calculated as described by Zhang *et al.* [$Z' = 1 - (3 \text{ SD positive control} + 3 \text{ SD negative control}) / (\text{mean positive control} - \text{mean negative control})$].¹⁸ In good agreement with previously reported IC_{50} values, staurosporine concentration response curves (CRC) yielded an IC_{50} of 7.4×10^{-9} M in the ASK1 signalosome inhibitor AlphaScreen assay (Fig. 3E).¹⁹ Importantly, the S/B and Z' factor values were ~11 and ~0.79, respectively, confirming that the ASK1 signalosome inhibitor AlphaScreen assay which we have developed is both robust and reproducible. Before initiating a pilot screen, we optimized assay parameters for HTS compatibility; the

final reaction conditions are provided in the methods section. First, we investigated the effect of DMSO concentration on the assay performance. As shown in Figure 4A, although the AlphaScreen signal tends to decrease in the presence of DMSO concentration $\geq 1\%$, the S/B ratio value remains ≥ 6 in the presence of DMSO concentrations ranging from 0.3% to 1% (v/v). In addition, IC_{50} values obtained for staurosporine remained unchanged in the presence of DMSO concentrations ranging from 0.3% to 2% (v/v). Together, these data indicate that the DMSO tolerance of the assay is well within the typical concentration range of HTS campaigns (0.1%–1% v/v). To determine batch-to-batch reagent quality, the assay was further monitored over the course of 3 days. Again, there were no significant differences in the performance of the assay when run on different days. The average and standard deviation (SD) of AlphaScreen signal for high ($n = 16$) and low controls (10 μ M staurosporine; $n = 16$) on each day are represented in Figure 4B. The S/B ratios for days 1, 2, and 3 were ~11, 7, and 8, respectively, all with Z' values >0.6 and coefficient of variation (CV) values $<6\%$. Finally, experiments were conducted to assess whether there may be issues with reagent stability during a “full deck” HTS campaign. As shown in Figure 4C, the average and SD of AlphaScreen signal for high ($n = 16$) and low controls ($n = 16$) as well as staurosporine IC_{50} and efficacy were not significantly altered when the assay was performed with substrate, enzyme, and DBs stored at room temperature for approximately 24 h. Collectively, our data suggest that the ASK1 inhibitor AlphaScreen assay is robust and suitable for HTS purposes.

LOPAC Screen Results

To determine the performance of our optimized ASK1 assay under semi-automated HTS conditions, we screened the Sigma LOPAC (Library of Pharmacologically Active Compounds; 1280 compounds) in duplicate on 2 different days at a final concentration of 15 μ M (0.6% DMSO). An IC_{100} of staurosporine was used as a positive control (100% inhibition), and DMSO was used as a negative control (0% inhibition). An activity scatterplot of all compounds tested, as well as positive and negative controls, is shown in Figure 5. Each plate contained high ($n = 16$) and low ($n = 16$) signal control wells, which were used to assess plate homogeneity and assay reproducibility. Each day, high-signal and low-signal average, SD, and the CV from the Sigma LOPAC plates were calculated (Supplementary Table S1). Overall, the CV values were $<10\%$, an acceptable variance level for a biochemical assay.²⁰ In addition, the ASK1 signalosome inhibitor AlphaScreen assay demonstrated a Z' factor value of 0.88 ± 0.04 for the pilot screen confirming assay robustness. Furthermore, the scatterplot of replicate measurements yielded a correlation coefficient of $r^2 = 0.89$, indicating high reproducibility in hit identification (Fig. 5B).

For each compound, the AlphaScreen signal was converted into percentage inhibition (%INH). Any compound greater than the assay average plus three times the SD (i.e., percentage inhibition greater than 50%) was considered a compound suitable for follow-up assay.²¹ Using this hit-cutoff criterion, we identified 18 hits (hit rate of 1.4%). Consistent with expectations, many of the hits were known protein kinase inhibitors (Supplementary Table S2). IC_{50} values for

Table 1. Conditions for the ASK1 Signalosome Inhibitor AlphaScreen Assay

Step	Parameter	Value	Description
1	Add ASK1/ATP mix	5 μ L	9 nM/60 μ M in kinase buffer
2	Add DMSO/compound	5 μ L	In kinase buffer
3	Incubate	10 min	Room temperature
4	Add biotinylated MKK6	5 μ L	90 nM in kinase buffer
5	Incubate	60 min	Room temperature
6	Add AlphaLISA beads mix	5 μ L	In DB
7	Incubate	16 h	Room temperature
8	Read AlphaScreen signal		

Step Notes

1. ASK1 and ATP in kinase buffer (25 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, and 0.01% Triton X-100) are added at 3 $\times$ in white 384-well Optiplates.
2. Compounds in kinase buffer are added at 3 $\times$, final DMSO concentration 0.6%.
3. MKK6 in kinase buffer is added at 3 $\times$.
4. Addition of DB stops the reaction. DB is prepared by mixing Protein A Acceptor beads (80 μ g/mL) and anti-phospho-MKK6 (1:200) in 25 mM Tris-HCl, 200 mM NaCl, 100 mM EDTA 0.3% BSA, and 0.01% Triton X-100. After a 1 h incubation period, donor beads were added (80 μ g/mL).
5. AlphaScreen signal is measured at the wavelength 520–620 nm using the Envision.

ASK1, apoptosis signal-regulating kinase 1; ATP, adenosine triphosphate; DB, detection buffer; DMSO, dimethyl sulfoxide.

Fig. 4. Dimethyl sulfoxide (DMSO) tolerance and reagent stability assessment. **(A)** Staurosporine IC₅₀ determinations in the presence of increasing concentrations of DMSO. **(B)** Average and SD of AlphaScreen signal for high and low controls (10 μ M staurosporine) on AlphaScreen assay run on three different days. **(C)** Variation of AlphaScreen signal and staurosporine IC₅₀ determinations using ASK1 assay reagents after storage at room temperature for the indicated period of time. Data are average $\pm$ SD.

Fig. 5. Scatter plot analysis of LOPAC pilot screen. **(A)** All data from all assay plates, including high and low controls, are displayed. Large separation in activity ($Z' = 0.88 \pm 0.04$) between wells dosed with staurosporine ("high control") and DMSO ("low control"), as well as a broad distribution of hits indicate that the ASK1 inhibitor AlphaScreen assay is robust. **(B)** Representative graph of compound activity correlating with plate replicates. $r^2 = 0.89$ indicates that AlphaScreen assay is reproducible.

the hits were determined in triplicate (Supplementary Table S2), and CRC for the four most potent are shown in Figure 6. To assess the contribution of false positives, we tested the 18 hits in duplicate using the AlphaScreen “TruHits” assay. IC₅₀ values for the compounds were determined using TruHits assay under conditions that generated an AlphaScreen signal similar to the ASK1 signalosome assay. Indirubin-3'-oxime and Reactive Blue 2 were found to inhibit the AlphaScreen signal by more than 50% (Supplementary Table S2). Reactive Blue 2 IC₅₀ was similar in the TruHits assay and in the ASK1 signalosome assay, flagging it as a false positive. In contrast, indirubin-3'-oxime exhibited an IC₅₀ of 22.77 and 0.78 μ M in the TruHits assay and in the ASK1 signalosome assay, respectively, and, as such, is likely to be an ASK1 signalosome inhibitor. Collectively, our data are consistent with the notion that all but one of the hits may have genuine ASK1 kinase inhibitory activity.

DISCUSSION

Extensive *ex vivo* and *in vivo* genetic studies have demonstrated that activation of ASK1 by stress-inducing signals is associated with the development of a number of human pathological conditions, including neurodegenerative disease, inflammation, and heart failure.²² Thus, ASK1 is well validated as a drug target for a variety of human diseases. To date, a few ASK1 selective inhibitors have been described in the literature. Here, we describe the development of a novel HTS-compatible homogenous biochemical assay for the direct detection of ASK1-mediated MKK6 phosphorylation.

In resting cells, inactive ASK1 constitutively forms a high-molecular-weight complex (~1,500 kDa), known as the ASK1 signalosome.² On stimulation, autophosphorylation of ASK1 (Thr838), along with intermolecular rearrangements and the interaction with specific accessory proteins within the ASK1 signalosome, contributes to ASK1 activation. Furthermore, in addition to the interaction between the ASK1 kinase catalytic ATP-binding domain and the substrate phosphoacceptor site of MKK6, docking interactions are essential for the phosphorylation of MKK6.²³ In this regard, we have demonstrated that oxidative stress rapidly increases ASK1 catalytic efficiency (k_{cat}/K_m) for MKK6 phosphorylation by increasing MKK6 binding affinity within the ASK1 signalosome.¹² Thus, assays performed using only the purified kinase domain and synthetic substrates are far less likely to recapitulate ASK1 activity *in vivo*. Moreover, we could not develop a robust AlphaScreen assay by using a commercially available recombinant kinase and

Fig. 6. IC₅₀ of most potent hits from the LOPAC screen. Data are average $\pm$ SD.

substrate. The regulation of ASK1 signalosome activity may be more complex than a simple on/off switch and is capable of sensing and integrating multiple and various stimuli. Indeed, a plethora of proteins associated with the signalosome orchestrate ASK1 signaling by modulating the catalytic activity and/or the stability of the enzyme. Therefore, to develop our assay, we used ASK1 signalosome purified from mammalian HEK293T cells after H₂O₂-induced activation. By using a post-translationally modified ASK1 signalosome, our assay more closely mimics the *in vivo* situation. Importantly, to ensure that our assay is specific to ASK1 and to rule out the possibility that co-purifying contaminating kinases were responsible for substrate phosphorylation, we demonstrated that an activated signalosome containing a kinase inactive mutant of ASK1 is unable to phosphorylate recombinant MKK6.¹² Therefore, we anticipate that our assay will identify specific ASK1 signalosome inhibitors. Furthermore, we anticipate that identified inhibitors might exhibit a distinct mechanism of action. Indeed, we anticipate that our assay will enable the discovery of not only compounds that are ATP competitive, but also molecules operating at ASK1 allosteric sites or at regulatory proteins located within the ASK1 signalosome complex.

Table 2. Most Potent Hits from LOPAC Pilot Screen

Compound structure	Compound name	Biochemical/physiological action	IC ₅₀ (μM)	% of inhibition
SP00125	Inhibitor of c-JNK ²⁴	0.39	95 ± 1
Indirubin-3'-oxime	Inhibitor of CDK5, ²⁵ GSK3 ²⁶	0.78	107 ± 1
Aurintricarboxylic acid	Inhibitor of protein-nucleic acid interactions ²⁷	1.59	82 ± 8
Myricetin	Inhibitor of Akt, ²⁸ PI3K ²⁹	2.23	98 ± 1

CDK, cyclin dependent kinase; c-JNK, c-Jun N-terminal kinase.

We previously characterized the kinetics of ASK1 activity using a radiometric assay.¹² While this radioactive assay is adaptable to an HTS format, it is burdened with all the liabilities associated with the use and disposal of radioactivity. Thus, an Amplified Luminescent Proximity Homogenous (AlphaScreen) assay platform provides a simpler and more user-friendly alternative to the radioactive assay. This non-radioactive proximity assay is based on singlet oxygen transfer between a donor and an acceptor bead. We choose this assay format, because it is more permissive in terms of the maximum molecular distance allowable between donor and acceptor labels when compared with TR-FRET. Indeed, laser excitation at 680 nm of the donor beads converts ambient oxygen to an excited singlet state that can react with the acceptor bead, resulting in light emission measured between 520 and 620 nm if the distance between the donor and acceptor beads is within a 200 nm distance. In this work, we used generic AlphaScreen reagents, full-length kinase, and natural substrate, along with specific anti-MKK6 and anti-phospho-MKK6 antibodies to develop a robust and HTS-compatible ASK1 assay. In order to reduce the amount of reagent, enzyme, and substrate, we

developed a four-step assay using low-volume 384-well Optiplates. Importantly, we demonstrated that staurosporine, a well-characterized kinase inhibitor, yielded IC₅₀ values of about 7.4×10^{-9} M, which is in good agreement with previous reports.¹⁹ In addition, we demonstrated that the assay is robust on a batch-to-batch basis, that staurosporine IC₅₀ value is affected neither by typical HTS DMSO concentration, nor by reagent storage conditions typically used in HTS campaigns. Importantly, the pilot screen, conducted in semi-automated conditions, produced excellent S/B, Z' values, and plate-to-plate reproducibility. In addition, the most potent compound that we identified, SP00125 (Table 2), shares a similar core structure with the recently reported ASK1 inhibitor NQDI 1 [Anthracen-9(10H)-one].¹⁶ Collectively, our data indicate that the ASK1 inhibitor AlphaScreen assay which we have developed is robust and suitable for screening large compound libraries. To help eliminate false positives, we counter screened the 18 initial hits using the TruHits assay. Importantly, Reactive Blue 2, the anthraquinone dye that constitutes the blue chromophore of blue dextran, a known AlphaScreen color quencher (www.perkinelmer.com), was identified (Supplementary Table S2), thus validating the TrueHits assay as a useful counterscreen; this indicates that our screening strategy can be used to discover inhibitors targeting stress-activated ASK1 signalosome. The next step will be to miniaturize the assay to a 1,536-well format for meeting the cost and throughput goals of screening.

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DISCLOSURE STATEMENT

No competing financial interests exist.

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Abbreviations Used

ASK1	= apoptosis signal-regulating kinase 1
ATP	= adenosine triphosphate
CDK	= cyclin-dependent kinase
c-JNK	= c-Jun N-terminal kinase
CRC	= concentration-response curves
CV	= coefficient of variation
DB	= detection buffer
DMSO	= dimethyl sulfoxide
HTS	= high-throughput screening
MAP3K	= mitogen-activated protein kinase kinase kinase
MKK	= MAP kinase kinase
PDE	= phosphodiesterase
PKC- α	= protein kinase C- α
S/B	= signal-to-background
SD	= standard deviation
SDS-PAGE	= sodium dodecyl sulfate-polyacrylamide gel electrophoresis