Development of an insect-cell-based assay for detection of kinase inhibition using NF-κB-inducing kinase as a paradigm

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Identification of small-molecule inhibitors by high-throughput screening necessitates the development of robust, reproducible and cost-effective assays. The assay approach adopted may utilize isolated proteins or whole cells containing the target of interest. To enable protein-based assays, the baculovirus expression system is commonly used for generation and isolation of recombinant proteins. We have applied the baculovirus system into a cell-based assay format using NIK [NF-κB (nuclear factor κB)-inducing kinase] as a paradigm. We illustrate the use of the insect-cell-based assay in monitoring the activity of NIK against its physiological downstream substrate IkB (inhibitor of NF-κB) kinase-1. The assay was robust, yielding a signal/background ratio of 2.1 and an average Z’ value of > 0.65 when used to screen a focused compound set. Using secondary assays to validate a selection of the hits, we identified a compound that (i) was non-cytotoxic, (ii) interacted directly with NIK, and (iii) inhibited lymphotoxin-induced NF-κB p52 translocation to the nucleus. The insect cell assay represents a novel approach to monitoring kinase inhibition, with major advantages over other cell-based systems including ease of use, amenability to scale-up, protein expression levels and the flexibility to express a number of proteins by infecting with numerous baculoviruses.

Key words: baculovirus expression system, cell-based assay, nuclear factor κB-inducing kinase (NIK), inhibitor of nuclear factor κB kinase-1 (1IKK1), nuclear factor κB (NF-κB).

INTRODUCTION

Kinases are key regulators of many biological processes, with aberrant activity of these proteins being associated with diseases such as cancer, inflammation and diabetes [1]. The unveiling of at least 500 distinct kinases through sequencing the human genome coupled with the recent successes of small-molecule kinase inhibitors [2,3] have led to an increase in drug research in this area second to G-protein-coupled receptors as drug-development targets [4]. A variety of approaches have been used to develop kinase assays for inhibitor identification. These involve utilizing purified protein, recombinant cell lines or primary cells. The strategy of choice can be divided into two categories: biochemical and/or cellular. A biochemical approach to monitoring kinase activity can be divided further into: (i) generic, and (ii) antibody-based formats that detect an epitope within the phosphorylated product. In both cases, suitable quantities of purified active kinase are required. This can often be challenging owing to protein expression yields, degradation, aggregation and low-activity protein upon purification. The majority of biochemical assays for kinases have used only the catalytic domain for HTS (high-throughput screening), thus identifying ATP site-directed inhibitors as the primary class of agents. Use of the catalytic domain can address some of the issues around yield, purity and aggregation of protein compared with full-length protein.

However, recent studies on the Akt family of kinases have demonstrated some of the benefits of using full-length protein by discovering a pharmacologically unique class of compounds [5].

More recently, there has been increased focus on cell-based assays, where in 2006 a survey had indicated that approx. half of HTS assays were cell-based [6]. This is partly due to the advances in suitable detection systems utilizing chemical or genetically encoded fluorescent probes and the development of sophisticated assay platforms such as microscope-based imaging systems or microplate cytometers [7,8]. Cells used in these types of assay can be primary cells or recombinant cell lines. Whereas primary cells offer major advantages such as physiological relevance of wild-type or disease states, they are difficult to obtain and prone to inter-individual variability. Stable cell lines offer reliability of protein expression and ease of continuous culture; however, there is a potential for the gene product to be toxic when expressed constitutively, and stable cell lines are time-consuming to generate and clone. Transient cell lines are used frequently, but can be costly and require large amounts of DNA that sometimes cause toxicity to cells. Modified bacloviruses termed BacMam have been developed to address some of these issues [9].

The baculovirus insect cell expression system is widely used as a tool for production of recombinant proteins. The advantages

Abbreviations used: APC, allophycocyanin; DTT, dithiothreitol; FP, fluorescence polarization; GST, glutathione transferase; HTS, high-throughput screening; IkB, inhibitor of nuclear factor κB; IKK, IkB kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MOI, multiplicity of infection; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; TEV, tobacco etch virus; TRAF, tumour-necrosis-factor-receptor-associated factor; TR-FRET, time-resolved fluorescence resonance energy transfer.

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of the baculovirus system over other expression systems are well documented [10,11]. Briefly, these include acceptable expression for a diverse range of proteins, the suitability of insect cells for post-translational modifications such as phosphorylation and ease of culture. Insect cells have been used to configure calcium flux assays for G-protein-coupled receptor targets [12], but, to our knowledge, there are currently no reports on the use of insect cells in a cellular assay format for any other target classes.

In the present paper, we describe the novel utility of baculovirus-infected insect cells to monitor kinase activity using human NIK [NF-κB (nuclear factor κB)-inducing kinase], also known as MAP3K (mitogen-activated protein kinase kinase kinase) 14, as a paradigm. We show that in baculovirus co-infections of insect cells, full-length NIK produced the highest phosphorylation as a paradigm. We show that in baculovirus co-infections of MAP3K (mitogen-activated protein kinase kinase) 14, robustness of the assay quantitatively is the used for screening purposes. The index that is used to measure the phosphorylation sites. The assay was sufficiently robust to be utilized a specific antibody against the NIK-mediated IKK1 (time resolved-florescence resonance energy transfer) format that developed an insect cell plate-based assay in a 384-well TR-FRET yielding average Z value for cell-based assays is greater than 0.4. The present assay developed an insect cell plate-based assay in a 384-well TR-FRET (time resolved-florescence resonance energy transfer) format that utilizes a specific antibody against the NIK-mediated IKK1 phosphorylation sites. The assay was sufficiently robust to be used for screening purposes. The index that is used to measure the robustness of the assay quantitatively is the Z’ value. This provides an important measure of the quality of an assay as it takes into account the signal window variability. The closer the Z’ value is to 1, the more robust the assay [13]; however, an acceptable Z’ value for cell-based assays is greater than 0.4. The present assay yielded average Z’ values greater than 0.65. Using this assay, a number of apparent NIK inhibitors were identified when screening a focused compound set. To validate these hits, a selection was analysed further, and we identified an inhibitor, compound A, that (i) was non-cytotoxic, (ii) interacted directly with NIK, and (iii) inhibited lymphotoxin-induced p52 translocation to the nucleus. This provided strong support that the insect cell system was able to identify bona fide kinase inhibitors. Thus the present study has set a precedent for the use of baculovirus-infected insect cells to detect kinase activity in a HTS-amenable system.

### MATERIALS AND METHODS

#### Cloning of expression constructs

Full-length and truncations of human NIK were cloned into a modified pFastBac vector containing a dual tag, FLAG–His, at the N-terminus followed by a NdeI restriction site. Cloning was performed by PCR amplification of the NIK of interest flanked with an N-terminal TEV (tobacco etch virus) protease cleavage site downstream of an NdeI site. An XhoI restriction site was incorporated into the reverse PCR primers at C-terminus. Upon ligation of vector and insert, the gene is in-frame with the N-terminal dual FLAG–His, tag, followed by the TEV protease cleavage site. PCR primers used for amplification of full-length or truncations of NIK are shown in Table 1. The mutations K429A/K430A and T559D were generated using the QuikChange® site-directed mutagenesis system (Stratagene) using the primers shown in Table 1. The deletion d78–84 was generated using overlap extension PCR with the primers also shown in Table 1. Human IKK1 (5–746)-pFastBac were generated using the QuikChange® site-directed mutagenesis system (Stratagene) using the primers shown in Table 1. All constructs were sequence-verified.

#### Baculovirus generation, protein expression and analysis by Western blotting

NIK (full-length and truncations) and IKK1-K44A-pFastBac constructs were used to generate P1-amplified baculoviruses using the Bac-to-Bac system (Invitrogen). NIK (full-length or truncations) and IKK1-K44A baculoviruses were mixed with Spodoptera frugiperda (Sf9) insect cells at a cell density of 10^6/ml and an MOI (multiplicity of infection) of 5–10 each. After incubating the cells for 48 h at 27°C while shaking at 100 rev/min, they were harvested by centrifugation at 805 g for 20 min. The cell pellets were recovered and lysed using 20 mM

### Table 1 Primers used in the present study

Underlined codons represent the reading frame, and residues in bold represent the start of NIK.

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<tr>
<th>Primer name</th>
<th>Sequence</th>
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Tris/HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100 and one tablet of protease inhibitor cocktail (Roche) per 10 ml. The lysates were placed on ice for 20 min then centrifuged at 17,500 g for 10 min at 4 °C. The supernatant fraction was mixed with SDS protein sample buffer (Invitrogen) supplemented with reducing agent (Invitrogen) and heated to 95 °C for 5 min. SDS/PAGE was used to separate proteins in 4–20% Tris/glycine gels (Invitrogen). The protein gels were then blotted onto to PVDF membranes using the semi-dry Western blotting apparatus (Bio-Rad Laboratories). The membranes were blocked in buffer containing 5% (w/v) non-fat dried skimmed milk powder (Marvel) in PBS and 0.1% Tween 20 (Sigma). The membranes were then treated with mouse anti-FLAG (Stratagene) and rabbit anti-phospho-IKK1 (Ser179/Ser181) (Cell Signaling Technology) diluted 1:1000 in blocking buffer, while shaking overnight at 4 °C. The membranes were washed five times for 3 min in blocking buffer then treated with anti-mouse and anti-rabbit antibodies (Odyssey) diluted 1:5000 in blocking buffer, while shaking at room temperature for 1 h. The membranes were then washed five times for 3 min in PBS/0.1% Tween 20 then twice for 3 min in PBS alone. Membranes were then visualized using the Odyssey imaging system with detection in both 700 nm (red) and 800 nm (green) channels.

Development of a NIK insect cell-based assay

Once the appropriate NIK and IKK1-K44A constructs were identified using the dual-detection Western blot methodology, we modified the assay so as to make it suitable for screening purposes, allowing the identification of compounds that inhibit NIK-mediated phosphorylation of IKK1-K44A. The infections of the Sf9 cells were performed in bulk solutions (typically 10–100 μl). Infected cells (5 μl, containing 5000–10000 cells) in culture medium were dispensed into 384-well plates that contained 50 nl of test compound in DMSO, yielding a final concentration of 6 μM for single concentration screening and 1% (v/v) DMSO. Plates were incubated at 27 °C for 24 and 48 h. Optimal conditions for the assay were determined by varying the number of cells per well, infection time, detergent type and concentration during the cell lysis step and antibody concentrations during the detection step. Assays were performed in black 384-well plates (Greiner). The optimized lysis buffer was 20 mM Tris/HCl, 150 mM NaCl, 2% (v/v) Triton X-100 and one protease inhibitor tablet per 10 ml of buffer. Assay signal was determined with cells ranging between 5000 and 10000 per well in the 384-well plates. The antibodies used in the detection step were 25 nM anti-FLAG–APC (allophycocyanin) (PerkinElmer), 2 nM LANCE® anti-rabbit europium-chelate (PerkinElmer) and 1 nM anti-phospho-IKK1/2 (Ser179/Ser181) rabbit monoclonal (Cell Signaling Technology). A mixture of the three antibodies was made in 50 mM Heps (pH 7.4), 1 mM CHAPS and 1 mM DTT (dithiothreitol). The detector used for readout was a PerkinElmer Viewlux. The Cell Titre Glo assay kit (Promega) was used in accordance with the manufacturer’s instructions and replicating the same conditions for infection and lysis as in the NIK/IKK1-K44A assay. The signal window for the assay was determined from assays in 384-well plate format that contained an entire column (16 wells) of dual NIK- and IKK1-K44A-infected Sf9 cells (high control) and another column containing cells infected with NIK-K429A/K430A and IKK1-K44A (low control). The quality and robustness of the assay, represented as Z’ [13] was calculated using eqn (1):

\[
Z' = 1 - \frac{3 \times S.D_{\text{highs}} + 3 \times S.D_{\text{lows}}}{\text{mean}_{\text{highs}} - \text{mean}_{\text{lows}}} \tag{1}
\]

Dose–response data were fitted to a four-parameter logistic equation using non-linear regression.

![Figure 1](image.png)

**Figure 1** Structure of the Rhodamine Green-labelled FP ligand

**NIK FP (fluorescence polarization) binding assay**

Purified NIK kinase domain (residues 318–947) was obtained as a GST (glutathione transferase)-fusion protein from Invitrogen. Rhodamine Green-labelled FP ligand was synthesized in-house (structure shown in Figure 1). Structurally related kinase FP ligands have been described previously [14]. Test compounds were diluted from 10 mM in DMSO in a 3-fold series of 11 points. Then, 100 nl of the dilution series were transferred to a 384-well low-volume plate (Greiner) using an Echo acoustic dispenser. GST–NIK and Rhodamine Green-labelled FP ligand were added to the assay plate to final concentrations of 20 and 5 nM respectively, in a total volume of 10 μl, using a Multidrop Combi dispenser. The plate was incubated at room temperature (21 °C) for 30 min, after which FP was measured using an Acquest plate reader (Molecular Devices). Data were converted into percentage displacement by normalization to high controls (enzyme-ligand mix) and low controls (ligand only) and fitted as described above.

**p52 nuclear translocation assay**

U2OS human osteosarcoma cells were seeded into clear-bottom poly-d-lysine-coated 384-well plates (Greiner) at 1500 cells in 45 μl of DMEM (Dulbecco’s modified Eagle’s medium) Ham’s F-12/10% (v/v) FBS (fetal bovine serum) (Invitrogen) per well, using a Multidrop Combi dispenser, and incubated at 37 °C under 5% CO2 for 24 h. Test compounds were diluted from 10 mM in DMSO in a 3-fold series of 11 points. Aliquots of 0.5 μl of the dilution series were transferred into the cell plate using a Cybiwell 384-well head pipetter. Then, 5 μl of a 3 μg/ml solution of lymphoxygen ζ1/2 (R&D Systems) in culture medium was added to each well, and the plate was incubated for a further 16–20 h. Cells were fixed for 10 min with 4% (w/v) formaldehyde in PBS, permeabilized with 0.1% (w/v) Triton X-100/PBS for 15 min, stained with anti-p52 antibody (Upstate Biotechnology), diluted 1:200 in PBS/0.5% BSA, for 1 h, and stained with secondary Alexa Fluor® 488-conjugated goat anti-mouse antibody (Invitrogen) (diluted 1:200) plus Hoechst 33342 (Invitrogen) (diluted 1:5000) in PBS/0.5% BSA for 1 h, with intermediate washing steps (PBS/0.5% BSA) between the staining steps. Plates were kept in PBS/0.5% BSA at 4 °C until analysis.

Images were collected on a PerkinElmer Opera system, using 405 and 488 nm laser excitation, and a 10× magnification objective. For image analysis by the ACapella software, nuclear and cytoplasmic areas were determined, intensities of the Alexa Fluor® 488 stain in these areas measured, and the ‘nuclear fraction’ of nuclear/(nuclear + cytoplasmic) intensities were calculated for each individual cell. Typically, 150–200 cells were analysed for each well. Since not all cells within a well responded to stimulus or showed the inhibition effect, usually approx. 70–80%, the most robust data were achieved by determining the percentage of responding cells within each well, with ‘responding
RESULTS
Detection of NIK-mediated IKK1 phosphorylation in co-infected insect cells

We examined the kinase activity of full-length and truncations of human NIK using the physiological downstream substrate IKK1. Reports show that IKK1 autophosphorylation occurs at the same sites as NIK-mediated phosphorylation, Ser\(^{176}/\text{Ser}^{180}\) [15]. In order to discriminate between these events, it was necessary to use a kinase-dead IKK1 mutant IKK1-K44A that renders the kinase incapable of autophosphorylation, thus allowing the detection of NIK-mediated IKK1 phosphorylation only [15,16].

Insect cells were co-infected with NIK and IKK1-K44A baculoviruses while shaking in flasks for 48 h, and the resulting product was detected by two-colour Western blotting. IKK1-K44A phosphorylation was detected in the green channel using a phospho-specific antibody recognizing phospho-Ser\(^{176}/\text{Ser}^{180}\). Both NIK and IKK1-K44A were tagged at the N-terminus with a FLAG epitope that was also probed and visualized in the red channel (Figure 2). As shown, both NIK (107 kDa full-length or smaller for truncations) and IKK1-K44A (85 kDa) protein was detected. An overlay of the green and red channels indicates the detection of phosphorylated protein at that given size and was visualized in yellow. The results show the detection of IKK1-K44A phosphorylation when co-infected with full-length NIK that was not observed in cells infected with IKK1-K44A alone (Figure 2). In addition, phosphorylation was mediated by the kinase activity of NIK, as a kinase-dead mutant NIK K429A/K430A [15] was unable to phosphorylate IKK1-K44A under the same conditions. A predicted constitutively active mutant of NIK T559D appeared incapable of phosphorylating IKK1-K44A. Also included was a construct containing a deletion in NIK from amino acids 78–84, associated with TRAF (tumour-necrosis-factor-receptor-associated factor) 3 binding, and reported to enhance expression yields [17]. By assessing the extent of product formation (percentage of phosphorylated IKK1-K44A relative to total IKK1-K44A protein, as determined from band intensities) full-length NIK confers the greatest activity, and truncations of the protein, including those predicted to contain the kinase domain, amino acids 389–658, exhibit either little or no kinase capability. These observations suggested that regions in NIK outside the kinase domain were important for the kinase potential of the protein. Thus full-length NIK and IKK1-K44A baculoviruses were the tools selected for development of an insect-cell-based assay.

Development of a medium/high-throughput assay to monitor NIK activity using baculovirus-infected insect cells

Once the in-flask co-infection studies had allowed the triage of appropriate constructs for NIK and IKK1-K44A, the desire was to develop an assay to screen against a library of compounds. The assay format that was considered was based on the principle of TR-FRET. This utilized commercially available antibodies which form a sandwich in the presence of phosphorylated FLAG–IKK1-K44A. A rabbit monoclonal antibody recognizing phospho-Ser\(^{176}/\text{Ser}^{180}\) of IKK1/2 becomes bound to the europium-labelled anti-rabbit IgG. A third antibody, the mouse monoclonal anti-FLAG–APC is bound to the FLAG epitope of IKK1-K44A (Figure 3). This forms a sandwich which, when excited by electromagnetic radiation at 320 nm, results in the excitation of the europium label which emits light at 615 nm. If the anti-FLAG–APC is in close proximity to the europium-labelled antibody (i.e. there exists the phosphorylated FLAG–IKK1-K44A), TR-FRET takes place, resulting in the excitation of the APC and subsequent
Insect-cell-based assay for detection of kinase inhibition

Figure 3  Schematic representation of TR-FRET assay for NIK

Detection of NIK-mediated phosphorylation of IKK1-K44A is measured using specific antibody interactions. (A) Cells were treated with baculoviruses and transferred from a typical shaking flask system to a static 384-well plate-based system. (B) Over the next 24 and 48 h, protein (NIK and IKK1-K44A) production occurs, followed by phosphorylation of IKK1-K44A. (C) Cells are lysed and the detection of the phospho-IKK1-K44A takes place using a TR-FRET system. The purple box indicates the FLAG tag. See the Materials and methods section for more details.

Figure 4  NIK assay signal/background ratio and pharmacology data

(A) Data showing that the assay window generated is dependent on NIK catalytic activity. The assay yields a low signal when using the NIK kinase-dead mutant (K429A/K430A) + IKK1-K44A and is the same when using an inhibitor (compound B). (B) Typical dose–response curve for the NIK/IKK1-K44A assay with compound B. Duplicate data are shown with IC50 values of 0.62 ± 0.05 μM. (C) Z’ values for 52 plates screened as part of the kinase-focused set of compounds. The assay yielded average Z’ values of >0.65, indicative of very good robustness.

emission of electromagnetic radiation at 665 nm. In the absence of phosphorylated FLAG–IKK1-K44A, a low signal would be obtained.

There were a number of key parameters that we addressed during the development of the insect cell assay. Some of these were necessary to understand optimal conditions required for expression of proteins in baculovirus-treated insect cells when miniaturizing from a typical shaking flask system to a static-plate-based system. Investigations were carried out to identify the optimal conditions for (i) the MOI of baculoviruses per cell for efficient expression in a 384-well plate, (ii) cell numbers per well, (iii) the infection period of insect cells with NIK and IKK1-K44A baculoviruses, (iv) detergent type/concentration for in-plate cell lysis, and (v) antibody concentrations to generate a robust assay that was screening-compatible. The final optimized assay in 384-well plate format contained 10000 cells/well that were infected with an MOI of 5 for each virus in a volume of 10 μl for a period of 48 h at 27 °C. After this period, the cells were lysed by the addition of 10 μl of lysis/detection solution. This was done using Tris/HCl buffer containing 2% (v/v) Triton X-100, 1 mM DTT, 25 nM anti-FLAG–APC, 2 nM europium-labelled anti-rabbit IgG and 2 nM anti-phospho-IKK1/2 (Ser176/Ser180). The final optimized assay yielded a signal/background ratio of ~2, which, although relatively low, provided an average Z’ value of ~0.65, owing to the fact that the assay produced low deviations in the values of the high and low controls (see Figure 4 for typical data quality).
A positive relationship was observed with the two datasets. The correlation coefficient was 0.95.

**Application of the NIK insect cell assay to a focused screening campaign**

Once a robust assay was configured, it was applied to screen a kinase-focused compound set to identify potential NIK inhibitors. Approx. 10 000 compounds were chosen to represent the diversity of GlaxoSmithKline kinase chemical space. This set of compounds was screened in duplicate against the NIK/IKK1-K44A assay at a concentration of 6 µM compound and a range of inhibitory responses were observed in the assay at this concentration. Figure 5 shows a correlation plot for the experimental data. The number of active compounds (being classified as those giving >50 % inhibition at 6 µM in duplicate) was 423 and they exhibited inhibition in the assay between 50 and 100 %. A total of 75 compounds were active on one of the two test occasions, equating to a 17 % false positive/negative hit rate. Of the 423 active compounds, care was needed to ensure that their mechanism of action was due to NIK inhibition and not, for example, cytotoxicity. Such compounds would be of no interest as the inhibition profile is essentially an artefact. In order to increase confidence that the inhibition in the cellular assay was not due to a NIK-independent process, the active compounds were investigated for their cytotoxic effects using the Promega Cell Titre Glo assay that measures intracellular ATP. To enable discrimination of the NIK-dependent active compounds from potential cytotoxicity effects, all active compounds were screened in this cytotoxicity assay. In total, 382 compounds were identified as exhibiting a potency in the NIK/IKK1-K44A assay pIC[50] [−log(IC[50])] > 4.5. Of these, a significant number exhibited cytotoxicity (343 in total). The remaining 39 compounds exhibited activity in the NIK/IKK1-K44A assay with pIC[50] > 4.5 and at least a 10-fold decrease in potency in the cytotoxicity assay (Figure 6). This 10-fold window indicated that the compounds blocked the NIK/IKK1-K44A assay independently of cytotoxicity and were thus considered to be ‘apparent NIK inhibitors’.

**Use of an FP assay to identify direct-binding NIK inhibitors**

To identify direct-binding inhibitors from among the apparent inhibitor set of compounds, an NIK FP binding assay was developed using a Rhodamine Green-labelled non-selective kinase inhibitor as an ATP-binding site probe. The principle of the FP assay as applied to kinases has been described elsewhere [18]. The source of kinase was purified truncated NIK protein (amino acids 318–947). The affinity of the ligand was shown to be 15 nM by titration, and the interaction was competitive with ATP. The binding assay was configured with an NIK concentration slightly in excess of the dissociation constant for the ligand, and a selection of apparent NIK inhibitors were tested for their ability to compete with this interaction. Figure 7 shows a normalized displacement curve for compound A, which yielded an average pIC[50] of 7.0 in duplicate determinations.

**Inhibition of lymphotoxin-induced p52 nuclear translocation**

In order to validate compounds further, a nuclear translocation assay was configured detecting the inhibition of lymphotoxin-induced migration of p52 into the nucleus. The antibody used for immunostaining recognizes both the NF-κB subunit p52 and its precursor p100. p52 translocates into the nucleus upon p100 processing [19], which can therefore be observed as a change...
Insect-cell-based assay for detection of kinase inhibition

**DISCUSSION**

We have developed an insect-cell-based assay monitoring kinase activity that involves infection, lysis and detection within a 384-well plate. We illustrated the utility of this assay through identification of apparent NIK inhibitors following a medium-throughput focused screen. A selection of these were characterized further, and we discovered a compound that bound directly to NIK and inhibited nuclear translocation of p52 in mammalian cells. The insect-cell-based assay demonstrated in the present study provides an additional tool to be considered when a screen is desired for identifying inhibitors of a given target. The major advantages of using this system over others include achieving adequate protein expression levels, ease of use, amenability to scale-up and the flexibility to express a number of proteins by infecting with numerous baculoviruses.

When considering which assay format to adopt for screening a number of factors are taken into account, not least the question of pursuing a biochemical or cell based approach or both. In some cases, the complexity of the biological target under study can influence this decision. In the present study, NIK was used as a paradigm. This kinase was first cloned over a decade ago and was identified as a member of the MAP3K family, comprising 947 amino acids including a kinase domain in the region 389–658 [20]. In the last 10 years, there have been significant advances in understanding the role of NIK in NF-κB activation. The transcription factor NF-κB plays a pivotal role in immune and inflammatory responses. Nuclear translocation of NF-κB is controlled by two main pathways: the classical and the alternative pathways. The classical pathway regulates innate and adaptive immune responses. In contrast, the alternative pathway appears to be activated by a more restricted set of signals principally involved in mounting a secondary adaptive immune response including those triggered by lymphotoxin β [21], B-cell-activating factor [22] and CD40 ligand [23]. It is now believed that NIK is an essential mediator of the alternative pathway, thus representing a novel target for autoimmune diseases, such as rheumatoid arthritis. Studies have shown that NIK activates IKK1 through phosphorylation of Ser176, leading to the processing of the NF-κB precursor protein p100. NIK has been shown to associate physically with IKK1 and p100, functioning as a `docking` molecule [24]. Other NIK protein interactions have been identified, including those with TRAF2 and TRAF3. The role that these interactions play in NIK activation and regulation is not fully understood. Owing to the complex nature of activation and function of this target, it is not surprising that, to date, there are no published reports demonstrating a biochemical assay of NIK activity using its physiological downstream substrate IKK1. We have observed that part-purified NIK showed insignificant activity against purified IKK1-K44A (results not shown) using the same constructs and detection system as that described for the insect cell assay. It is tempting to speculate that the diminished activity was due to...
the loss of an essential component as a consequence of purification.

The Western blotting data had shown that regions outside of the kinase domain of NIK (residues 389–658) were required to achieve the highest phosphorylation of IKK1 (Figure 2). Previous reports have linked some of these regions to association with IKK1 [25] and TRAF2 [21]. Furthermore, it has been reported that recombinant NIK spontaneously forms oligomers [25], possibly induced through its interaction with TRAF2. Reports show that in an assessment of a series of truncated NIK constructs multiple regions within NIK contribute to the oligomerization of the kinase. Only the relatively long 200–947 construct from the series of truncations retained significant, although reduced, stimulatory activity of NF-κB in a luciferase reporter assay. It is possible that the truncated NIK proteins described in the present paper fail to form suitable oligomers, thus affecting catalytic function (Figure 2). Also, the lack of activity observed in purified NIK may have been caused by the disruption of oligomers. A number of kinases have been reported to form homo-oligomers within cells [26], thus isolating these proteins in a purified system may not be a feasible approach to assay design or representative of the mechanism in vivo.

Using the Western blotting method, we also investigated the potential for NIK to phosphorylate kinase-dead IKK2 (K44A). Reports show that NIK preferentially phosphorylates IKK1 over IKK2 [15]. However, we observed similar levels of phosphorylation for both substrates (results not shown). One possible explanation is that the mechanisms involved in controlling substrate specificity for endogenous protein break down in the recombinant baculovirus expression system because of the high protein levels of kinase and substrate. This is not unique to insect cells, and extrapolation from any recombinant system to physiological systems will be limited because of high protein levels.

Cell-based assays commonly utilize recombinant systems such as stable or transiently transfected mammalian cells, or primary cells. Successful assay design using these systems relies on an enabling level of protein expression, reproducibility and scalability. Any one of these can be limiting. The higher expression levels typically observed in a baculovirus expression system are well documented for numerous target classes [10]. Insect cells are robust and relatively easy to use. Baculovirus generation is simple and induced protein expression is usually reproducible. In a typical screening campaign, a large number of microtitre (384-well) or nanotitre (1536-well) plates are used to minimize the requirements for reagents. One of the main objectives of such a screen (focused or high-throughput, comprising compound numbers in the tens of thousands to millions respectively) carried out on a target of interest is in discovering compounds that have the potential for further development in the drug-discovery process. In order to make an assay amenable for screening, a number of key requirements need to be fulfilled. These will include the cost of the assay per well. In order to achieve this with potentially expensive reagents, miniaturization is an integral part of assay development. We have successfully miniaturized the insect cell assay to 5 μl of cells per well and 5 μl of detection reagent. In addition, the large numbers of compounds involved also necessitates sizeable quantities of cell supply to resource a screening exercise. In a typical mammalian assay system, this can be labour-intensive, and can suffer from cell stability issues over the course of a screening day. The insect cell system is scalable and robust, with a 1 litre culture providing sufficient cell numbers for at least 100 000 wells. Another important aspect for development of a screening assay is to optimize conditions to obtain a high signal/background ratio. This would enable compounds that are genuine inhibitors of the assay to be identified with confidence, away from noise within the signal window. The current assay format offers a signal/background ratio of 2:1 and a very good average Z’ value of >0.65, thus providing confidence that compounds identified using this assay are statistically significant inhibitors.

As with other cellular assay systems, it was important to incorporate further assays to determine the specificity of apparent inhibitors, and gain confidence that the inhibitory effects are due to specific inactivation of NIK rather than off-target effects. Using a cell cytotoxicity assay, we have discriminated between those compounds that cause cytotoxicity and those that are apparent inhibitors of NIK. In addition to cell cytotoxicity, however, there are other compound activities that are important to avoid, particularly in the insect cell assay described in the present paper. This is because compound addition is made at the point of infection thus providing multiple opportunities for compounds to inhibit the processes of infection, transcription and translation as well as the effect of interest, phosphorylation. Inhibition of one or all of these processes would produce a low assay signal and appear as NIK inhibition. Therefore, in order to validate the inhibitors from the insect cell assay further, a selection were assessed in a direct-binding FP assay using purified truncated NIK protein. At least one compound showed binding with a pIC50 of 7.0 (compound A). Furthermore, of those apparent NIK inhibitors tested, the majority blocked the lymphotixin-induced translocation of p52 into the nucleus. We determined the intracellular localization of p100/p52 as a measure for activation or inhibition of the endogenous NIK–p100 pathway in U2OS cells. Compound A demonstrated clear effects in this pathway assay (Figure 8). Thus compound A, an inhibitor identified from the insect cell assay (with a pIC50 of 6.3) was (i) non-cytotoxic (pIC50 < 4.5), (ii) showed direct binding to purified NIK protein with a pIC50 of 7.0, and (iii) illustrated activity in a NIK-dependent pathway in mammalian cells.

We also developed an insect cell assay detecting wild-type IKK1 autophosphorylation (results not shown) to identify (i) off-target compound activities, and (ii) selective NIK inhibitors. This used the same detection reagents as the NIK/IKK1-K44A assay, thus allowing the identification of compounds that were format-specific in addition to those inhibiting baculovirus infection and/or protein expression, as well as IKK1 autophosphorylation. Compound A inhibited IKK1 autophosphorylation with similar potency to NIK inhibition (pIC50 of 6.3) in this assay. Furthermore, inhibition of IKK1 activity against IκBα using compound A was also observed in vitro (results not shown). The lack of selectivity of compound A was not surprising as the compound set screened is kinase-focused and it is common to identify non-selective kinase inhibitors from this set. It is anticipated that a full-diversity screen against a complete compound library (millions of compounds) could potentially yield selective inhibitors. The downstream assays that we used to validate hits from the primary screen highlights the importance of incorporating these types of assay in a screening path given the potential for false positives with the insect- (or any other) cell-based screening system. With the appropriate downstream assays in place (cytotoxicity, direct binding, format-dependent specificity and pathway assays), we demonstrate that the insect-cell-based assay can identify compounds which are active in a NIK-dependent pathway assay in mammalian cells.

The present study has set a precedent for the development of a robust cellular kinase assay using insect cells expressing the kinase of interest. NIK was used to illustrate the potential of this system; however, this approach could be applied to other kinases, and potentially other target classes.

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