Tavare, J. M., Fletcher, L. M., and Welsh, G. I. (2001). Using green fluorescent protein to study intracellular signaling. *J. Endocrinol.* **170,** 297–306.

Thomas, N., and Goodyer, D. (2003). Stealth sensors: Real-time monitoring of the cell cycle. *Targets* **2,** 26–33.

Trask, O. J., Jr., Baker, A., Williams, R. G., Kickischer, D., Kandasamy, R., Laethem, C., Johnston, P. A., and Johnston, P. A. (2006). Assay development and case history of a 32K-biased library high-content MK2-EGFP translocation screen to identify p38 MAPK inhibitors on the ArrayScan 3.1 imaging platform. *Methods Enzymol.* **414** (this volume).

Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4,** 67–73.

Zu, Y. L., Ai, Y., and Huang, C. K. (1995). Characterization of an autoinhibitory domain in human mitogen-activated protein kinase-activated protein kinase 2. *J. Biol. Chem.* **270,** 202–206.

[22] Development and Implementation of Three Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway Imaging Assays to Provide MAPK Module Selectivity Profiling for Kinase Inhibitors: MK2-EGFP Translocation, c-Jun, and ERK Activation

By DEBRA NICKISCHER, CARMEN LAETHEM, OSCAR J. TRASK, JR., RHONDA GATES WILLIAMS, RAMANI KANDASAMY, PATRICIA A. JOHNSTON, and PAUL A. JOHNSTON

Abstract

This chapter describes the development and implementation of three independent imaging assays for the major mitogen-activated protein kinase (MAPK) signaling modules: p38, JNK, and ERK. There are more than 500 protein kinases encoded in the human genome that share an ATP-binding site and catalytic domain conserved in both sequence and structure. The majority of kinase inhibitors have been found to be competitive with ATP, raising concerns regarding kinase selectivity and potency in an environment of millimolar intracellular concentrations of ATP, as well as the potential for off-target effects via the many other cellular proteins that bind and/or utilize ATP. The apparent redundancy of the kinase isoforms and functions in the MAPK signaling modules present additional challenges for kinase

METHODS IN ENZYMOLOGY, VOL. 414 0076-6879/06 $35.00

DOI: 10.1016/S0076-6879(06)14022-7

inhibitor selectivity and potency. Imaging assays provide a method to address many of these concerns. Cellular imaging approaches facilitate analysis of the targets expressed in the context of their endogenous substrates and scaffolding proteins and in a complex environment for which subcellular localization, cross talk between pathways, phosphatase regulatory control, and intracellular ATP concentrations are relevant to the functions of the kinase. The assays described herein provide a strategy to profile kinase inhibitors for MAPK pathway selectivity while simultaneously providing information on cell morphology or toxicity. Results suggest that the MAPK pathways are indeed susceptible to nonselective kinase inhibitors such as staurosporin and inhibitors that inhibit upstream MAPK Kinase Kinases (MKKKs) and MAPK Kinases (MKKs) in the MAPK signaling pathway, especially those involved in cross talk between the pathways. However, selective MAPK inhibitors were identified that exhibited pathway selectivity as evidenced by significantly lower IC_{50} values for their respective p38, JNK, or ERK signaling pathway assays.

Introduction

There are more than 500 protein kinases encoded in the human genome that share an ATP-binding site catalytic domain that is conserved in both sequence and structure (English and Cobb, 2002; Noble *et al.*, 2004). Not surprisingly, the majority of kinase inhibitors identified to date interact within the ATP-binding site and have been found to be competitive with ATP (English and Cobb, 2002; Noble *et al.*, 2004). The degree of conservation of the ATP-binding site raises concerns over the ability to build selective inhibitors (English and Cobb, 2002; Noble *et al.*, 2004). Another significant hurdle for ATP-competitive kinase inhibitors is their ability to exhibit potent activity in cellular environments where concentrations of ATP are reportedly in the millimolar range (English and Cobb, 2002; Noble *et al.*, 2004). In addition to kinases, numerous other cellular proteins bind and/or utilize ATP, each providing the potential for off-target binding of ATP-competitive kinase inhibitors leading to adverse effects (English and Cobb, 2002; Noble *et al.*, 2004).

The emergence of automated cell-based imaging assays has provided a powerful tool to interrogate the inhibition of kinases and signaling pathways in the cellular context (Almholt *et al.*, 2004; Giuliano *et al.*, 1997; Mitchison, 2005; Trask *et al.*, 2006; Williams *et al.*, 2006). Imaging assays may be configured to measure multiple kinase targets or signaling pathway readouts, thereby providing a selectivity profile (Almholt *et al.*, 2004; Giuliano *et al.*, 1997; Mitchison, 2005) Image analysis algorithms also

provide multiple features and image-based parameters that provide information on cell morphology and cytotoxicity (Giuliano *et al.*, 1997). Thus, by mining ''high-content'' data it is possible to extract additional information on the effects of compound treatment of cells beyond the single target readout typical of most other assay formats (Giuliano *et al.*, 1997; Mitchison, 2005).

Williams *et al.* (2006) and Trask *et al.* (2006) described the development and implementation of a mitogen-activated protein kinase-activated protein kinase-2 (MK2) translocation assay to screen for inhibitors of the p38 mitogen-activated protein kinase (MAPK) signaling pathway. This chapter describes the development of two additional imaging assays to identify inhibitors of extracellular signal-regulated kinases (ERK) and stress-activated protein (c-jun N-terminal, JNK) mitogen-activated signaling pathways. Furthermore, it describes the implementation of these three independent MAPK signal transduction pathway assays to assess the hits from a p38 inhibitor MK2-EGFP translocation screen (Trask *et al.*, 2006) and to profile compounds arising from two lead optimization efforts focused on the construction of selective p38 and JNK inhibitors.

Definition of the Cell Model

Mitogen-activated protein kinases are members of the signaling cascades for diverse extracellular stimuli that regulate fundamental cellular processes, including embryogenesis, differentiation, mitosis, apoptosis, movement, and gene expression (Cowan and Storey, 2003; English and Cobb, 2002; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). Four distinct MAP kinase families have been described: extracellular signal-regulated kinases (ERKs), c-jun N-terminal (JNK) or stress-activated protein kinases (SAPK), ERK5/big MAP kinase 1 (BMK1), and the p38 group of protein kinases (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002; Ono and Han, 2000). MAP kinases phosphorylate other protein kinases, phospholipases, transcription factors, and cytoskeleton proteins (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). The core unit of a MAPK pathway is a three-member protein kinase cascade (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). MAP kinases are regulated by phosphorylation and serve as substrates for MAPK kinases (MKKs). The third component of the phospho-relay system are the MKKKs that phosphorylate and activate specific MKKs (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). Specificity of MAPK responses is achieved by the activation of

distinct MKKK-MKK-MAPK signaling modules in response to different stimuli (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). The importance of MAPK signaling pathways as potential drug targets is indicated by the large number of patent applications that have been submitted by numerous pharmaceutical companies describing small molecule modulators of these pathways (English and Cobb, 2002; Fabbro *et al.*, 2002; Noble *et al.*, 2004; Regan *et al.*, 2002). A number of small molecule inhibitors of the MAPK signaling modules have exhibited efficacy in animal disease models, and several have advanced into human clinical trials for cancer and inflammation therapies (English and Cobb, 2002; Fabbro *et al.*, 2002; Noble *et al.*, 2004; Regan *et al.*, 2002).

The p38 (reactivating kinases, RKs, or p40) kinase module is known to mediate stress responses and is activated by heat shock, ultraviolet light, bacterial lipopolysaccharide, or the proinflammatory cytokines interleukin (IL)-1β or tumor necrosis factor (TNF)-α (Cowan and Storey, 2003; Garrington and Johnson, 1999; Ono and Han, 2000). Activation of the p38 pathway results in the phosphorylation of downstream kinases, transcription and initiation factors that affect cell division, apoptosis, and invasiveness of cultured cells and the inflammatory response (Cowan and Storey, 2003; Garrington and Johnson, 1999; Ono and Han, 2000). In addition to p38a (CSBP, MPK2, RK, Mxi2), there are three p38 homologues: p38β, p38γ (ERK6, SAPK3), and p38δ (SAPK4) (Ono and Han, 2000). p38α and p38β are expressed ubiquitously, whereas p38γ is expressed predominantly in skeletal muscle, and p38δ is enriched in lung, kidney, testis, pancreas, and small intestine (Ono and Han, 2000). Upstream kinases acting on p38 include MKK3 and MKK6, which in turn are activated by MLKs and ASK1 (MKKKs).

The ERK MAPK module responds primarily to growth factors and mitogens by stimulating transcriptional responses involved in cell division, migration, and survival (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). ERK1 and ERK2 are widely expressed and are activated upstream by MEK1 and MEK2, which are in turn activated by Raf (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002).

The JNK MAPK module responds to a wide variety of stress signals, including heat shock, osmotic stress, proinflammatory cytokines, ischemia, and ultraviolet exposure (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). JNK1 and JNK2 are widely expressed in many tissues, whereas JNK3 is brain specific (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). JNK1 and JNK2 are activated upstream by MKK4 and MKK7, which are in

turn activated by a variety of MKKKs: MEKKs 1–4, ASK1, and MLKs (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002).

Thus, the MAPK signaling pathways are cascades composed of at least three kinases: MKKK-MKK-MAPK. This signaling cascade is exquisitely sensitive to regulatory input at multiple levels involving several mechanisms (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). On the basis of sequence homology and function, 12 members of the MAPK family have been identified together with 7 MKKs and 14 MKKKs (Garrington and Johnson, 1999; Johnson and Lapadat, 2002). The MKKKs are diverse in structure and have a variety of regulatory motifs not found in MKKs or MAPKs: pleckstrin homology domains, proline-rich sequences for binding Src-homlogy domains, leucine-zipper dimerization sequences, and binding sites for GTP-binding proteins (Garrington and Johnson, 1999). The diversity of regulatory domains in different MKKKs may provide the flexibility that allows the MAPK modules to respond to diverse stimuli. The MKK family has the fewest members in the MAPK module and has the highest specificity for their MAPK substrates (Garrington and Johnson, 1999). However, there is a considerable degree of cross talk between the MAPK modules that may be involved in modulating or fine-tuning the response to stimuli (Cowan and Storey, 2003; Garrington and Johnson, 1999). For example, each MKK can be phosphorylated by multiple MKKKs, and several MAPK substrates, such as Elk1 and MK3, can be phosphorylated by all three MAPK signaling pathways (Cowan and Storey, 2003; Garrington and Johnson, 1999).

Given the apparent redundancy within MAPK signaling modules and cross talk between pathways, how then may specificity be achieved? One level of specificity may be provided at the level of cell/tissue specific and/or developmental expression patterns of the different isoforms that comprise the MAPK modules (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). Kinase activity can also be regulated by subcellular location, thus specificity and regulation of MAPK pathways may therefore be provided by scaffolding or anchoring proteins that bring together specific kinases for the selective activation, sequestration, and localization of signaling complexes (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). Because MAPKs are regulated by phosphorylation, it therefore follows that phosphatases will be a key element of their control, and a family of dual specificity (Ser/Thr/Tyr) MAPK phosphatases (MKP1, MKP2, and MKP3) has been implicated (Cowan and Storey, 2003). Imaging assays provide a means to investigate kinase targets in the intracellular milieu where these regulatory mechanisms are intact.

Cellomics ArrayScan Automated Imaging Platform

The ArrayScan platform marketed by Cellomics (Fisher Scientific, Hampton, NH) is one of the most widely deployed high-content screening (HCS) systems. The studies described in this chapter and in Williams *et al.* (2006) and Trask *et al.* (2006) are performed on an ArrayScan II that has the software upgraded to a 3.1 version. The ArrayScan 3.1 houses a Zeiss Axiovert S100 inverted microscope outfitted with 5×/0.25 NA, 10×/0.3NA, and 20×/0.4 NA Zeiss objectives. Illumination is provided by a Xe/Hg arc lamp source (EXFO, Quebec, Canada), and fluorescence is detected by a 12-bit high sensitivity −20°-cooled CCD camera (Photometrics Quantix). The ArrayScan 3.1 provides the capability of imaging multiple fluorescent probes by acquiring wavelength channels sequentially in which each fluorophore is excited separately and then collected through single- or multiband-pass filter sets and detected on the chip of a monochromatic CCD camera. Channel selection is accomplished using a fast excitation filter wheel combined with single or multiband emission filter. The system comes with filter sets designed for the common fluorescent probes and can distinguish up to four labels in a single preparation with minimal cross talk between channels. The ArrayScan 3.1 is a wide-field imaging system that illuminates a ''large'' area of the specimen and directly images that area all at once. It uses an image-based autofocus system that images a fluorescent label in cells, typically fluorescently stained nuclei, but any feature of interest could be used, and an algorithm measures the relative sharpness of the image. It should be noted that any fluorescent particle could potentially be focused upon. The ArrayScan 3.1 was integrated with a Zymark Twister Robot and 80 plate stacker for fixed end point assays.

The cytoplasm-to-nuclear translocation algorithm developed by Cellomics may be used to quantify the relative distribution of a fluorescently tagged target between two cellular compartments, namely the cytoplasm and the nucleus (Giuliano *et al.*, 1997). Labeling with a nucleic acid dye such as Hoechst 33342, DAPI, DRAQ5, or other fluorophore identifies the nuclear region, and this signal is used to focus the instrument and to define a nuclear mask. The mask is eroded to reduce cytoplasmic contamination within the nuclear area, and the final reduced mask is used to quantify the amount of target channel fluorescence within the nucleus. The nuclear mask is then dilated to cover as much of the cytoplasmic region as possible without going outside the cell boundary. Removal of the original nuclear region from this dilated mask creates a ring mask that covers the cytoplasmic region outside the nuclear envelope. The ''Cytonuc'' difference measurement is calculated as the difference of the average nuclear intensity minus the average cytoplasmic ring intensity on a per cell basis or may be reported as an overall well average value (Giuliano *et al.*, 1997).

Development of the JNK MAPK Signaling Pathway Assay

To complement the p38 MAPK assay described elsewhere (Trask *et al.*, 2006; Williams *et al.*, 2006), we set out to develop a c-Jun activation assay leveraging a similar HCS approach. The c-Jun activation assay was developed in the HeLa adenocarcinoma cervical cell line (ATCC-CCL2) using a commercially available screening kit (Hitkit) purchased from Cellomics. To confirm that the SAPK signaling pathway is intact in the HeLa cell line, cells are either left untreated or are treated with 100 n*M* of the protein synthesis inhibitor anisomycin for 30 min. Cells are then solubilized in SDS sample buffer, their total cellular proteins are separated on 10% SDS-PAGE gels, transferred to nitrocellulose, and the resulting blots are probed with specific antibodies for the total and phosphorylated forms of JNK1, JNK2, and c-Jun (Fig. 1A). The total JNK1, JNK2, and c-Jun signals detected on the Western blots appear unaffected by anisomycin treatment. However, activation of the JNK/SAPK signaling pathway by anisomycin treatment dramatically increases the phosphorylation signals for JNK1, JNK2, and c-Jun relative to untreated controls (Fig. 1A).

The c-Jun activation kit from Cellomics utilizes a combination of a primary mouse monoclonal antibody to phospho-c-Jun (Ser 63) and an Alexa 488-conjugated goat anti-mouse IgG secondary antibody to measure the amount of phospho-c-Jun in cells by indirect immunofluorescence. HeLa cells (5×10^3) per well were seeded in EMEM + 10% fetal bovine serum (FBS) and 2 m*M* L-glutamine and were incubated overnight at 37° and 5% CO_2. Medium, with or without 100 n*M* anisomycin, was added and the plates were incubated at 37° and 5% CO_2 for 25 min. The cells were fixed with formaldehyde, permeabilized, incubated with the primary mouse antibody against phoshorylated c-Jun, washed, and then incubated with the goat anti-mouse secondary antibody conjugated with Alexa Fluor 488 containing Hoechst dye. The Cellomics ArrayScan 3.1 is used to capture images of the phospho-c-Jun in fields of view of untreated and anisomycin-treated HeLa cells (Fig. 1B). The level of phospho-c-Jun fluorescent signal is significantly brighter in anisomycin-treated cells than in untreated cells, and the majority of the staining of both populations appears localized within the nucleus. Labeling nuclei with Hoechst 33342 identifies the nuclear region, and this signal is used to focus the instrument and in defining a nuclear mask. The cytoplasm-to-nuclear translocation algorithm measures the relative distribution of the target, in this case c-Jun, between two cellular compartments, the cytoplasm and the nucleus. Bit maps of individual cells, from both nuclear and c-Jun channels for untreated and treated cells, are also shown (Fig. 1B). As determined by the algorithm, the nuclear masks are indicated in blue and the cytoplasmic ring masks are indicated in green. While the

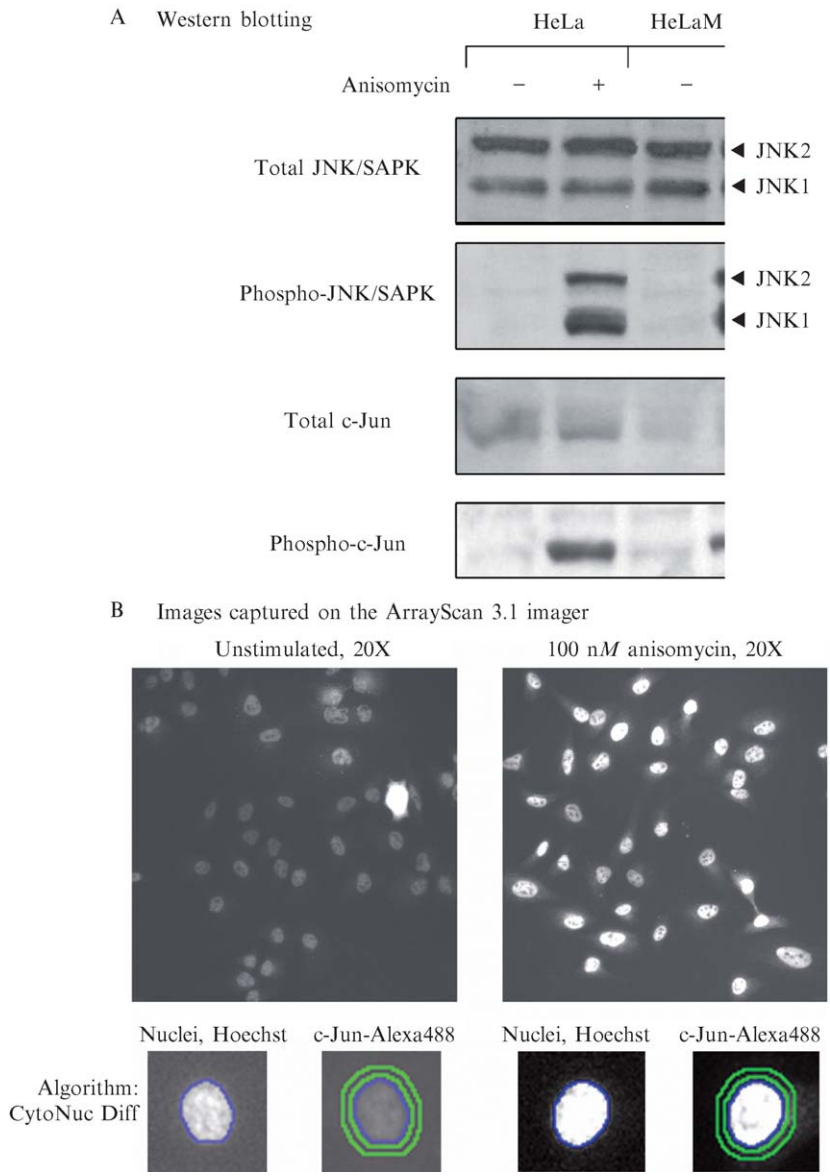


FIG. 1. Anisomycin-induced SAPK pathway activation: Western blotting and images captured on the ArrayScan 3.1 imager. (A) HeLa cells either were left untreated or were treated with 100 n*M* of the protein synthesis inhibitor anisomycin for 30 min. Cells were then solubilized in SDS sample buffer, their total cellular proteins were separated on 10% SDS-PAGE gels, and they were transferred to nitrocellulose, and the resulting blots were probed

nuclear staining appears equivalent in both cells, the phospho-c-Jun fluorescent signal is significantly brighter in the nuclear region of the anisomycin-treated cell than in the untreated cell. The ''Cytonuc'' difference (average nuclear intensity – average cytoplasmic ring intensity) for c-Jun staining will therefore be significantly higher in anisomycin-treated cells.

A series of experiments was conducted to optimize the c-Jun activation assay and to provide a robust and reproducible readout of the JNK/SAPK pathway activation (Figs. 2 and 3). To evaluate the c-Jun activation responses of HeLa cells, several stimuli were examined in dose–response experiments: IL-1β, platelet-derived growth factor, phorbol ester (PMA), and anisomycin (Fig. 2A). Even though anisomycin, IL-1, and PMA all produce dose-dependent c-Jun activation responses, anisomycin was selected for further assay development, as it exhibits the largest signal window. Cell density effects were examined as follows: 2.5, 5.0, and 10.0×10^3 HeLa cells per well are seeded in EMEM + 10% FBS and incubated overnight at 37° and 5% CO_2. Medium, with or without 100 n*M* anisomycin, was added and the plates were incubated at 37° and 5% CO_2 for 25 min. The ArrayScan imager and cytoplasm-to-nuclear translocation algorithm are able to adequately quantify the c-Jun activation response at all three seeding densities (Fig. 2B). Data presented are the means from at least 250 valid objects (cells), or 10 fields, whichever came first. A seeding density of 5.0×10^3 cells per well was selected for further assay development to reduce both the cell culture demands for the assay and the number of fields required to obtain a valid object count greater than 250 while maintaining good physical separation between cells. To identify the optimal stimulation time, HeLa cells were plated at 5.0×10^3 cells per well; after overnight culture, medium containing the indicated concentrations of anisomycin was added and plates were incubated for the indicated time points prior to fixation and staining (Fig. 2C). Although there is a measurable response to anisomycin

with specific antibodies for the total and phosphorylated forms of JNK1, JNK2, and c-Jun. (B) HeLa cells (5×10^3) per well were seeded in EMEM + 10% FBS and incubated overnight at 37° and 5% CO_2. Medium, with or without 100 n*M* anisomycin, was added and the plates were incubated at 37° and 5% CO_2 for 25 min. The cells were fixed with formaldehyde, permeabilized, incubated with the primary mouse antibody against phosphorylated c-Jun, washed, and then incubated with the goat antimouse secondary antibody conjugated with Alexa Fluor 488 and containing Hoechst dye. The Cellomics ArrayScan 3.1 was used to capture images of the phospho-c-Jun in fields of view of untreated and anisomycin-treated HeLa cells. The cytoplasm-to-nuclear translocation algorithm measures the relative distribution of the target, in this case c-Jun, between two cellular compartments, the cytoplasm and the nucleus. Bit maps of individual cells, from both nuclear and c-Jun channels for untreated and treated cells, are also shown; nuclear masks are indicated in blue and the cytoplasmic ring masks are indicated in green.

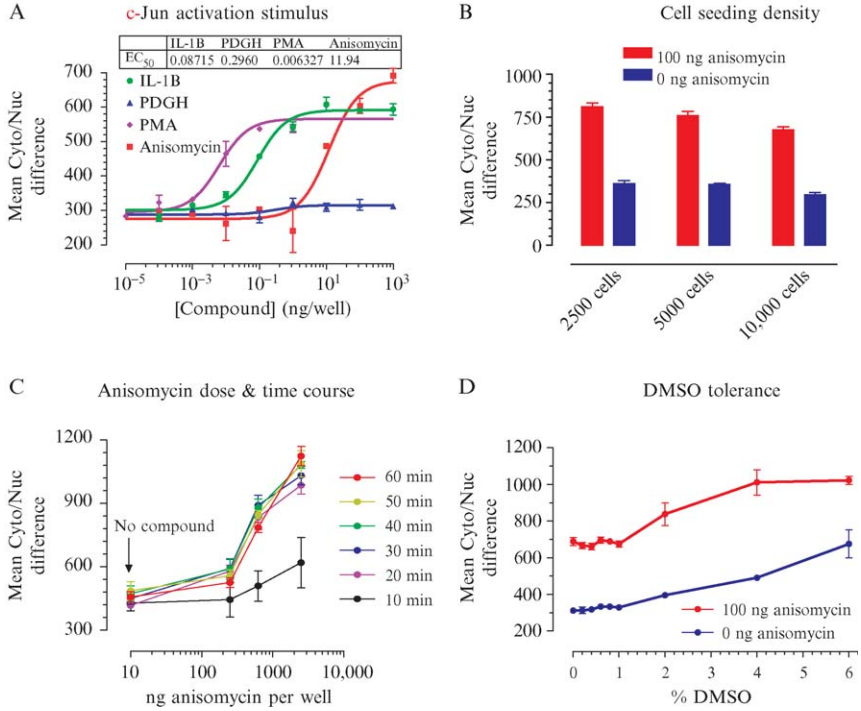


FIG. 2. c-Jun activation assay development. (A) HeLa cells (5.0×10^3) per well were seeded in EMEM + 10% FBS and incubated overnight at 37° and 5% CO_2. The c-Jun activation dose responses of Hela cells treated with the indicated doses of interleukin-1β (IL-1), platelet-derived growth factor (PDGH), or phorbol ester (PMA) were compared to anisomycin-induced responses quantified by the cytoplasm-to-nuclear translocation algorithm from images captured on the ArrayScan. (B) HeLa cells (2.5, 5.0, and 10.0×10^3) per well were seeded in EMEM + 10% FBS and incubated overnight at 37° and 5% CO_2. Medium, with or without 100 n*M* anisomycin, was added and the plates were incubated at 37° and 5% CO_2 for 25 mins. The c-Jun activation responses were quantified by the cytoplasm-to-nuclear translocation algorithm from images captured on the ArrayScan. (C) HeLa cells (5.0×10^3) per well were plated and, after overnight culture, medium containing the indicated concentrations of anisomycin was added and plates were incubated for the indicated time points prior to fixation and staining. The c-Jun activation responses were quantified by the cytoplasm-to-nuclear translocation algorithm from images captured on the ArrayScan. (D) HeLa cells (5.0×10^3) per well were plated and, after overnight culture, were treated for 30 min with medium or 100 ng/ml anisomycin at the indicated DMSO concentrations. The c-Jun activation responses were quantified by the cytoplasm-to-nuclear translocation algorithm from images captured on the ArrayScan.

treatment after 10 min of incubation, at least 20 min is required for a maximal response. The response appears stable between 20 and 60 min, and a 30-min incubation period was selected for further assay development. Finally, compound screening libraries are typically solubilized in dimethyl sulfoxide (DMSO), and we wanted to evaluate the DMSO tolerance of the c-Jun activation response in HeLa cells. HeLa cells were plated at 5.0×10^3 cells per well and after overnight culture, were treated for 30 min with media or 100ng/mL anisomycin at the indicated DMSO concentrations (Fig. 2D). The c-Jun activation response appears unaffected at DMSO concentrations $\leq 1.0\%$. However, at DMSO concentrations $>1.0\%$, the Cytonuc difference increases with the DMSO concentration, independently of the treatment conditions. We therefore decided that for the c-Jun activation assay, compounds would be diluted such that DMSO concentrations would not exceed 1.0%. Having completed these assay development experiments enabled us to finalize a c-Jun activation protocol.

c-Jun Activation Protocol

Materials

HeLa cells (ATCC)
Formaldehyde (Sigma F1268)
EMEM (BioWhittaker) containing 10% FBS (JRH Biosciences), 2 m*M* L-glutamine (BioWhittaker), and 10 m*M* HEPES (BioWhittaker)
Packard 96-well ViewPlates
c-Jun Activation Hit Kit (Cellomics)

Buffers and Working Solutions

1× wash buffer: Add 20 ml of 10× wash buffer (from Hit Kit) to 160 ml with deionized water, adjust pH to 7.2, and bring to a final volume of 200 ml. May be stored for several days at 4°.
Fixation solution: In a fume hood, add 2.2 ml 37% formaldehyde to 19.8 ml 1× wash buffer. Prepare fresh for each assay and warm to 37° before use.
Permeabilization buffer: Add 4 ml of 10× permeabilization buffer (Hit Kit) to 30 ml deionized water, adjust pH to 7.2, and bring to a final volume of 40 ml. May be stored for several days at 4°.
Detergent buffer: Add 8 ml of 10× detergent buffer (from Hit Kit) to 60 ml of deionized water, adjust pH to 7.2, and bring to a final volume of 80 ml. May be stored for several days at 4°.
Primary antibody solution: Add 27.5 μl of c-Jun primary antibody (from Hit Kit) to 5.5 ml 1× wash buffer. Prepare fresh daily.

Staining solution: Add 55 μl of secondary antibody (from Hit Kit) and 2.75 μl Hoechst Dye (from Hit Kit) to 5.5 ml 1× wash buffer. Prepare fresh daily.

Protocol

1. Culture HeLa cells to approximately 80% confluency in EMEM plus 2 m*M* glutamine and 10% FBS. (Split 1:5 every 3 to 4 days.)
2. Harvest cells with 0.05% trypsin-EDTA, adjust the cell number to 5×10^4 cells/ml in media, and plate 100 μl per well (5000 cells per well).
3. Incubate plates overnight at 37° and 5% CO_2.
4. Add 20 μl of medium containing 7× compounds and controls in 7% DMSO.
5. Add 20 μl of medium containing 5 μg/ml anisomycin.
6. Incubate 30 min at 37° and 5% CO_2.
7. Aspirate culture medium and add 200 μl of prewarmed fixation solution.
8. Incubate in fume hood for 10 min at room temperature (all remaining steps are performed at room temperature).
9. Aspirate fixation solution and add 200 μl of 1× wash buffer.
10. Aspirate wash buffer and add 200 μl of permeabilization buffer and incubate 90 s.
11. Aspirate permeabilization buffer and add 200 μl of 1× wash buffer.
12. Aspirate wash buffer, add 50 μl of primary antibody solution, and incubate 1 h.
13. Aspirate primary antibody solution, add 200 μl detergent buffer, and incubate 5 min.
14. Aspirate detergent buffer and add 200 μl of 1× wash buffer; perform procedure twice.
15. Add 50 μl of staining solution and incubate 1 h.
16. Aspirate staining solution, add 200 μl of detergent buffer, and incubate 5 min.
17. Aspirate detergent buffer and add 200 μl of 1× wash buffer; perform procedure twice.
18. Add 200 μl of wash buffer, seal plates, and read on the ArrayScan.

c-Jun Activation Signal Window and Reproducibility

To evaluate the reproducibility of the c-Jun activation response in HeLa cells, cells were plated at 5.0×10^3 cells per well and on the following day are treated with the indicated doses of anisomycin in three independent experiments run on separate days (Fig. 3A). The EC_{50} for anisomycin-induced c-Jun activation ranged from 52 to 114 n*M* and produced on

average an EC_{50} of 87 ± 32 nm, indicating a reproducible assay. To evaluate the ability of the c-Jun activation response in HeLa cells to identify SAPK pathway inhibitors, cells were plated and on the next day the indicated doses of the JNK 1/2 inhibitor SP 600125 were added simultaneously with 100 ng/ml anisomycin (final) and cells were incubated for 30 min (Fig. 3B). In three independent experiments, the IC_{50} for the JNK 1/2 inhibitor SP 600125 ranged from 3.0 to 5.7 μM and produced on average an IC_{50} of 4.77 ± 1.51 μM. To further evaluate the robustness and reproducibility of the c-Jun activation response in HeLa cells, two full 96-well plates were treated under the following conditions, one plate per condition: medium alone (blue squares) and 100 ng/ml anisomycin (green circles) (Fig. 3C). In addition to the population scatter plot, plate heat map views of the two plates are also presented (Fig. 3D). The average Cytonuc difference for the medium control plate was 356, and the average Cytonuc difference for the anisomycin-treated control plate was 1580, producing an average assay signal to background window of 4.4-fold. A comparison of medium control plate variability data to anisomycin-treated plate variability data produced a Z factor of 0.77, indicating that the assay would definitely be compatible with HTS (Zhang *et al.*, 1999).

Development of the ERK MAPK Signaling Pathway Assay

To complement our p38 and JNK MAPK signaling pathway HCS assays, an ERK activation assay was developed in the HeLa adenocarcinoma cervical cell line (ATCC-CCL2) using antiphospho-p44/42 MAPK (ERK1/2) antibodies. To confirm that the ERK signaling pathway is intact in the HeLa cell line, cells were left untreated or were treated with either 10 ng/ml oncostatin M (OSM) or 50 ng/ml PMA for 6 min. Cells were then solubilized in SDS sample buffer, their cellular proteins separated on 10% SDS-PAGE gels, transferred to nitrocellulose, and the resulting blot was probed with specific antibodies for the total and phosphorylated forms of ERK1 and ERK2 (Fig. 4A). The total ERK1 and ERK2 signals detected on the Western blots appear unaffected by OSM and PMA treatment. However, activation of the ERK signaling pathway by both OSM and PMA treatment dramatically increases the phosphorylation signals for ERK1 and ERK2 relative to untreated controls (Fig. 4A).

The ERK activation imaging assay utilizes a combination of a primary polyclonal rabbit anti-phospho-p44/42 MAPK (Thr202/Try204) (ERK1/2) antibody purchased from Cell Signaling Technologies and an Alexa 488-conjugated goat antirabbit IgG secondary antibody from Molecular Probes to measure the amount of phospho-ERK1/2 in cells by indirect immunofluorescence (Fig. 4B). HeLa cells (5×10^3) per well were seeded in DMEM

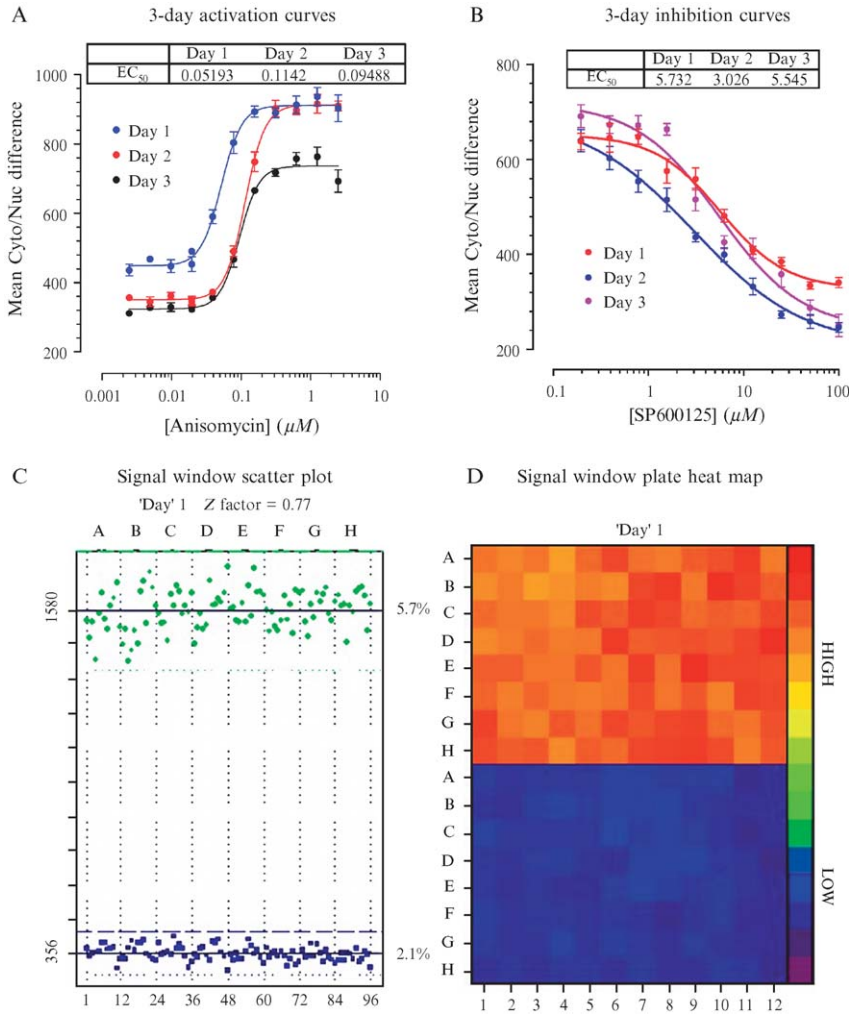


FIG. 3. c-Jun activation reproducibility and variability. (A) HeLa cells (5.0×10^3) per well were plated and on the following day were treated with the indicated doses of anisomycin in three independent experiments run on separate days. (B) HeLa cells (5.0×10^3) per well were plated and, on the next day, the indicated doses of the JNK1/2 inhibitor SP 600125 were added simultaneously with 100 ng/ml anisomycin (final) and cells were incubated for 30 min. Three independent inhibition experiments were run on separate days. (C) HeLa cells (5.0×10^3) per well were plated and, on the next day, two full 96-well plates were treated under the following conditions, one plate per condition: media alone (blue squares) and 100 ng/ml anisomycin (green circles). The Z factor was calculated according to the method of Zhang *et al.* (1999). (D) A plate heat map view of the two plates is also presented. The c-Jun activation responses were quantified by the cytoplasm-to-nuclear translocation algorithm from images captured on the ArrayScan.

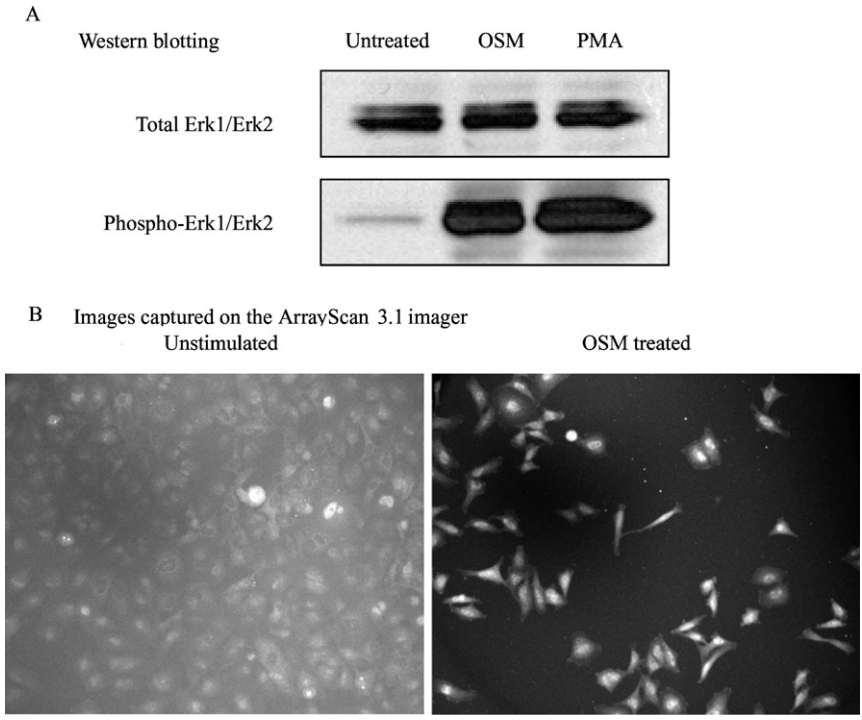
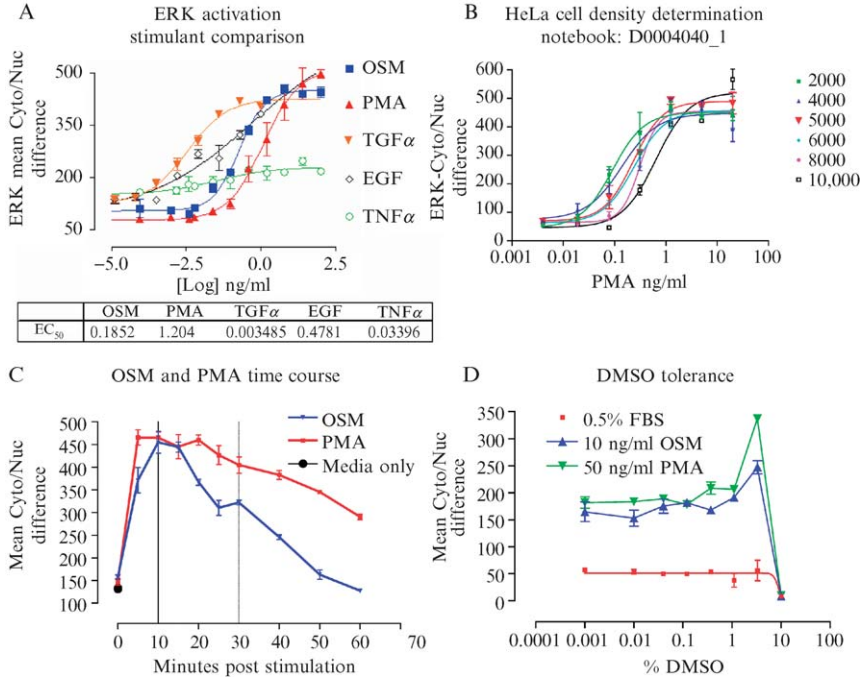


FIG. 4. ERK signaling pathway activation: Western blotting and images captured on the ArrayScan 3.1 imager. (A) HeLa cells were left untreated or were treated with either 10 ng/ml oncostatin M (OSM) or 50 ng/ml phorbol ester (PMA) for 6.0 min. Cells were then solubilized in SDS sample buffer, their total cellular proteins were separated on 10% SDS-PAGE gels, and they were transferred to nitrocellulose, and the resulting blots were probed with specific antibodies for the total and phosphorylated forms of ERK1 and ERK2 . (B) HeLa cells (5×10^3) per well were seeded in DMEM + 0.5% FBS and incubated overnight at 37° and 5% CO_2. Medium, with or without 10 ng/ml OSM, was added, and the plates were incubated at 37° and 5% CO_2 for 10 min. Cells were fixed with formaldehyde, permeabilized, incubated with the primary polyclonal rabbit antiphospho-p44/42 MAPK antibody, washed, and then incubated with the goat antirabbit secondary antibody conjugated with Alexa Fluor 488 and containing Hoechst dye. The Cellomics ArrayScan 3.1 was used to capture images of the phospho-ERK1/2 in fields of view of untreated and OSM-treated HeLa cells.

+ 0.5% FBS and were incubated overnight at 37° and 5% CO_2. Medium, with or without 10 ng/ml OSM, was added and the plates were incubated at 37° and 5% CO_2 for 10 min. The cells were fixed with formaldehyde, permeabilized, incubated with the primary polyclonal rabbit anti-phospho-p44/42 MAPK antibody, washed, and then incubated with the goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 and containing Hoechst dye. The Cellomics ArrayScan 3.1 was used to capture images of

the phospho-ERK1/2 in fields of view of untreated and OSM-treated HeLa cells (Fig. 4B). In untreated HeLa cells, the phospho-ERK1/2 fluorescent signal is relatively dim and appears to be localized predominantly in the cytoplasm. In contrast, the level of phospho-ERK1/2 fluorescent signal is significantly brighter in OSM-treated cells, and the majority of the staining appears localized within the nucleus. The cytoplasm-to-nuclear translocation algorithm measures the relative distribution of the target, in this case phospho-ERK1/2, between two cellular compartments, the cytoplasm and the nucleus. The ''Cytonuc'' difference (average nuclear intensity - average cytoplasmic ring intensity) for phospho-ERK1/2 staining will therefore be significantly higher in OSM-treated cells.

A series of assay development experiments was conducted to optimize the ERK activation assay and provide a robust and reproducible readout of the ERK pathway activation (Figs. 5 and 6). Because the ERK MAPK module responds to a variety of growth factors and mitogens, a number of different stimuli were tested in the ERK1/2 activation assay: EGF, TNFa, PMA, OSM, and TGFα (Fig. 5A). EGF, PMA, OSM, and TGFα all induced robust dose-dependent ERK1/2 activation responses as quantified by the cytoplasm-to-nuclear translocation algorithm from images captured on the ArrayScan (Fig. 5A). TNFα treatment, however, only produced a weak activation of phospho-Erk1/2 when measured on the ArrayScan. OSM and PMA are the stimuli selected for further assay development. To evaluate the effects of seeding density, HeLa cells were seeded at a variety of densities between 2 and 10×10^3 cells per well in DMEM + 0.5% FBS and are incubated overnight at 37° and 5% CO_2. Medium, with or without the indicated doses of PMA, was added and the plates were incubated at 37° and 5% CO_2 for 30 min (Fig. 5B). The ArrayScan imager and cytoplasm-to-nuclear translocation algorithm are able to adequately quantify the ERK activation response at all six seeding densities (Fig. 5B). Data presented are the means from at least 250 valid objects (cells), or 10 fields, whichever came first. A seeding density of 5.0×10^3 cells per well was selected for further assay development to reduce both the cell culture demands for the assay and the number of fields required to obtain a valid object count $\geq$250. To identify the optimal stimulation time, HeLa cells were plated 5.0×10^3 cells per well and, after overnight culture, medium containing either 10 ng/ml OSM or 50 ng/ml PMA was added and plates were incubated for the indicated time points prior to fixation and staining (Fig. 5C). The ERK activation induced by PMA or OSM treatment is relatively rapid and reaches a maximal response at 5 and 10 min, respectively (Fig. 5C). With OSM treatment the maximal activation of ERK1/2 appears stable between 10 and 15 min post-treatment and then declines to pre-stimulation levels in a roughly linear fashion over the next 45 min. For



	OSM	PMA	TGFα	EGF	TNFα
EC_{50}	0.1852	1.204	0.003485	0.4781	0.03396

FIG. 5. ERK activation assay development. (A) HeLa cells (5.0×10^3) per well were plated and, after overnight culture, were treated with the indicated doses of EGF, TNFα, PMA, OSM, or TGFα. (B) HeLa cells were seeded at a variety of densities between 2 and 10×10^3 cells per well in DMEM + 0.5% FBS and incubated overnight at 37° and 5% CO_2. Medium, with or without the indicated doses of PMA, was added, and the plates were incubated at 37° and 5% CO_2 for 30 min. (C) HeLa cells (5.0×10^3) per well were plated and, after overnight culture, medium containing either 10 ng/ml OSM or 50 ng/ml PMA was added and plates were incubated for the indicated time points prior to fixation and staining. (D) HeLa cells (5.0×10^3) per well were plated and, after overnight culture, were treated with either 10 ng/ml OSM or 50 ng/ml PMA for 10 min at the indicated DMSO concentrations. Cells were fixed with formaldehyde, permeabilized, incubated with the primary polyclonal rabbit anti-phospho-p44/42 MAPK antibody, washed, and then incubated with the goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 and containing Hoechst dye. The Cellomics ArrayScan 3.1 was used to capture images of the phospho-ERK1/2, and ERK activation responses were quantified by the cytoplasm-to-nuclear translocation algorithm.

PMA treatment, the maximal activation of ERK1/2 appears stable between 5 and 20 min posttreatment and then declines to ~50% of prestimulation levels in a roughly linear fashion over the next 40 min. A treatment period of 10 min was selected for further assay development with both OSM and PMA. Finally, because compound libraries are typically solubilized in DMSO, we wanted to evaluate the DMSO tolerance of the ERK activation

response. HeLa cells were plated 5.0×10^3 cells per well and, after overnight culture, were treated with either 10 ng/ml OSM or 50 ng/ml PMA for 10 min at the indicated DMSO concentrations (Fig. 5D). The ERK activation response appears unaffected at DMSO concentrations $\leq 1.0\%$. At 5% DMSO there is an apparent increase in the Cytonuc difference for both OSM and PMA, but at 10% DMSO there are so few cells left in the wells that no values can be measured. We therefore decided that for the ERK activation assay, compounds would be diluted such that DMSO concentrations would not exceed 0.5%. Based on the results of these assay development experiments we were able to compose an ERK activation protocol.

ERK1/2 Activation Protocol

Materials

HeLa cells (ATCC)
Formaldehyde (Sigma F1268)
DMEM (BioWhittaker) containing 10% FBS (JRH Biosciences), 2 m*M* L-glutamine (BioWhittaker), 10,000 units penicillin/streptomycin (BioWhittaker), and 10 m*M* HEPES (BioWhittaker)
Dulbecco's PBS Mg^{2+} and Ca^{2+} free (BioWhittaker)
Packard 96-well View Plates
Phospho-p44/42 map kinase (Thr202/Try204) antibody (Cell Signaling)
Alexa 488 goat anti-rabbit IgG (Molecular Probes)
Hoechst 33342 (Sigma)
Triton X-100 (Roche)
Tween-20 (Roche)
Phorbol Ester (PMA) (Sigma)
Oncostatin M (OSM) (R&D)

Buffers and Working Solutions

Wash buffer: Dulbecco's PBS Mg^{2+} and Ca^{2+} free
Permeabilization buffer: PBS/Triton X-100, 0.5%
Blocking Buffer: PBS/Tween 20, 0.1%
Formaldehyde, 3.7%: Add 1.2 ml 37% formaldehyde to 10.8 ml wash buffer. Keep warmed to 37° until ready for use. (Prepare formaldehyde solution in a fume hood.)
Primary antibody solution: 1 μg/ml (final) phospho-p44/42 map kinase antibody in PBS
Secondary antibody solution: 10 μg/ml (final) Alexa 488 goat anti-rabbit IgG and 2 μg/ml (final) Hoechst 33342 dye in PBS

Protocol

1. Harvest HeLa cells and seed 5000 cells per well into 96-well plate(s) in medium.
2. Culture cells at 37°, 5% CO_2 overnight.
3. Add 25 μl of compound (inhibitor) to the wells and incubate for 15 min at 37°, 5% CO_2.
4. Add 25 μl of 10 ng/ml OSM activation stimulus to the wells and incubate for 10 min at 37°, 5% CO_2.
5. Aspirate medium and add 100 μl of prewarmed fixation solution (3.7% formalin) and incubate for 10 min at room temperature.
6. Wash once with 100 μl wash buffer, aspirate wash buffer, add 100 μl of permeabilization buffer, and incubate for 90 s.
7. Aspirate permeabilization buffer and add 100 μl wash buffer.
8. Aspirate buffer and add 50 μl of primary antibody; 1 μg/ml (final) phospho-p44/42 MAP kinase (Thr202/Try204) antibody, and incubate for 1 h at room temperature.
9. Aspirate primary antibody, wash once with blocking buffer, and incubate for 15 min at room temperature.
10. Aspirate and wash once with 100 μl PBS and aspirate.
11. Add 50 μl secondary antibody and stain; 10 μg/ml (final) Alexa 488 goat anti-rabbit IgG and 2 μg/ml (final) Hoechst 33342 dye in PBS and incubate for 1 h at room temperature in the dark.
12. Aspirate and wash once with 100 μl blocking buffer and incubate for 10 min at room temperature.
13. Aspirate buffer and wash once with 100 μl wash buffer.
14. Aspirate buffer, add 100 μl of wash buffer, and seal plate.
15. Read plate on the ArrayScan.

ERK1/2 Activation Signal Window and Reproducibility

To evaluate the reproducibility of the ERK activation response in HeLa cells, cells were plated and on the following day are treated with the indicated doses of OSM or PMA in three independent experiments run on separate days (Fig. 6A and B). The EC_{50} for OSM-induced ERK activation ranges from 0.1 to 0.22 ng/ml and produces on average an EC_{50} of 0.169 $\pm$ 0.062 ng/ml, indicating a reproducible assay (Fig. 6A). The EC_{50} for PMA-induced ERK activation ranges from 0.81 to 0.95 ng/ml and produces, on average, an EC_{50} of 0.884 $\pm$ 0.068 ng/ml, indicating a reproducible assay (Fig. 6B). To evaluate the ability of the ERK activation response in HeLa cells to identify ERK pathway inhibitors, cells were plated and on the next day the indicated doses of the MEK1/2 inhibitor U0126 were added 15 min prior to the addition

of 10 ng/ml OSM and cells were incubated for an additional 10 min (Fig. 6C). In three independent experiments, the IC_{50} for the MEK1/2 inhibitor U0126 ranged from 0.489 to 0.711 μM and produced, on average, an IC_{50} of 0.616 $\pm$ 0.115 μM. To further evaluate the robustness and reproducibility of the ERK activation response in HeLa cells, three full 96-well plates were treated under the following conditions, one plate per condition: medium alone (green circles), 10 ng/ml OSM (red triangles), and 10 ng/ml OSM + 10 μM staurosporin (blue squares)(Fig. 6D). The average Cytonuc difference for the media control plate was 71, and the average Cytonuc difference for the anisomycin-treated control plate was 177, producing an average assay signal-to-background window of 2.49-fold. A comparison of medium control plate variability data to anisomycin-treated plate variability data produced a Z factor of 0.54, indicating that the assay would be compatible with HTS (Zhang *et al.*, 1999).

MAPK Pathway Inhibitor Test Cassette

To evaluate the three independent MAPK signaling pathway imaging assays that we had developed and to determine their ability to discriminate selectivity profiles across the MAPK family, we assembled a test cassette of known MAPK pathway inhibitors (Table I). In addition to five "selective" p38 inhibitors, we included the JNK-1/2 inhibitor SP 600125, the MEK-1/2 inhibitors UO126 and PD 98059, the PI3-kinase inhibitor wortmannin, and staurosporin as a nonselective kinase inhibitor. We also selected two activation stimuli for each MAPK pathway assay: anisomycin and TNFα for the p38 pathway, anisomycin and IL-1β for the JNK pathway, and OSM and PMA for the ERK pathway (Table I).

All five p38 inhibitors produced IC_{50} values for inhibition of anisomycin- and TNFα-induced MK2-EGFP translocation $<$100 nM. UO126 and PD 98059, the MEK-1/2 inhibitors, produced IC_{50} values in the 20 to 39 μM range for both activating stimuli, whereas the nonselective kinase inhibitor staurosporin produced submicromolar IC_{50} values for both stimuli (Table I). Neither the PI3-kinase inhibitor wortmannin nor the JNK-1/2 inhibitor SP 600125 inhibited anisomycin- or TNFα-induced MK2-EGFP translocation. Overall, the MK2-EGFP translocation assay appears exquisitively sensitive to p38 inhibitors relative to other kinase inhibitors.

In the c-Jun activation assay, the JNK-1/2 inhibitor SP 600125 and staurosporin produced IC_{50} values of 8.6 and 1.4 μM, respectively, with IL-1ß as the stimulus , and IC_{50} values of 14 and 7.2 μM, respectively, with anisomycin as the stimulus (Table I). Three of the five "selective" p38 inhibitors, the Merck p38 inhibitor, RWJ 68354, and SB203580, produced IC_{50} values between 20 and 40 μM with anisomycin as the activating stimulus, whereas only the Merck

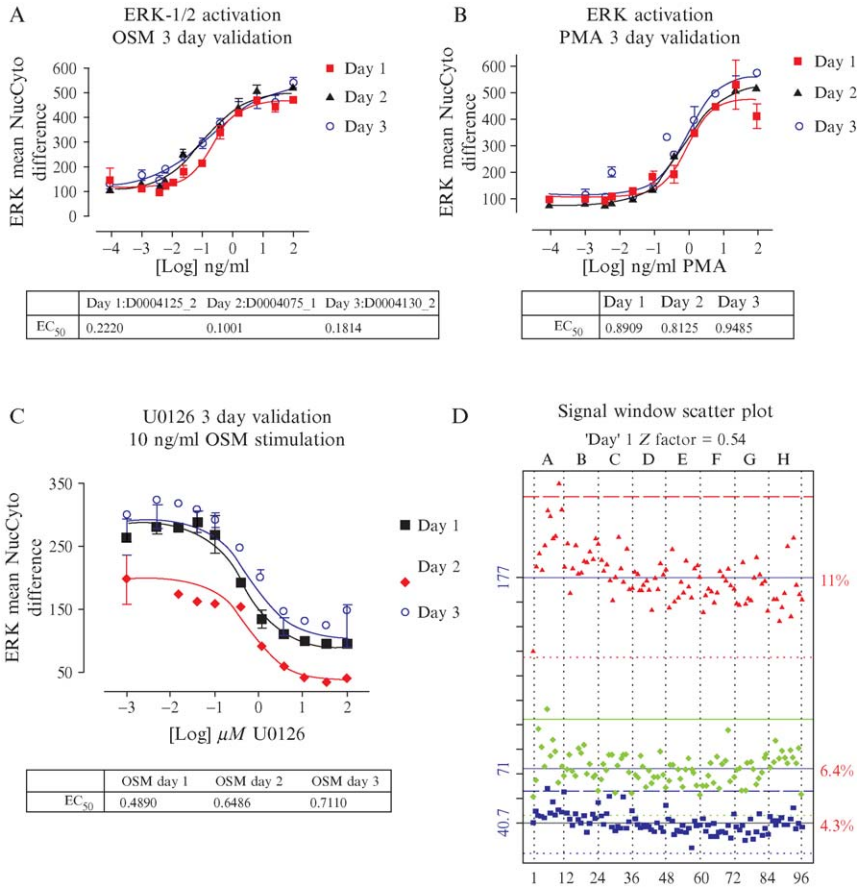


FIG. 6. ERK activation reproducibility and variability. (A) HeLa cells (5.0×10^3) per well were plated and, after overnight culture, were treated with the indicated doses of OSM in three independent experiments run on separate days. (B) HeLa cells (5.0×10^3) per well were plated and, after overnight culture, were treated with the indicated doses of PMA in three independent experiments run on separate days. (C) HeLa cells (5.0×10^3) per well were plated and on the next day the indicated doses of the MEK1/2 inhibitor U0126 were added 15 min prior to the addition of 10 ng/ml OSM and cells were incubated for an additional 10 min. Three independent experiments were run on separate days. (D) HeLa cells (5.0×10^3) per well were plated and, after overnight culture, three full 96-well plates were treated under the following conditions, one plate per condition: medium alone (green circles), 10 ng/ml OSM (red triangles), and 10 ng/ml OSM + 10 μM staurosporin (blue squares). The Z factor was calculated according to the method of Zhang *et al.* (1999). Cells were fixed with formaldehyde, permeabilized, incubated with the primary polyclonal rabbit anti-phospho-p44/42 MAPK antibody, washed, and then incubated with the goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 and containing Hoechst dye. The Cellomics ArrayScan 3.1 was used to capture images of the phospho-ERK1/2, and the ERK activation responses were quantified by the cytoplasm-to-nuclear translocation algorithm.

TABLE I
PROFILING OF A SELECTED KINASE INHIBITOR TEST CASSETTE IN DISTINCT MAPK SIGNALING PATHWAY IMAGING ASSAYS[a]

Test cassette of commercially available kinase inhibitors												
	MK2-GFP translocation				cJun activation				ERK activation			
	Anisomycin		TNFα		Anisomycin		IL-1β		OSM		PMA	
Compound	% Inhib at 50 μM	IC_{50} μM	% Inhib at 50 μM	IC_{50} μM	% Inhib at 50 μM	IC_{50} μM	% Inhib at 50 μM	IC_{50} μM	% Inhib at 50 μM	IC_{50} μM	% Inhib at 50 μM	IC_{50} μM
SP600125	29.5	>50	36.5	>50	95.5	14	107	8.6	15	>50	−52	>50
VX-745	146	0.045	119	0.050	22.5	>50	−10	>50	0	>50	−36	>50
Merck p38 inhib	−30.5	0.007	−99.5	0.011	214	19	235.5	21	117	10.95	117.5	12.5
RWJ 68354	122	0.087	97	0.074	105	20.5	**48**	38.5	−0.5	>50	12	>50
SB242235	96.5	0.028	81	0.103	**41**	>50	12	>50	−7.5	>50	5	>50
SB203580	64	0.053	16	0.220	59.5	41	−10	>50	**37.5**	>50	75.5	26
PD98059	58.5	39.000	78	24.500	26.5	>50	−8.5	>50	**44**	>50	−0.5	>50
U0126 # 9903	64	33.000	67.5	28.000	26.5	>50	**26**	44	96.5	1.275	94	6.95
Wortmannin	33.5	>50	38.5	>50	−57.5	>50	−123.5	>50	−25.5	>50	−73	>50
Staurosporin	180.5	0.755	282	0.505	173	7.2	196.5	1.4	85	0.003	83	0.003

[a] Data represent the mean of duplicate independent determinations.

p38 inhibitor and RWJ 68354 inhibited c-Jun activation by IL-1ß. Two of the five ''selective'' p38 inhibitors, VX-745 and SB242235, did not inhibit c-Jun activation by either stimulus. Neither of the MEK-1/2 inhibitors UO126 and PD 98059 nor the PI3-kinase inhibitor wortmannin inhibited c-Jun activation by anisomycin, although U0126 inhibited IL-1ß-induced activation (Table I). Clearly the c-Jun activation assay was inhibited by the JNK-1/2 kinase inhibitor; however, it was also susceptible to three of the five p38 inhibitors, albeit at >100-fold less sensitivity that the MK2-EGFP translocation p38 MAPK pathway assay.

The MEK-1/2 inhibitor U0126 produced IC_{50} values in the 1 to 7 μM range for both ERK activation stimuli, whereas the nonselective kinase inhibitor staurosporin produced nanomolar IC_{50} values for both stimuli, and the Merck p38 inhibitor produced IC_{50} values in the 11 to 12 μM range for both stimuli (Table II). SB203580 produced an IC_{50} of 26 μM with PMA as the activating stimulus, but failed to inhibit OSM-induced ERK activation. None of the other compounds inhibited ERK activation by either stimulus: the MEK-1/2 inhibitor PD 98059, three of the ''selective'' p38 inhibitors, VX-745, RWJ 68354, and SB242235, the PI3-kinase inhibitor wortmannin, and the JNK-1/2 inhibitor SP 600125 (Table I). The ERK activation assay was the least susceptible to inhibition by compounds that are reportedly selective for the other MAPK signaling pathways (Table I).

Staurosporin is a potent inhibitor of numerous kinases *in vitro* that is active in many cell-based assays. The ability of staurosporin to inhibit all three MAPK signaling pathways assays (Table I) is likely due to its ability to bind to the ATP-binding sites of multiple kinases in these pathways. The Merck p38 inhibitor also produced IC_{50} values in all three assays, but the ability to inhibit the JNK and ERK signaling pathways (Table I) was likely due to the apparent cytotoxicity, or significantly reduced adherence of the HeLa cells, observed at the two highest concentrations of the compound (Williams *et al.*, 2006). Images revealed that there were much fewer cells in the fields of view captured from wells treated with 50 and 16.6 μM of the Merck p38 inhibitor compared to the 5.5 μM dose, which produced obvious outliers in several cell number and morphology parameters derived by the image analysis algorithm (Williams *et al.*, 2006). The high-content nature of image-derived data therefore provides additional information that may be a consequence of target inhibition or may be due to off-target effects of the compound. The Merck p38 inhibitor produced an $IC_{50} \sim 7$–10 nM in the MK2-EGFP translocation p38 MAPK pathway assay and IC_{50} values of 20 and 10 μM in the JNK and ERK signaling pathway assays, respectively. A number of the p38 inhibitors, including the Merck p38 inhibitor, have been withdrawn from clinical trials because of adverse toxicity profiles (English and Cobb, 2002; Fabbro *et al.*, 2002; Noble *et al.*, 2004; Regan *et al.*, 2002).

TABLE II
PROFILING OF SELECTED MK2-EGFP TRANSLOCATION INHIBITOR HIT COMPOUNDS IN DISTINCT MAPK SIGNALING PATHWAY IMAGING ASSAYS[a]

MK2-EGFP translocation MTS hit assessment						
	MK2-GFP		cJun activation		ERK activation	
Compound	Mean % stimu at 50 μM	Mean IC_{50} μM	Mean % inhib at 50 μM	Mean IC_{50} μM	Mean % inhib at 50 μM	Mean IC_{50} μM
1	125.5	0.119	125	2	−7	>50
2	152.5	0.711	31	>50	5	>50
3	129	0.746	60	18	24	>50
4	140	0.780	30	8	−13	>50
5	109	1.028	117	4	−3	>50
6	96.5	1.331	53	40	20	>50
7	87.5	1.782	131	1	−10	>50
8	207.5	4.589	11	40	−86	>50
9	128.5	7.037	113	38	25	>50
10	110.5	14.123	9	>50	36	>50
11	50	14.916	−12	>50	−8	>50
12	10.5	>50	−21	>50	5	>50
13	12.5	>50	−2	>50	−8	>50
14	23	>50	−2	>50	−24	>50
15	16.5	>50	−9	>50	16	>50
16	15	>50	−26	>50	−11	>50

[a] Data represent the mean of duplicate independent determinations.

In contrast to staurosporin and the Merck p38 MAPK inhibitor, the PI3-kinase inhibitor wortmannin did not inhibit any of the three MAPK signaling pathway assays (Table I), and the JNK-1/2 inhibitor SP 600125 only inhibited the c-Jun activation assay, perhaps indicating some degree of MAPK pathway selectivity (Table I). The MEK-1/2 inhibitor UO126 produced IC_{50} values ranging between 1.2 and 6.9 μM in the ERK activation assay and an IC_{50} of 44 μM in the IL-1β-induced c-Jun activation assay. In contrast, the MEK-1/2 inhibitor PD 98059 did not inhibit either the c-Jun or the ERK activation assay. The MEK-1/2 inhibitors UO126 and PD 98059 produced IC_{50} values ranging between 25 and 39 μM in the MK2-EGFP translocation p38 MAPK assay, perhaps indicating that ERK1/2 may be capable of phosphorylating MK2 as well as MK1 or perhaps reflecting the cross talk between the different modules of MAPK signaling pathways (Cowan and Storey, 2003; Garrington and Johnson, 1999). All five p38 inhibitors produced IC_{50} values for inhibition of anisomycin- and TNFα-induced MK2-EGFP translocation <200 nM (Table I). As discussed

earlier, the Merck p38 inhibitor also inhibited the c-Jun and ERK activation assays with IC_{50} values of 20 and 10 μM, respectively, likely due to the apparent cytotoxicity at these concentrations interfering with the image analysis algorithm. The RWJ 68354 p38 inhibitor inhibited anisomycin- and TNFα-induced MK2-EGFP translocation with IC_{50} values between 74 and 87 nM and the c-Jun activation assay with IC_{50} values between 20 and 38 μM. The SB203580 p38 inhibitor inhibited anisomycin- and TNFα-induced MK2-EGFP translocation with IC_{50} values between 53 and 220 nM and the c-Jun and ERK activation assays with IC_{50} values of 41 and 26 μM, respectively. In general, therefore, selective MAPK inhibitors exhibited pathway selectivity by producing significantly lower IC_{50} values for their respective ERK, JNK, or p38 signaling pathway assays. However, each of the MAPK pathways assays was also susceptible to nonselective kinase inhibitors such as staurosporin and inhibitors that inhibit upstream MKKKs and MKKs in the MAPK signaling pathway, especially those involved in the cross talk between different modules of the MAPK signaling pathways.

p38 Inhibitor Hit Assessment

Williams *et al*. (2006) and Trask *et al*. (2006) described the development and optimization of the MK2-EGFP translocation assay and the subsequent use of this assay to screen a 32K kinase biased library to identify p38 MAPK inhibitors. As part of the follow-up hit assessment for the screen, selected hits and related structural analogs were profiled in the three MAPK signaling pathway assays described here (Table II). Eleven of the 16 hits and related analogs produced an IC_{50} in the anisomycin-induced MK2-EGFP translocation assay: 4 in the submicromolar range, 5 in the 1 to 10 μM range, and 2 at $\sim$14 μM (Table II). Five of the 16 hits and related analogs produced an IC_{50} in the anisomycin-induced c-Jun activation assay: 3 in the 1 to 10 μM range, 1 at $\sim$18 μM, and 1 at 38 μM (Table II). None of the 16 hits and related analogs produced an IC_{50} in the OSM-induced ERK activation assay (Table II). Most of the hits produced significantly lower IC_{50} values in the MK2-EGFP translocation assay relative to the c-Jun activation assay, and 3 of the hit scaffolds identified in the MK2-EGFP translocation HCS were selected for p38a inhibitor hit-to-lead chemistry structure activity relationship (SAR).

p38a Inhibitor Profiling

To further evaluate the ability of the three MAPK signaling pathway assays to discriminate activity across these pathways, we compiled a set of

40 compounds directed at p38 inhibition. All 40 compounds produced IC_{50} values in the p38 cell-based MK2-EGFP translocation assay: 29 in the submicromolar range, 10 in the 1 to 5 μM range, and 1 at ~21.7 μM (Table III). Three of the 40 tested produced IC_{50} values in the cell-based ERK activation assay: 3 in the 30 to 40 μM range (Table III), suggesting little or no interference with the ERK MAPK pathway. In contrast, 22 of the 40 p38 inhibitors also inhibited the c-Jun activation assay, albeit with significantly less potency than in the MK2-EGFP translocation assay: 1 in the submicromolar range, 5 in the 1 to 10 μM range, and 16 in the 10 to 40 μM range (Table III). Although most of the compounds exhibited low nanomolar potency against p38 isoforms *in vitro*, a number of these compounds also exhibited submicromolar or low nanomolar potency against JNK isoforms *in vitro* (data not shown). In the majority of cases, however, despite exhibiting low to mid-nanomolar potency in the MK2-EGFP translocation assay, these mixed p38-JNK inhibitors typically only produced 5 to 30 μM potencies in the c-Jun activation assay. Because all three MAPK pathway assays were performed in the HeLa cell background, it is unlikely that differential cell permeability is contributing to the apparent differences in cellular potencies between mixed p38-JNK inhibitors with similar *in vitro* potencies. In general, the apparent MAPK selectivity profile from the *in vitro* kinase assays was reflected in the cell-based MAPK assays; however, the apparent p38 selectivity of the compounds was typically more pronounced in the cell-based assays.

JNK Inhibitor Profiling

To further characterize the selectivity of the three MAPK signaling pathway assays, a set of 40 JNK inhibitors was assembled (Table IV). Twenty-three of the 40 compounds produced IC_{50} values in the c-Jun activation cell-based assay: 4 in the sub-micromolar range, 3 in the 1 to 10 μM range, and 16 in the 10 to 40 μM range (Table IV). However, despite exhibiting potencies in the mid-nanomolar or sub-micromolar IC_{50} values in the JNK *in vitro* kinase assays (data not shown), with only four exceptions the compounds typically produced cellular activity in the 5 to 40 μM range. Fifteen of the 40 tested also produced IC_{50} values in the cell-based ERK activation assay: 3 in the 1 to 10 μM range and 12 in the 10 to 40 μM range (Table IV). The ERK MAPK module responds primarily to growth factors and mitogens and is involved in the regulation of cell division, migration, and survival (Cowan and Storey, 2003; Garrington and Johnson, 1999; Johnson and Lapadat, 2002), and inhibition of this pathway may have an adverse effect that could impact the therapeutic index of these JNK inhibitors. Conversely, there appears to be relatively little interference of the p38 MAPK signaling

TABLE III
PROFILING OF SELECTED p38α INHIBITOR COMPOUNDS IN DISTINCT MAPK SIGNALING PATHWAY IMAGING ASSAYS[a]

Selected p38α inhibitors			
	Cell-based imaging assay IC_{50}		
Compound	MK2-EGFP	cJun	ERK
1	0.011	17.91	>50
2	0.024	31.07	>50
3	0.024	>50	>50
4	0.036	31.20	>50
5	0.038	10.25	>50
6	0.043	0.05	>50
7	0.044	5.02	>50
8	0.076	10.10	>50
9	0.077	32.95	>50
10	0.092	14.04	>50
11	0.118	29.63	>50
12	0.127	15.55	>50
13	0.147	30.15	38.32
14	0.207	>50	>50
15	0.227	5.37	>50
16	0.255	5.05	>50
17	0.259	14.94	>50
18	0.262	15.34	>50
19	0.269	>50	>50
20	0.284	>50	>50
21	0.354	23.36	>50
22	0.386	37.24	>50
23	0.452	>50	>50
24	0.469	2.02	>50
25	0.477	18.85	>50
26	0.487	>50	>50
27	0.790	>50	>50
28	0.853	19.92	>50
29	0.987	>50	>50
30	1.259	>50	>50
31	1.354	9.25	>50
32	1.390	>50	>50
33	1.564	>50	>50
34	1.648	>50	>50
35	2.061	>50	>50
36	2.865	>50	38.67
37	3.177	>50	>50
38	3.675	>50	>50
39	4.277	>50	>50
40	21.730	>50	33.50

[a] Cell-based data represent the mean of duplicate independent determinations.

TABLE IV
PROFILING OF SELECTED JNK INHIBITOR COMPOUNDS IN DISTINCT MAPK SIGNALING PATHWAY IMAGING ASSAYS[a]

Selected JNK inhibitor compounds						
Cell-based imaging assays						
	cJun activation		MK2-GFP		ERK activation	
Compound	Mean % inhib at 50 μM	Mean IC_{50} μM	Mean % stimu at 50 μM	Mean IC_{50} μM	Mean % inhib at 50 μM	Mean IC_{50} μM
1	24	0.575	17	>50	99.5	14.080
2	109	10.335	36.5	>50	−40	>50
3	157	0.707	98	19.789	111	7.503
4	−2.5	>50	−1.5	>50	−38.5	>50
5	136	0.508	65	11.951	94	4.581
6	114	5.891	22	>50	−20	>50
7	138	0.830	116	9.742	98	1.931
8	99	10.957	24	>50	−50.5	>50
9	17	>50	4.5	>50	−12.5	>50
10	68	32.009	−3	>50	94.5	31.939
11	101	9.110	53	40.208	86.5	15.777
12	61	26.373	25	>50	34.5	>50
13	84	16.446	6	>50	78	27.825
14	73	21.465	8	>50	45.5	>50
15	46.5	9.413	21	>50	71	36.620
16	25.5	>50	−1.5	>50	37.5	34.098
17	68.5	27.925	32.5	>50	93.5	15.332
18	29	>50	21	>50	88.5	19.944
19	−18.5	>50	15	>50	14	>50
20	99	12.776	46	24.543	4	>50
21	109	10.769	24.5	>50	21.5	43.802
22	88.5	13.439	40	43.156	70.5	38.125
23	56	40.990	92.5	24.965	65.5	35.000
24	61	40.089	28	>50	−62	>50
25	84	18.839	58	38.146	1	>50
26	40	>50	21	>50	−42	>50
27	46	34.161	54	35.865	−23.5	>50
28	21	>50	54	28.192	−15	>50
29	−5.5	>50	10	>50	−25	>50
30	2	>50	11.5	>50	15.5	>50
31	61	40.842	17	>50	−48	>50
32	25	>50	5	>50	8	>50
33	17	>50	24	>50	−33.5	>50
34	0	>50	18	>50	−1	>50
35	−21	>50	18	>50	−3.5	>50
36	11	>50	24	>50	−17.5	>50
37	1	>50	16	>50	−0.5	>50
38	20	>50	24	>50	10.5	40.846
39	8	>50	19	>50	−11.5	>50
40	60	19.354	25	>50	−4	>50

[a] Cell-based data represent the mean of duplicate independent determinations.

pathway by these JNK inhibitors, with only 10 of the 40 tested producing IC_{50} values in the cell-based MK2-EGFP translocation p38 assay: 1 in the 1 to 10 μM range and 9 in the 10 to 40 μM range (Table IV).

Discussion

This chapter and ones by Williams *et al*. (2006) and Trask *et al*. (2006) described the development and implementation of the three distinct high-content cell-based MAPK signaling pathway assays to profile kinase inhibitors for MAPK module selectivity. The apparent redundancy of the kinase isoforms and functions in the MAPK signaling modules presents a challenge for kinase inhibitor selectivity and potency. For ATP-competitive inhibitors, these selectivity concerns may be exacerbated further by the degree of conservation of the ATP-binding site within the kinase family, the millimolar intracellular concentrations of ATP that may significantly affect cellular potency, and the potential for off-target effects due to binding to many other cellular proteins that bind and/or utilize ATP (English and Cobb, 2002; Noble *et al.*, 2004). The development of imaging assays for the major MAPK signaling modules, p38, JNK, and ERK, provided a strategy to profile kinase inhibitors for signaling pathway selectivity while simultaneously measuring effects on cell morphology or toxicity end points. These assays provided a mechanism to screen kinase inhibitors against targets that were expressed in the context of their endogenous substrates and scaffolding proteins in the appropriate cellular localization and that had to perform their functions in a complex environment involving cross talk between signaling pathways, phosphatase regulatory control, and intracellular ATP concentrations. The MAPK pathways assays were susceptible to nonselective kinase inhibitors such as staurosporin and inhibitors that inhibit upstream MKKKs and MKKs in the MAPK signaling pathway, especially those involved in cross talk between the pathways. In general, however, selective MAPK inhibitors exhibited pathway selectivity as evidenced by significantly lower IC_{50} values for their respective p38, JNK, or ERK signaling pathway assays.

References

Almholt, D. L., Loechel, F., Nielsen, S. J., Krog-Jensen, C., Terry, R., Bjorn, S. P., Pedersen, H. C., Praestegaard, M., Moller, S., Heide, M., Pagliaro, L., Mason, A. J., Butcher, S., and Dahl, S. W. (2004). Nuclear export inhibitors and kinase inhibitors identified using a MAPK-activated protein kinase 2 redistribution screen. *Assay Drug Dev. Technol.* **2,** 7–20.

Cowan, K. J., and Storey, K. B. (2003). Mitogen-activated protein kinases: New signaling pathways functioning in cellular responses to environmental stress. *J. Exp. Biol.* **206,** 1107–1115.

English, J. M., and Cobb, M. H. (2002). Pharmacological inhibitors of MAPK pathways. *Trends Pharmacol. Sci.* **23,** 40–45.

Fabbro, D., Ruetz, S., Buchdunger, E., Cowan-Jacob, S. W., Fendrich, G., Liebetanz, J., Mestan, J., O'Reilly, T., Traxler, P., Chaudhuri, B., Fretz, H., Zimmermann, J., Meyer, T., Caravatti, G., Furet, P., and Manley, P. W. (2002). Protein kinases as targets for anticancer agents: From inhibitors to useful drugs. *Pharmacol. Ther.* **93,** 79–98.

Garrington, T. P., and Johnson, G. L. (1999). Organization and regulation of mitogen activated protein kinase signaling pathways. *Curr. Opin. Cell Biol.* **11,** 211–218.

Giuliano, K. A., DeBiasio, R. L., Dunlay, R. T., Gough, A., Volosky, J. M., Zock, J., Pavlakis, G. N., and Taylor, D. L. (1997). High-content screening: A new approach to easing key bottlenecks in the drug discovery process. *J. Biomol. Screen.* **2,** 249–259.

Johnson, G. L., and Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK and p38 protein kinases. *Science* **298,** 1911–1912.

Mitchison, T. J. (2005). Small-molecule screening and profiling by using automated microscopy. *Chembiochem.* **6,** 33–39.

Noble, M. E. M., Endicott, J. A., and Johnson, L. N. (2004). Protein kinase inhibitors: Insights into drug design and structure. *Science* **303,** 1800–1805.

Ono, K., and Han, J. (2000). The p38 signal transduction pathway, activation and function. *Cell. Signal.* **12,** 1–13.

Regan, J., Breitfelder, S., Cirillo, P., Gilmore, T., Graham, A. G., Hickey, E., Klaus, B., Madwed, J., Moriak, M., Moss, N., Pargellis, C., Pav, S., Proto, A., Swinamer, A., Tong, L., and Torcellini, C. (2002). Pyrazole urea-based inhibitors of p38 MAP kinase: From lead compound to clinical candidate. *J. Med. Chem.* **45,** 2994–3008.

Trask, O. J., Jr., Baker, A., Williams, R. G., Kickischer, D., Kandasamy, R., Laethem, C., Johnston, P. A., and Johnston, P. A. (2006). Assay development and case history of a 32K-biased library high-content MK2-EGFP translocation screen to identify p38 MAPK inhibitors on the ArrayScan 3.1 imaging platform. *Methods Enzymol.* **414** (this volume).

Williams, R. G., Kandasamy, R., Nickischer, D., Trask, O. J., Jr., Laethem, C., Johnston, P. A., and Johnston, P. A. (2006). Generation and characterization of a stable MK2-EGFP cell line and subsequent development of a high-content imaging assay on the Cellomics ArrayScan platform to screen for p38 mitogen-activated protein kinase inhibitors. *Methods Enzymol.* **414** (this volume).

Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4,** 67–73.