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

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SYNOPSIS

Identification of small molecule inhibitors by High Throughput Screening necessitates the development of robust, reproducible and cost-effective assays. The assay approach adopted may utilise 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 NF κ B inducing kinase (NIK) as a paradigm. We illustrate the use of the insect cell based assay in monitoring activity of NIK against its physiological downstream substrate I κ B kinase-1. The assay was robust yielding a signal:background of 2:1 and an average Z' of greater than 0.65 when used to screen a focussed compound set. Using secondary assays to validate a selection of the hits, we identified a compound that was a) non-cytotoxic, b) interacted directly with NIK and c) inhibited lymphotoxin induced NF- κ B 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 to include ease of use, amenability to scale-up, protein expression levels, and the flexibility to express a number of proteins by infecting with numerous baculoviruses.

Keywords: NF κ B inducing kinase, I κ B kinase-1, baculovirus expression system, cell-based assay.

Abbreviations: NIK, NF κ B inducing kinase; IKK, I κ B kinase-1, TR-FRET, time-resolved fluorescence resonance energy transfer; FP, fluorescence polarisation; sf9, *Spodoptera frugiperda*; HTS, high-throughput screening.

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 utilising 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 further divided 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 due 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 high-throughput screening (HTS), 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 to full-length. 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 approximately 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 type of assays can be primary cells or recombinant cell-lines. While 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 frequently used, but can be costly and require large amounts of DNA that sometimes cause toxicity to cells. Modified baculoviruses termed BacMam have recently 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 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 capability 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.

Herein we describe the novel utility of baculovirus-infected insect cells to monitor kinase activity using human NF-kappa B inducing kinase (NIK/MAP3K14) as a paradigm. We show that in baculovirus co-infections of insect cells, full-length NIK produced the highest phosphorylation of its downstream physiological substrate I κ B kinase-1 (IKK1). These baculoviruses were then used to develop an insect cell plate based assay in a 384-well Time Resolved-Florescence Resonance Energy Transfer (TR-FRET) format that utilizes a specific antibody to 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 to 1, the more robust the assay [13] however an acceptable Z' value for cell-based assays is greater than 0.4. The current assay yielded average Z' values greater than 0.65. Using this assay, a number of apparent NIK inhibitors were identified when screening a focussed compound set. To validate these hits a selection were further analysed and we identified an inhibitor, compound A, that was a) non-cytotoxic, b) interacted directly with NIK and c) 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 this report has set precedence 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-6xHis at the N-terminus followed by the restriction site *NdeI*. Cloning was performed by PCR amplification of NIK of interest flanked with an N-terminal TEV protease cleavage site downstream of an *NdeI* site. The restriction site *XhoI* 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 tag FLAG-6xHis followed by the TEV protease cleavage site. PCR primers used for amplification of full-length or truncations of NIK are shown below (underlined codon represents the reading frame, and those in bold represent the start of NIK):

Forward primers:

NIK (a.a 1): gggaattccatatgggagaacctgtacttccagggtatggccgctcatggaatggcc

NIK (a.a 48): gggaattccatatgggagaacctgtacttccagggttctccagttctctcggaaag

NIK (a.a 233): gggaattccatatgggagaacctgtacttccagggtctccaatgtctcaatcacgtg

NIK (aa 327): gggaattccatatgggagaacctgtacttccagggtg**cg**cacgaaaattcagtgtg

NIK (aa 393): gggaattccatatgggagaacctgtacttccagggt**tacc**gcgaggaagtccac

NIK (aa 397): gggaattccatatgggagaacctgtacttccagggt**gtt**actgggctaccaccaattg

Reverse Primers:

NIK (end): ccgccgctcgagttatgggcggttttcgagctg

NIK (aa903): ccgccgctcgagTTATGTTACCAAGCTGAAAGCGGCAGC

NIK (aa658): ccgccgctcgagTTAGACTTGCTGCAGGGCGCGATTAAC

The mutations KK429/430AA and T559D were generated using the Quickchange site-directed mutagenesis (SDM) system (Stratagene) using the primers detailed below:

For KK429/430AA:

Forward: ggcttccaatgtgccgtggctgccgtcctgaggtcttc

Reverse: gaagacctccagacggacggcagccacggcacattggaagcc

For T559D:

Forward: ggcgactacatccctggagacgagaccacatggctccag

Reverse: ctggagccatgtgggtctcgtctccagggatgtagtcgcc

The deletion d78-84 was generated using overlap extension PCR with the primers outlined below:

Forward 1: ctgaggccggtccagcgggtgaatgcgagaactcgcaagaattc

Reverse: gaattcttgcgagttctcgcattcagccgctggaccggcctcag

Human IKK1 (5-746)-pFastBac 1 containing an N-terminal FLAG tag upstream of a TEV protease cleavage site was kindly provided by Angela Bridges (GSK, Harlow).

This construct was used as template to generate the K44A kinase dead mutant using the Quickchange SDM system (Stratagene) and the primers below:
Forward: CTTGATCTCAAATAGCAATTGCCTCTTGTCGCCTAGAGCTAAG
Reverse: CTTAGCTCTAGGCGACAAGAGGCAATTGCTATTTTGAGATCAAG
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 1×10^6 /ml and an MOI of 5-10 each. After incubating the cells for 48hrs at 27°C while shaking at 100rpm, they were harvested by centrifugation at 805g for 20 minutes. The cell pellets were recovered and lysed using 20mM Tris-HCl pH7.5, 150mM NaCl 1% v/v triton-X100 and protease inhibitor cocktail (Roche, 1 tablet per 10ml). The lysates were placed on ice for 20 minutes then centrifuged at 17,500g for 10 minutes at 4°C. The supernatant fraction was mixed with sodium dodecyl sulphate (SDS) protein sample buffer (Invitrogen) supplemented with reducing agent (Invitrogen) and heated to 95°C for 5 minutes. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins in 4-20% Tris-Glycine gels (Invitrogen). The protein gels were then blotted onto PVDF membranes using the semi-dry western blotting apparatus (Bio-Rad). The membranes were blocked in buffer containing 5% w/v milk (Marvel) in phosphate buffered saline (PBS) and 0.1% v/v Tween-20 (Sigma). The membranes were then treated with mouse anti-FLAG (Stratagene, 1:1000) and rabbit anti-phospho IKK1 ser176/180 (Cell signalling, 1:1000) diluted in blocking buffer, while shaking overnight at 4°C. The membranes were washed 5x3 minutes in blocking buffer then treated with anti-mouse and anti-rabbit antibodies (Odyssey, 1:5000 each) diluted in blocking buffer, while shaking at room temperature for 1hr. The membranes were then washed 5x3minutes in PBS/0.1% v/v Tween-20 then 2x3minutes in PBS alone. Membranes were then visualised using the Odyssey imaging system detecting 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 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 10-100ml). Infected cells (5µl, containing 5,000-10,000 cells) in culture media were dispensed into 384 well plates that contained 50nl of test compound in 100% v/v DMSO yielding a final concentration of 6µM for single concentration screening and 1% v/v DMSO. Plates were incubated at 27°C for 24 and 48hrs. 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 784076). The optimised lysis buffer was 20mM Tris/HCl, 150mM NaCl, 2% v/v Triton & 1 protease inhibitor tablet per 10ml buffer. Assay signal was determined with cells ranging between 5,000-10,000 per well in the 384 well plates. The antibodies used in the detection step were 25nM anti-FLAG-APC (Perkin Elmer, AD0059F), 2nM LANCE anti-rabbit Eu chelate (Perkin Elmer, AD0082) and 1nM anti-phospho IKK1/2, S176/180 in IKK sequence, rabbit

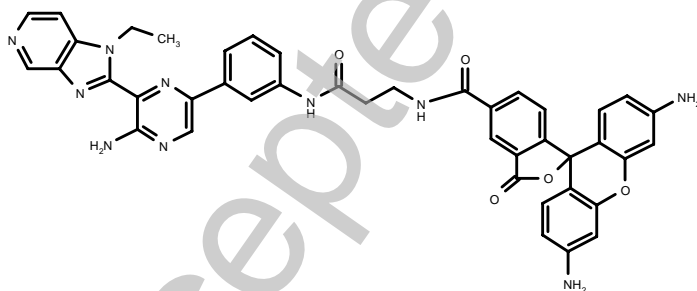
monoclonal (Cell Signalling 2697). A mixture of the three antibodies was made in 50mM HEPES, 1mM CHAPS, 1mM DTT, pH 7.4. The detector used for readout was a Perkin Elmer Viewlux. The Cell Titre Glo assay kit (Promega, G7570) was used in accordance with manufacturer's instructions and replicating the same conditions for infection and lysis as 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 KK429/430AA and IKK1-K44A (low control). The quality and robustness of the assay, represented as Z' [13] was calculated using equation (1):

$$Z' = 1 - \frac{(3 \times S.D._{highs} + 3 \times S.D._{lows})}{(\text{mean}_{highs} - \text{mean}_{lows})} \quad (1)$$

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

NIK fluorescence polarisation binding assay

Purified NIK kinase domain (318-947) was obtained as a GST fusion from Invitrogen (PV4902). Rhodamine green labelled FP ligand was synthesised in-house (structure shown below). Structurally related kinase FP ligands have been described previously [14]. Test compounds were diluted from 10mM in DMSO in a 3-fold series of 11 points. 100nL of the dilution series were transferred to a 384-well low volume plate (Greiner 784076) using an Echo acoustic dispenser. GST-NIK and rhodamine green labelled FP ligand were added to the assay plate to final concentrations of 20nM and 5nM respectively, in a total volume of 10uL, using a Multidrop Combi. The plate was incubated at room temperature for 30 minutes after which fluorescence polarisation was measured using an Acquest plate reader (Molecular Devices). Data were converted to % displacement by normalisation to high controls (enzyme-ligand mix) and low controls (ligand only) and fitted as described above.



Structure of the Rhodamine green labelled FP ligand

p52 nuclear translocation assay

U2OS human osteosarcoma cells were seeded into clear bottom PDL-coated 384-well plates (Greiner 781948) at 1500 cells in 45µl DMEM Ham's F-12/10% FBS (Invitrogen) per well, using a Multidrop Combi dispenser, and incubated at 37°C/5% CO₂ for 24h. Test compounds were diluted from 10mM in DMSO in a 3-fold series of 11 points. 0.5 µl of the dilution series were transferred into the cell plate using a Cybiwell 384-well head pipetter. 5 µl of a 3 µg/ml solution of lymphotoxin a1/b2 (R&D Systems 678-LY) in culture media were added to each well, and the plate incubated for a further 16-20h. Cells were fixed for 10 mins with 4% formaldehyde in

PBS, permeabilised with 0.1% Triton X 100/PBS (15 min), stained with anti-p52 antibody (Upstate 05-361) diluted 1:200 in PBS/0.5% BSA (1h), and stained with secondary goat anti-mouse Alexa488 antibody (Invitrogen A11001) (1:200) plus Hoechst 33342 (Invitrogen H-3570) (1:5000) in PBS/0.5% BSA (1h), with intermediate wash steps (PBS/0.5% BSA) between each of these steps. Plates were kept with PBS/0.5% BSA at 4°C until analysis.

Images were collected on a Perkin Elmer Opera system, using 405nm and 488nm laser excitation, and a 10x magnification objective. For image analysis by the ACella software, nuclear and cytoplasmic areas were determined, intensities of the Alexa488 stain in these areas measured, and the 'nuclear fraction' of nuclear/(nuclear+cytoplasmic) intensities 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, but usually around 70-80%, the most robust data were achieved by determining the 'percentage of responding cells' within each well, with 'responding cell' being defined as a cell with a 'nuclear fraction' value greater than 0.5.

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, Serine 176/180 [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 only of NIK mediated IKK1 phosphorylation [15, 16].

Insect cells were co-infected with NIK and IKK1-K44A baculoviruses while shaking in flasks for 48 hours, and the resulting product detected by two colour western blotting. IKK1-K44A phosphorylation was detected in the green channel using a phospho-specific antibody recognizing phospho-Serine 176/180. Both NIK and IKK1-K44A were tagged at the N-terminus with a FLAG epitope that was also probed and visualised in the red channel (Figure 1). As shown both NIK (107kDa full-length or smaller for truncations) and IKK1-K44A (85kDa) protein was detected. An overlay of the green and red channels indicates the detection of phosphorylated protein at that given size and was visualised 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 1). In addition, phosphorylation was mediated by the kinase activity of NIK as a kinase dead mutant NIK KK429/430AA [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 TRAF3 binding, and reported to enhance expression yields [17]. By assessing 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 389-658 exhibit either little or no kinase capability. These observations suggested that regions in NIK outside of 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 Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET). This utilized commercially available antibodies which form a sandwich in the presence of phosphorylated FLAG-IKK1-K44A. A rabbit monoclonal antibody recognizing the phospho-Serine 176/180 of IKK1/2 becomes bound to the europium (Eu) labeled anti-rabbit IgG. A third antibody, the mouse monoclonal anti-FLAG-Allophycocyanin (APC) is bound to the FLAG epitope of IKK1-K44A (Figure 2). This forms a sandwich which when excited by electromagnetic radiation at 320nm results in the excitation of the Eu-label which emits light at 615nm. If the anti-FLAG-APC is in close proximity to the Eu-labelled antibody (i.e. there exists the phosphorylated FLAG-IKK1-K44A) TR-FRET takes place resulting in the excitation of the APC and subsequent emission of electromagnetic radiation at 665nm. 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 miniaturising from a typical shaking flask system to a static plate based system. Investigations were carried out to identify the optimal conditions for a) the multiplicity of infection (MOI) of baculoviruses per cell for efficient expression in a 384-well plate b) cell numbers per well c) the infection period of insect cells with NIK and IKK1-K44A baculoviruses, d) detergent type/concentration for in plate cell lysis and e) antibody concentrations to generate a robust assay that was screening compatible. The final optimised assay in 384-well plate format contained 10,000 cells/well that were infected with an MOI of 5 for each virus in a volume of 10 μ l for a period of 48hrs at 27°C. After this period the cells were lysed by the addition of 10 μ l of lysis/detection solution. This was done using Tris/HCl buffer containing Triton-X 100 at 2% v/v, 1mM DTT, 25nM anti-FLAG-APC, 2nM europium labeled anti-rabbit IgG and 2nM anti phospho-Serine 176/180 of IKK1/2. The final optimized assay yielded a Signal:Background (S:B) of ~2 which although is relatively low provided an average Z' >0.65 due to the fact that the assay produced low deviations in the values of the high and low controls (see Figure 3 for typical data quality).

Application of the NIK insect cell assay to a focussed screening campaign

Once a robust assay was configured it was applied to screen a kinase focussed compound set to identify potential NIK inhibitors. Approximately 10,000 compounds were chosen to represent the diversity of GSK kinase chemical space. This set of compounds was screened in duplicate against the NIK/IKK1-K44A assay at a concentration of 6 μ M compound and a range of inhibitory responses were observed in the assay at this concentration. Figure 4 shows a correlation plot for the experimental data. The number of active compounds (being classified as those giving >50% inhibition at 6 μ M in duplicate) was 423 and they exhibited inhibition in the assay between 50-100%. 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 needed to be taken to ensure 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 actives from potential cytotoxicity effects, all actives were screened in this cytotoxicity assay. In total 382 compounds were identified as exhibiting a potency in the NIK/IKK1-K44A assay $pIC_{50} > 4.5$. 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 5). This 10-fold window indicated that the compounds blocked the NIK/IKK1-K44A assay independent of cytotoxicity and were thus considered 'apparent NIK inhibitors'.

Use of a fluorescence polarisation assay to identify direct binding NIK inhibitors

To identify direct binding inhibitors from amongst the apparent inhibitor set of compounds a NIK fluorescence polarisation binding assay was developed using a rhodamine green labelled non-selective kinase inhibitor as an ATP binding site probe. The principle of the fluorescence polarisation assay as applied to kinases has been described elsewhere [18]. The source of kinase was purified NIK truncate protein (318-947). The affinity of the ligand was shown to be 15nM by titration, and the interaction was competitive with ATP. The binding assay was configured with a 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 6 shows a normalised 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 recognises both the NFkB subunit p52 and its precursor p100. p52 translocates into the nucleus upon p100 processing [19], which therefore can be observed as a change of staining pattern with a p100/p52 antibody. Following immunocytochemical analysis of p100/p52 localisation in various cell lines, U2OS cells were found to exhibit a reproducible response. Treated with lymphotoxin $\alpha 1/\beta 2$, the majority of cells show higher intensity of p100/p52 staining in the nucleus compared with cytoplasm (Figure 7A). In the control experiment, the staining intensity is higher in the cytoplasm (Figure 7B). When cells are treated with lymphotoxin $\alpha 1/\beta 2$ in the presence of compound A, the p52 nuclear translocation effect is inhibited (Figure 7C). Out of 12 selected apparent NIK inhibitors, 9 showed activity in this assay, and quantitative image analysis (as described in the Materials and Methods section) gave pIC_{50} values in the range of 4 to 6 (data not shown).

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 focussed screen. A selection of these were characterised 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 here, 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 this study NIK was used as a paradigm. This kinase was first cloned over a decade ago and identified as a member of the mitogen-activating protein (MAP) kinase kinase kinase (MAP3K) family, comprising 947 amino acids including a kinase domain in the region 389-658 [20]. In the past 10 years there have been significant advances in understanding the role of NIK in NF- κ B activation. The transcription factor NF κ B plays a pivotal role in immune and inflammatory responses. Nuclear translocation of NF κ B 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 Serine-176 leading to the processing of the NF κ B precursor protein p100. Recently NIK was shown to physically associate 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. Due 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 (data 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 (389-658) were required to achieve highest phosphorylation of IKK1 (Figure 1). 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 oligomerisation of the kinase. Only the relatively long 200-947 construct from the series of truncations retained significant, although reduced stimulatory activity of NF- κ B in a luciferase reporter assay. It is possible that the truncated NIK proteins described in this report fail to form suitable oligomers thus affecting catalytic function (Figure 1). 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 homooligomers 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 (data 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 due to 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 due to high protein levels.

Cell-based assays commonly utilise 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 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 (focussed 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 successfully miniaturised the insect cell assay to 5 μ l of cells per well and 5 μ l 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 up to 100,000 wells. Another important aspect for development of a screening assay is to optimize conditions to obtain a high S:B 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 S:B ratio of 2:1 and a very good average Z' of >0.65 , thus providing confidence that compounds identified in the current 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 though, there are other compound activities important to avoid particularly in the insect cell assay described here. 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, 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 NIK truncate 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 lymphotoxin induced translocation of p52 into the nucleus. We determined the intracellular localisation 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 7). Thus

compound A, an inhibitor identified from the insect cell assay (with pIC₅₀ 6.3) was a) non-cytotoxic (pIC₅₀ < 4.5) b) showed direct binding to purified NIK protein with a pIC₅₀ 7.0 and c) illustrated activity in a NIK-dependent pathway in mammalian cells.

We also developed an insect cells assay detecting wild type IKK1 autophosphorylation (data not shown) to identify a) off target compound activities and b) 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 6.3) in this assay. Furthermore, inhibition of IKK1 activity against IκB alpha using compound A was also observed *in vitro* (data not shown). The lack of selectivity of compound A was not surprising as the compound set screened is kinase focussed 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 we used to validate hits from the primary screen highlights the importance of incorporating these type assays in a screening path given the potential for false positives with the insect cell (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 assay can identify compounds which are active in a NIK-dependent pathway assay in mammalian cells.

This work has set precedence 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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FIGURE LEGENDS

Figure 1 Full-length NIK is required for maximal IKK1-K44A phosphorylation

Insect cells were co-infected with IKK1-K44A in the presence or absence of full-length, mutant or truncations of NIK baculovirus. The construct lengths are shown in the schematic above the blot. A deletion of 7 amino acids 78-84 is described as d78-84. Mutant constructs are described by positions of mutants and the substituted amino acids, in single letter form. KD and CA are the kinase dead and predicted constitutively active forms respectively. Following a 48 hour incubation period of viruses with cells at 27°C, a sample of cells was lysed and the products separated by SDS-PAGE. Expressed NIK and IKK1-K44A protein was detected by probing for the N-terminal FLAG epitope (red). Simultaneously, phosphorylation of IKK1-K44A at serine 176/180 was detected using a specific antibody (green). The red and green channels were superimposed in this visualisation. Thus phosphorylated IKK1-K44A appears as a yellow band running at the expected molecular weight of 85kDa. The molecular weights for NIK are indicated below the blot.

Figure 2 Schematic representation of TR-FRET assay for NIK

Detection of NIK mediated phosphorylation of IKK1 is measured using specific antibody interactions. In (a) cells were treated with baculoviruses and transferred from a typical shaking flask system to a static 384-well plate based system. During the next 24 and 48hrs, (b) protein (NIK and IKK1-K44A) production occurs followed by phosphorylation of IKK1-K44A, (c) the cells are lysed and the detection of the phospho-IKK1-K44A takes place using a TR-FRET system. See the Materials and Methods for more details.

Figure 3 NIK assay signal: background 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 (KK429/430AA) + 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 is shown with IC_{50} values in the assay of $0.62 \pm 0.05 \mu M$, (C) Z' values for 52 plates screened as part of the kinase focussed set of compounds. The assay yielded average $Z' > 0.65$, indicative of a very good robustness.

Figure 4 Correlation plot for n=1 vs n=2 data set for the NIK/IKK1-K44A assay

A positive relationship is observed with the two data sets. Correlation coefficient was 0.95.

Figure 5 pIC50 NIK/IKK1-K44A vs pIC50 cell cytotoxicity

The open circles highlight the 39 compounds that were at least 10-fold more active in the NIK/IKK1-K44A than the Cell Titre Glo cytotoxicity assay. pIC50 determined using n=2 for each assay.

Figure 6 NIK fluorescence polarisation binding assay

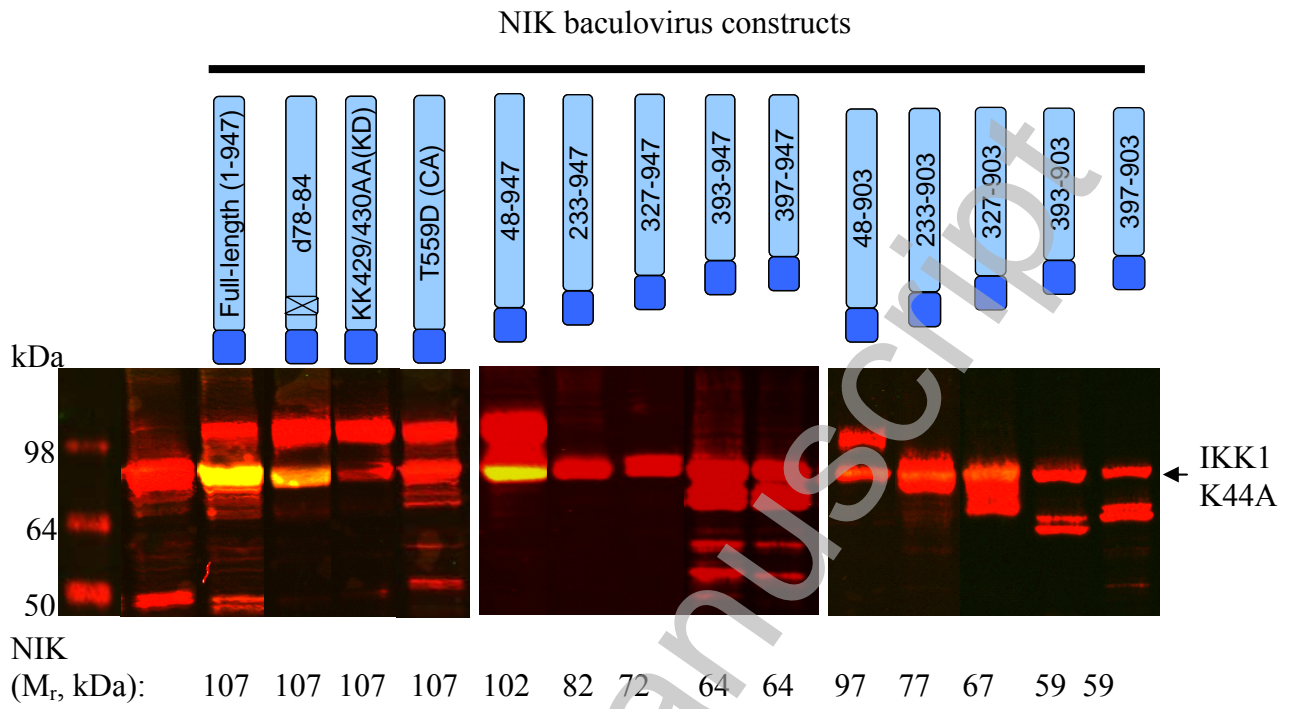
A range of concentrations of compound A was incubated for 30 minutes with GST-NIK (20nM) and rhodamine green labelled FP ligand (5nM). Fluorescence polarisation was recorded, and converted to % displacement by reference to high controls (enzyme-ligand mix) and low controls (ligand only). Resulting data were fitted as described in the text to yield a pIC₅₀ for compound A of 7.0.

Figure 7 Inhibition of lymphotoxin induced p52 nuclear translocation

(A) Lymphotoxin $\alpha 1/\beta 2$ (0.3 $\mu\text{g}/\text{ml}$) induces translocation of p52 into the nucleus in U2OS cells. (B) Without lymphotoxin stimulation, staining for p52/p100 is higher in the cytoplasm for the majority of cells. (C) Adding compound A (3 μM) together with lymphotoxin $\alpha 1/\beta 2$ (0.3 $\mu\text{g}/\text{ml}$) prevents p52 translocation. (D) Assay window and standard deviation determined from 16 repeat wells per condition.

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Figure 1

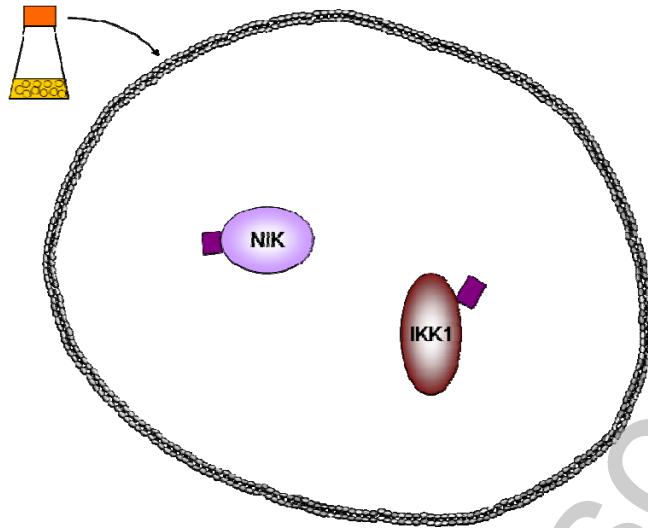


THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20081646

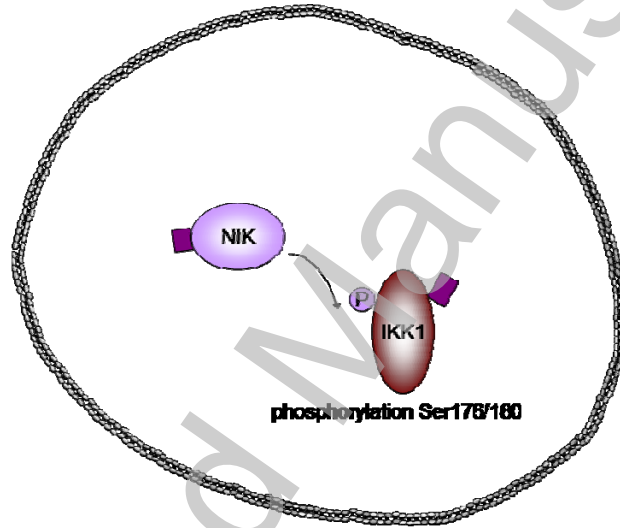
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Figure 2

A



B



C

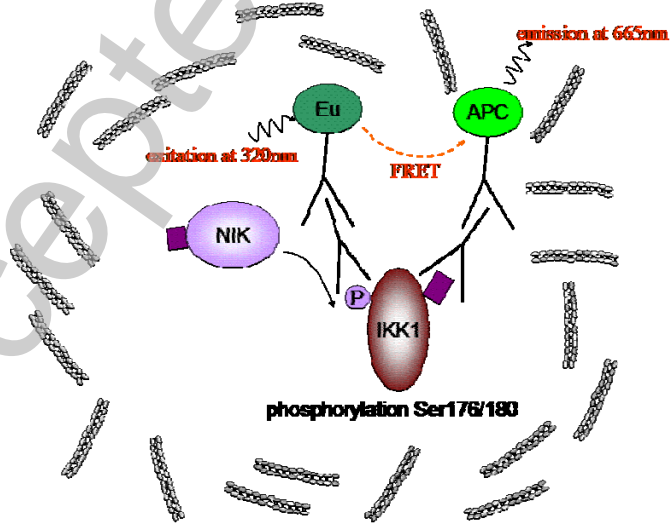


Figure 3A

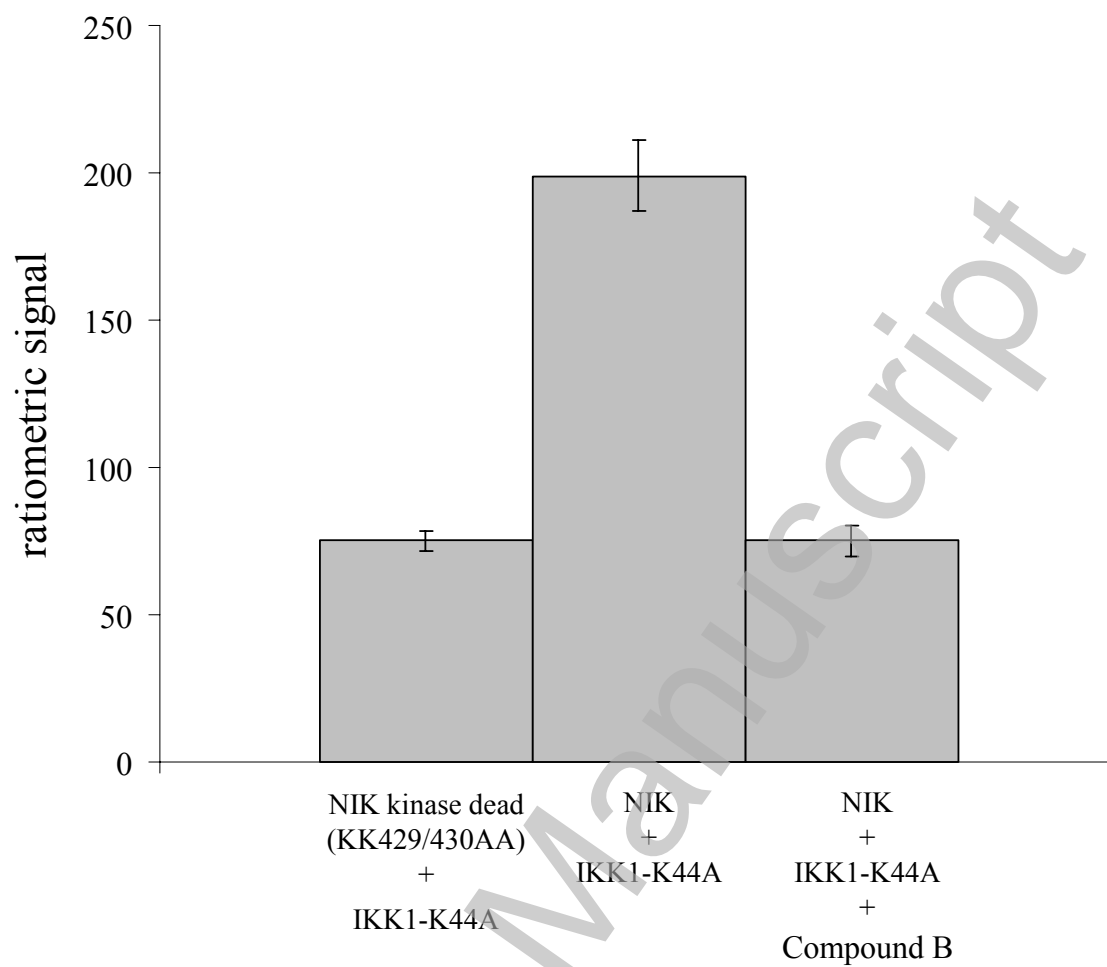


Figure 3B

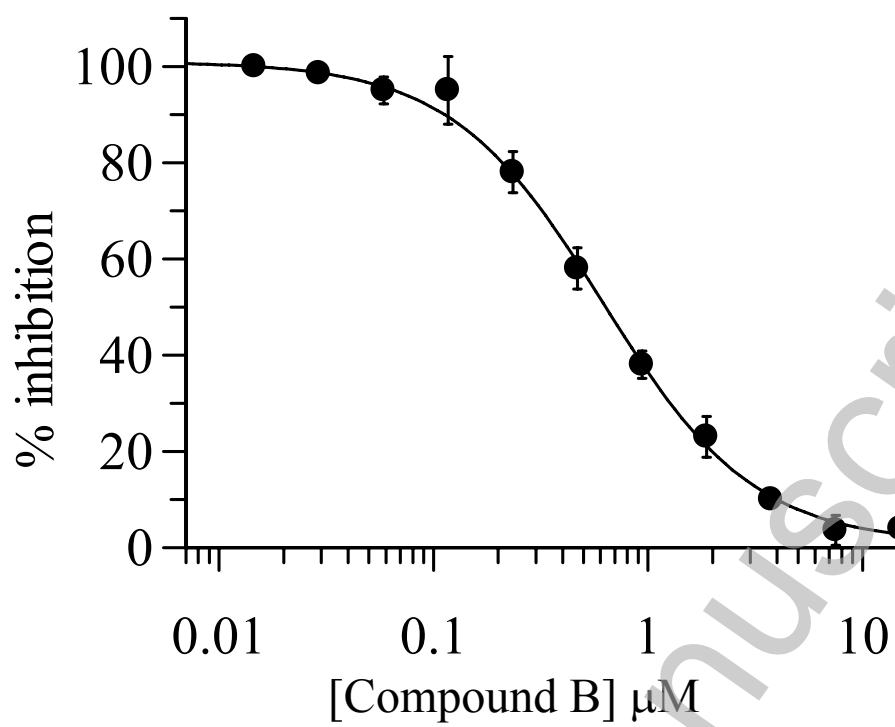


Figure 3C

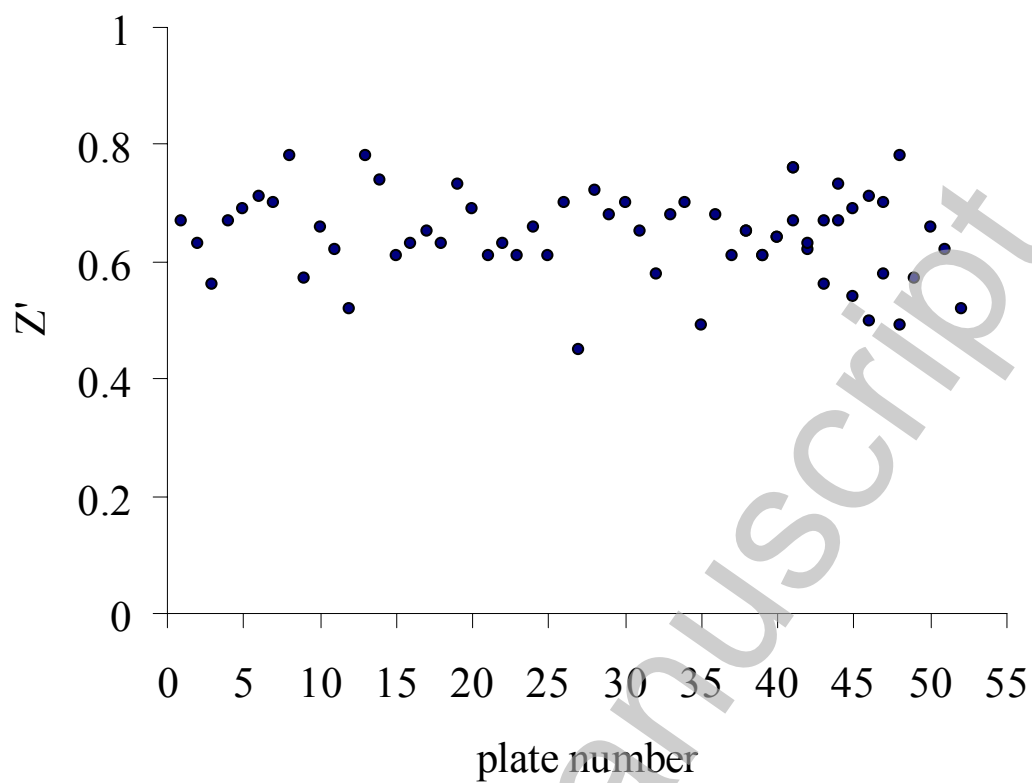


Figure 4

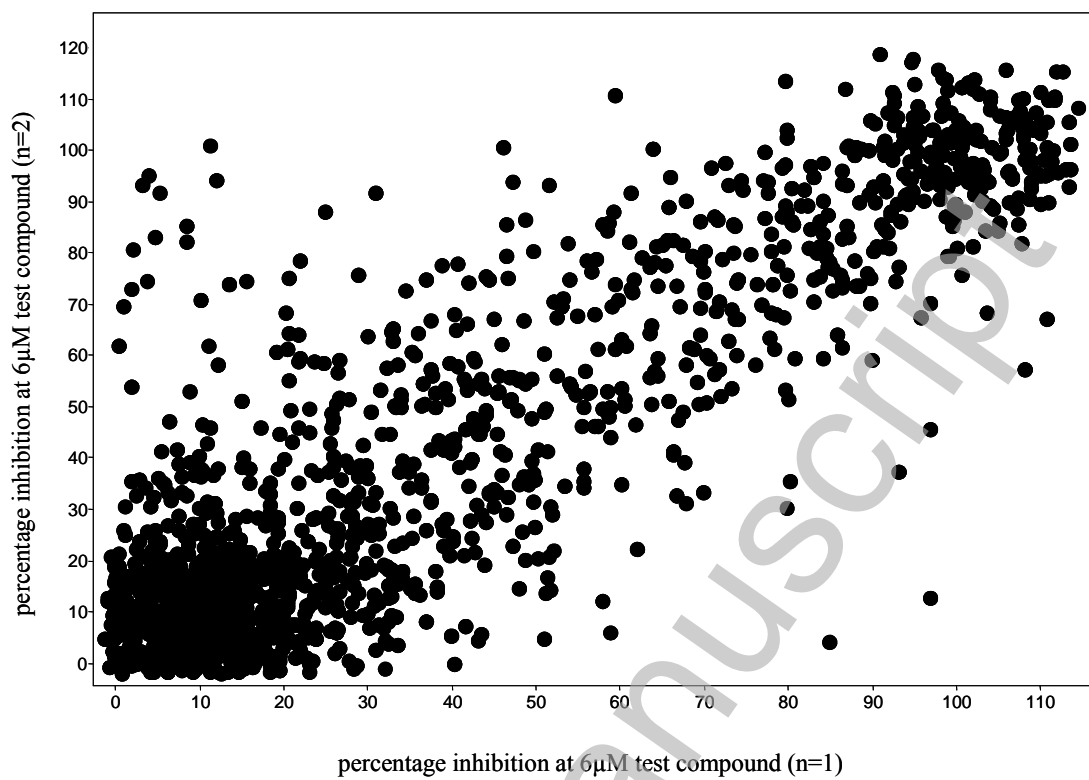


Figure 5

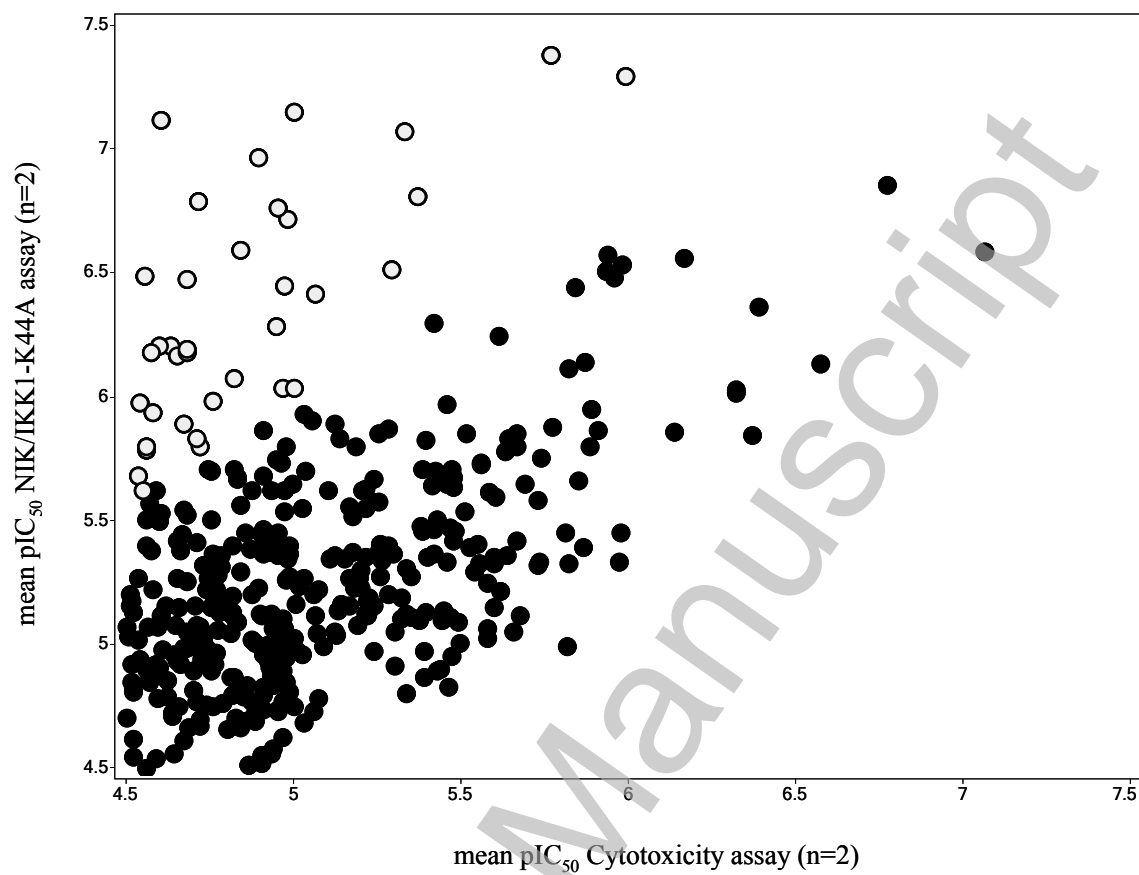
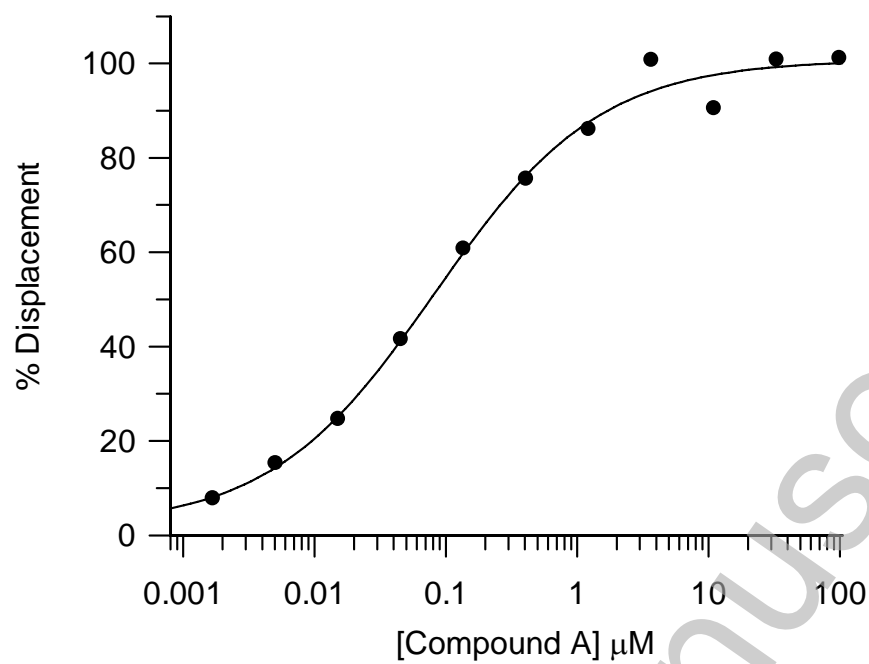


Figure 6



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Figure 7

