[23] Assay Development and Case History of a 32K-Biased Library High-Content MK2-EGFP Translocation Screen to Identify p38 Mitogen-Activated Protein Kinase Inhibitors on the ArrayScan 3.1 Imaging Platform

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Abstract

This chapter describes the conversion and assay development of a 96-well MK2-EGFP translocation assay into a higher density 384-well format high-content assay to be screened on the ArrayScan 3.1 imaging platform. The assay takes advantage of the well-substantiated hypothesis that mitogen-activated protein kinase-activating protein kinase-2 (MK2) is a substrate of p38 MAPK kinase and that p38-induced phosphorylation of MK-2 induces a nucleus-to-cytoplasm translocation. This chapter also presents a case history of the performance of the MK2-EGFP translocation assay, run as a ''high-content'' screen of a 32K kinase-biased library to identify p38 inhibitors. The assay performed very well and a number of putative p38 inhibitor hits were identified. Through the use of multiparameter data provided by the nuclear translocation algorithm and by checking images, a number of compounds were identified that were potential artifacts due to interference with the imaging format. These included fluorescent compounds, or compounds that dramatically reduced cell numbers due to cytotoxicity or by disrupting cell adherence. A total of 145 compounds produced IC_{50} values <50.0 μM in the MK2-EGFP translocation assay, and a cross target query of the Lilly-RTP HTS database confirmed their inhibitory activity against *in vitro* kinase targets, including p38a. Compounds were confirmed structurally by LCMS analysis and profiled in cell-based imaging assays for MAPK signaling pathway selectivity. Three of the hit scaffolds identified in the MK2-EGFP translocation HCS run on the ArrayScan were selected for a p38a inhibitor hit-to-lead structure activity relationship (SAR) chemistry effort.

Introduction

The ability to automate the capture and analysis of fluorescent images of thousands of cells in tens of thousands of wells of microtiter plates has

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made fluorescence microscopy, one of the premier tools of cell biology, compatible with drug discovery (Almholt *et al.*, 2004; Giuliano *et al.*, 1997; Lundholt *et al.*, 2005; Mitchison, 2005; Oakley *et al.*, 2002; Ramm *et al.*, 2003; Thomas and Goodyer, 2003). All high-content screening (HCS) platforms require a process for the input and output of multiple microtiter plates, mechanisms to position plates on a stage, the ability to position wells over the optics with precision and reproducibility, a method to capture quality images, image analysis applications (algorithms), data review tools, and a protocol for data storage and management (Berlage, 2005; Giuliano *et al.*, 1997). Reagent selection for sample preparation and staining is also critical (Giuliano *et al.*, 1997). However, HCS platforms vary in the degree to which these components have been integrated. Many of the large high-throughput screening (HTS) instrument vendors have entered the HCS market by acquiring smaller imaging platform manufacturers to provide their offerings in the HCS field. For example, Cellomics, Inc. (Pittsburgh, PA), the company that introduced the ArrayScan, one of the first HCS platforms to penetrate the drug discovery market, was recently acquired by Fisher Scientific (Hampton, NH).

Williams *et al*. (2006) described the generation and characterization of a stable MK2-EGFP HeLa cell line and the subsequent development of a 96-well high-content imaging assay on the Cellomics ArrayScan platform to screen for p38 MAPK inhibitors. The assay took advantage of the well-substantiated hypothesis that mitogen-activated protein kinase-activating protein kinase-2 (MK2) is a substrate of p38 MAPK kinase and that p38-induced phosphorylation of MK2 induces a nucleus-to-cytoplasm translocation (Engel *et al.*, 1998; Neininger *et al.*, 2001; Zu *et al.*, 1995). Through a process of heterologous expression of a MK2-EGFP fusion protein in HeLa cells using retroviral infection, antibiotic selection, and flow sorting, we were able to isolate an MK2-EGFP-HeLa cell line in which the MK2-EGFP translocation response could be robustly quantified on the Cellomics ArrayScan HCS platform to provide a cell-based model for identifying p38 inhibitors (Williams *et al*., 2006). This chapter describes the assay development process to convert this assay from a 96- to a 384-well format for screening a 32K kinase-biased library for novel p38 inhibitors.

Cellomics ArrayScan Automated Imaging Platform

The ArrayScan platform marketed by Cellomics (Fisher Scientific, Hampton, NH) is one of the most widely deployed HCS systems. The studies described in this chapter 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 multiwavelength fluorescence by acquiring wavelength channels sequentially in which each fluorophore is excited separately and detected on the chip of a monochromatic CCD camera. Channel selection is accomplished using a fast excitation filter wheel combined with a multiband emission filter, although single band emission filters can be used to improve selectivity. 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. 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, or DRAQ5 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).

Conversion of the 96-Well MK2-EGFP Translocation Assay to a 384-Well Format Assay on the Arrayscan® Imager

Williams *et al*. (2006) described the development and optimization of a 96-well MK2-EGFP translocation assay on the ArrayScan 3.1 HCS platform that could quantify the activation and inhibition of the p38 MAPK pathway using the cytoplasm-to-nuclear translocation algorithm.

To increase the throughput for screening and reduce the demands on cell culture, we decided to convert the assay to the higher-density 384-well format. To establish the optimal cell seeding density, MK2-EGFP-A4 cells are seeded in 384-well Matrical glass plates at 2.5, 5.0, and 10.0×10^3 cells per well and on the following day are treated with 100 ng/ml anisomycin or are left untreated. Other wells are pretreated with the p38 inhibitor SB203580 at 1 μM for 12 min, followed by the addition of 100 ng/ml anisomycin (final) and a further incubation for 25 min. The ArrayScan imager was used to capture images (Fig. 1A), and the cytoplasm-to-nuclear translocation algorithm was used to quantify the MK2-EGFP translocation response at all three seeding densities (Fig. 1B). The ArrayScan imager and cytoplasm-to-nuclear translocation algorithm are able to adequately quantify the MK2-EGFP translocation at all three seeding densities (Fig. 1B). A seeding density of 2.5×10^3 cells per well was selected for further assay development to reduce the cell culture demands for the assay and to assist with the image analysis segmentation to identify discrete cells.

We next wanted to examine the time course for anisomycin-induced MK2-EGFP translocation and whether the length of preincubation of cells with p38 inhibitors prior to the addition of a maximum dose of anisomycin might affect their ability to inhibit the MK2-EGFP translocation response (Fig. 2). The amount of MK2-EGFP that translocates from the nucleus to the cytoplasm, indicated by the Cytonuc difference, appears to decrease from >300 to below 100 in a roughly linear fashion over time for up to 20 to 25 min and then remains stable for up to 60 min (Fig. 2A). Between 60 and 90 min there appears to be a small gradual increase in the Cytonuc difference, implying that the MK2-EGFP may be returning to the nucleus. However, the Cytonuc difference had not returned to the pretreatment or untreated values by 125 min, the longest time point measured (Fig. 2A). Forty minutes was selected as the standard incubation time for all further assay development. The ability of the p38 inhibitor SB203580 to inhibit anisomycin-induced MK2-EGFP translocation is not influenced by preincubation with the inhibitor prior to the addition of the activating stimulus (Fig. 2B). Indeed, co-addition of SB303580 with the anisomycin is just as effective as prior incubation with the inhibitor.

Compound screening libraries are typically solubilized in dimethyl sulfoxide (DMSO), and we wanted to evaluate the DMSO tolerance of the MK2-EGFP translocation response in HeLa-MK2-EGFP-A4 cells treated for 40 min with media or 100 ng/ml anisomycin at the indicated DMSO concentrations (Fig. 3B). The MK2-EGFP translocation response appears unaffected at DMSO concentrations $\leq 0.65\%$. However, at DMSO concentrations $\geq 1.25\%$, the Cytonuc difference increases with the DMSO concentration, independently of the treatment conditions. At DMSO

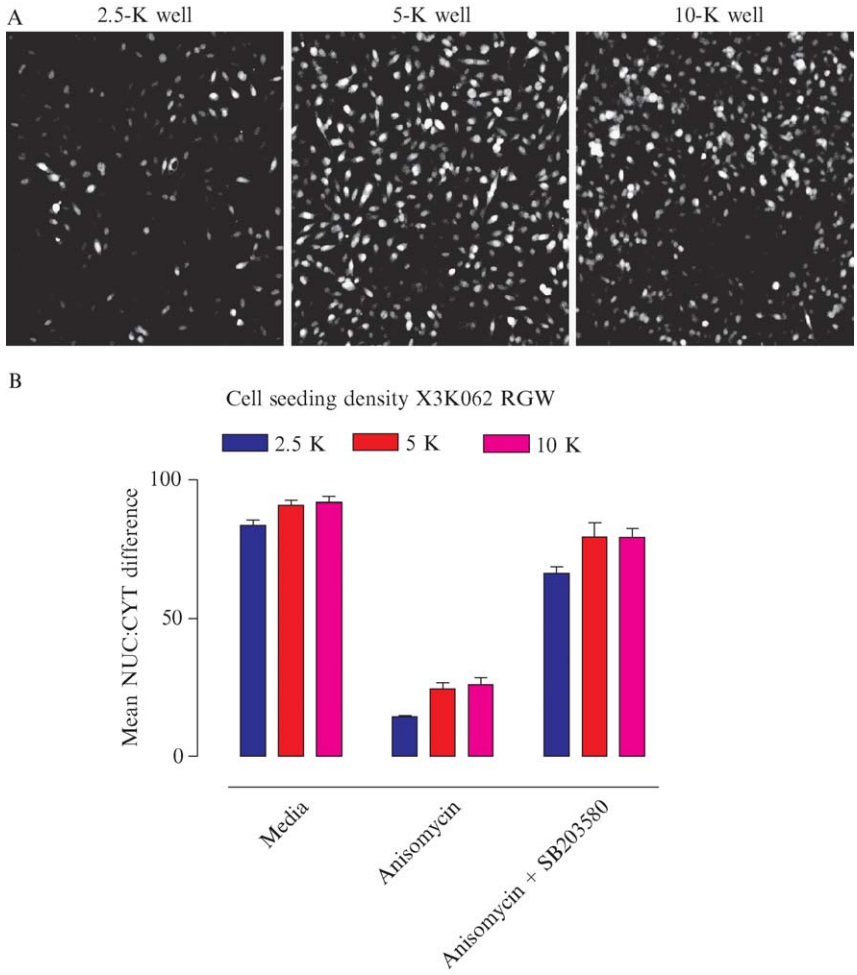


FIG. 1. Three hundred eighty-four-well MK2-EGFP translocation assay development: Cell seeding density. MK2-EGFP-A4 cells were seeded in 384-well Matrical glass plates at 2.5, 5.0, and 10.0×10^3 cells per well and on the following day were treated with 100 ng/ml anisomycin or were left untreated. Other wells were pretreated with the p38 inhibitor SB203580 at 1 μM for 12 min, followed by the addition of 100 ng/ml anisomycin (final) and a further incubation for 25 min. The ArrayScan imager was used to capture images (A), and the cytoplasm-to-nuclear translocation algorithm was used to quantify the MK2-EGFP translocation response at all three seeding densities (B).

concentrations $\geq$1.25%, the majority of the HeLa-MK2-EGFP-A4 population assumes a rounded cell morphology rather than the more typical well-attached, flat cell morphology (Fig. 3A). The rounded A4 cells have a much

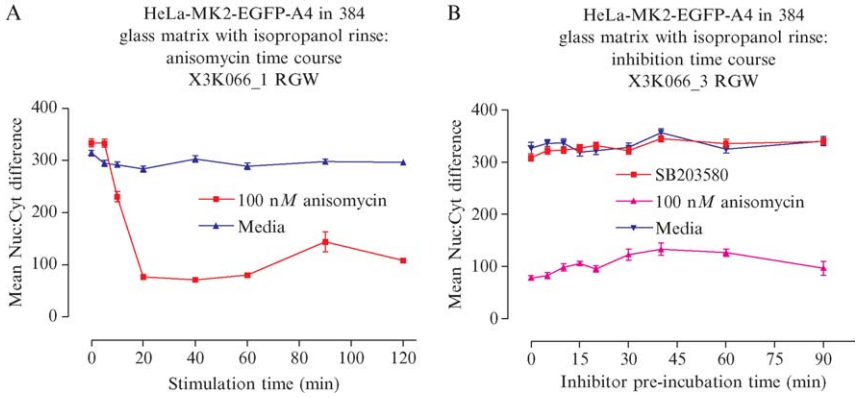


FIG. 2. Three hundred eighty-four-well MK2-EGFP translocation assay development: Activation and inhibition time courses. (A) Activation time course. HeLa-MK2-EGFP cells (2.5×10^3) from the HeLa-MK2-EGFP-A4 clone were seeded into each of the 384 wells of Matrical glass plates in EMEM + 10% FBS and incubated overnight at 37° and 5% CO_2. Cells were treated ± 200 ng/ml anisomycin for the indicated times and fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, fluorescent images were collected on the ArrayScan imager, and the cytoplasm-to-nuclear translocation algorithm was used to quantify the MK2-EGFP translocation response. (B) Inhibition time course. HeLa-MK2-EGFP cells (2.5×10^3) from the HeLa-MK2-EGFP-A4 clone were seeded into each of the 384 wells of Matrical glass plates in EMEM + 10% FBS and were incubated overnight at 37° and 5% CO_2. The p38 inhibitor SB203580 was preincubated with the cells for the indicated times prior to the addition of 200 ng/ml anisomycin. Plates were incubated for 40 min and fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, fluorescent images were collected on the ArrayScan imager, and the cytoplasm-to-nuclear translocation algorithm was used to quantify the MK2-EGFP translocation response.

smaller cytoplasm area than the normal well spread and attached cells, and the cytoplasm-to-nuclear translocation algorithm therefore has difficulty segmenting the cytoplasm from the nuclear areas to make the difference calculation. For analysis of rounded cells, the parameters of the algorithm would have to be modified.

Having transferred the MK2-EGFP translocation assay from a 96- to a 384-well format and completed the optimization of the assay conditions for the ArrayScan imager and nuclear translocation algorithm, we wanted to confirm the quality of the images (Fig. 4). MK2-EGFP-A4 cells were seeded in 384-well Matrical glass plates at 2.5×10^3 cells per well and on the following day were treated with the indicated doses of anisomycin and incubated for 40 min (Fig. 4). The quality of the images (Fig. 4) indicates that a 384-well MK2-EGFP translocation assay has been developed and optimized for image capture on the ArrayScan imager.

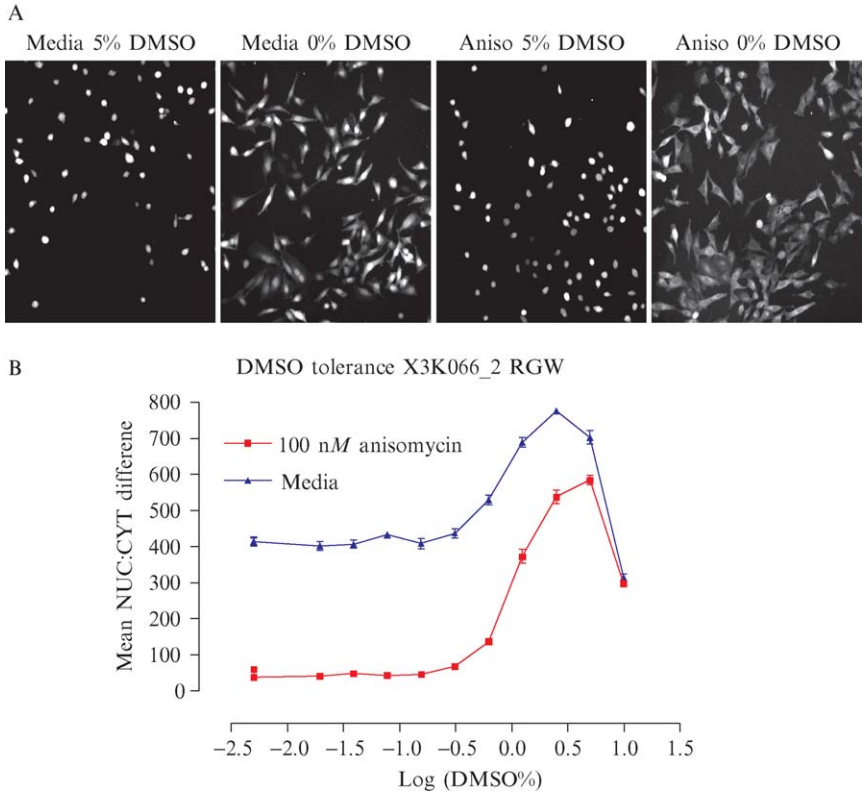


FIG. 3. Three hundred eighty-four-well MK2-EGFP translocation assay DMSO tolerance. HeLa-MK2-EGFP-A4 cells (2.5×10^3) were seeded in 384-well Matrical glass plates in EMEM + 10% FBS and were incubated overnight at 37° and 5% CO_2. Cells were treated ± 200 ng/ml anisomycin containing the indicated concentrations of DMSO for 40 min and fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye. The fluorescent images (A) were captured on the ArrayScan, and the cytoplasm-to-nuclear translocation algorithm was used to quantify the MK2-EGFP translocation response (B).

MK2-EGFP Translocation Assay Reproducibility and Signal Widow Evaluation

To evaluate the reproducibility of the MK2-EGF translocation response in HeLa-MK2-EGFP-A4 cells, cells were seeded at 2.5×10^3 cells per well and on the following day were treated with the indicated doses of anisomycin in three independent experiments run on separate days (Fig. 5). In three independent experiments, the EC_{50} for anisomycin-induced MK2-EGFP translocation ranged from 25 to 30 n*M* and produced on average an EC_{50} of 27.6 ± 2.3 nm, indicating a very reproducible assay.

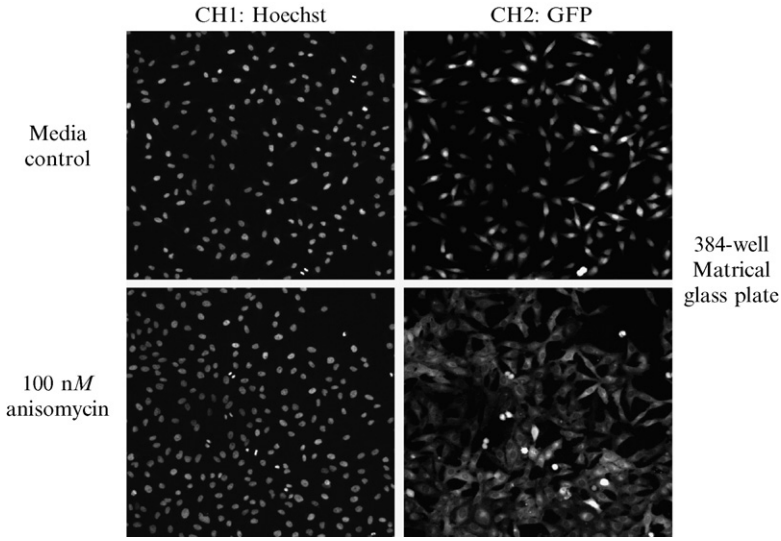
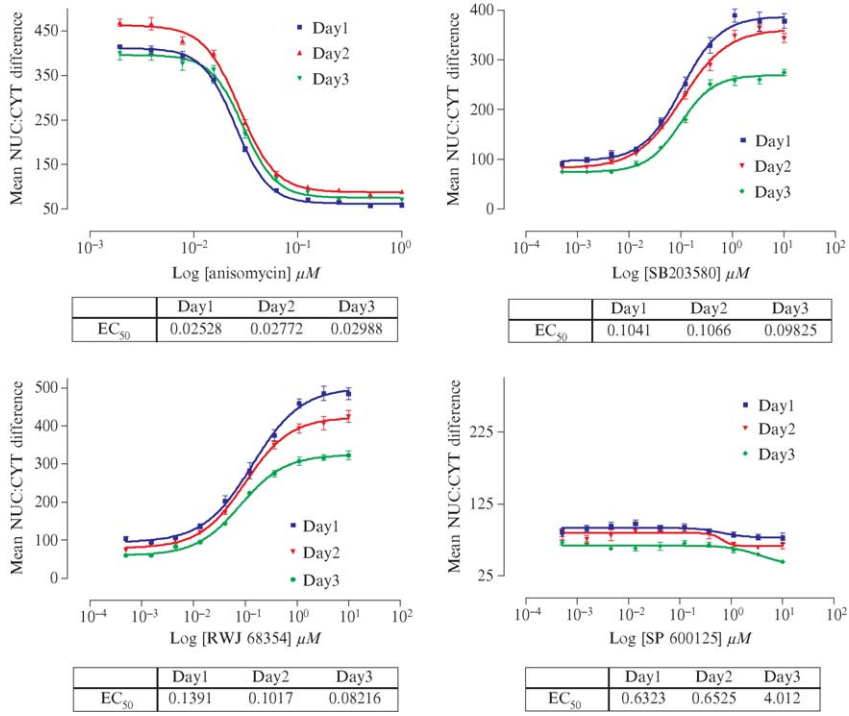


FIG. 4. Three hundred eighty-four-well MK2-EGFP translocation assay. HeLa-MK2-EGFP-A4 images captured on the ArrayScan 3.1 platform. HeLa-MK2-EGFP cells (2.5×10^3) from the HeLa-MK2-EGFP-A4 clone were seeded into each of the 384 wells of Matrical glass plates in EMEM + 10% FBS and were incubated overnight at 37° and 5% CO_2. Cells were treated ± 200 ng/ml anisomycin or were left untreated for 40 min and were fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, and fluorescent images were collected on the ArrayScan imager. Images of Hoechst-stained nuclei collected in channel 1 or MK2-EGFP collected in channel 2 are both presented.

To evaluate the ability of the MK2-EGF translocation response in HeLa-MK2-EGFP-A4 cells to identify p38 inhibitors and examine the reproducibility of the assay, three independent inhibition experiments were run on separate days. Cells were seeded at 2.5×10^3 cells per well and on the next day the indicated doses of the p38 inhibitors SB203580 and RWJ 68543 or the JNK1/2 inhibitor SP 600125 were added simultaneously with 200 ng/ml anisomycin (final) and cells were incubated for 40 min (Fig. 6). In three independent experiments, the IC_{50} for the p38 inhibitor SB203580 ranged from 98 to 106 n*M* and produced on average an IC_{50} of 103 ± 4.2 nm. In three independent experiments, the IC_{50} for the p38 inhibitor RWJ 68543 ranged from 82.6 to 139 n*M* and produced on average an IC_{50} of 107.8 ± 28.7 nm. In three independent experiments, the JNK1/2 inhibitor SP 600125 failed to inhibit the anisomycin-induced MK2-EGFP translocation and produced an average IC_{50} of >10 μM, indicating the selectivity of the assay for p38 inhibitors.



	Day1	Day2	Day3
EC_{50}	0.02528	0.02772	0.02988

	Day1	Day2	Day3
EC_{50}	0.1041	0.1066	0.09825

	Day1	Day2	Day3
EC_{50}	0.1391	0.1017	0.08216

	Day1	Day2	Day3
EC_{50}	0.6323	0.6525	4.012

FIG. 5. Three hundred eighty-four-well MK2-EGFP translocation assay and 3-day activation and inhibition curves. Three-day EC_{50} curves: 2.5×10^3 HeLa-MK2-EGFP A4 cells were seeded in 384-well Matrical glass plates in EMEM + 10% FBS and were incubated overnight at 37° and 5% CO_2. For activation of the response, cells were treated with the indicated doses of anisomycin for 40 min and fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, and fluorescent images were collected on the ArrayScan. Data are presented from three independent experiments, each performed in triplicate wells and run on separate days. Three-day IC_{50} curves: 2.5×10^3 HeLa-MK2-EGFP A4 cells were seeded in 384-well Matrical glass plates in EMEM + 10% FBS and were incubated overnight at 37° and 5% CO_2. For inhibition of the response, the indicated doses of SB203580, RWJ 68354, or SP 600125 were added simultaneously with the addition of 200 ng/ml anisomycin (final) and plates were incubated for 40 min. Plates were fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, and fluorescent images were collected on the ArrayScan. Data are presented from three independent experiments, each performed in triplicate wells and run on separate days. The cytoplasm-to-nuclear translocation algorithm was used to analyze the images captured on the ArrayScan and quantify the anisomycin induction and/or inhibition of the MK2-EGFP translocation response.

To further evaluate the robustness and reproducibility of the MK2-EGF translocation response in HeLa-MK2-EGFP-A4 cells, six full 384-well plates are treated under the following conditions, two plates per

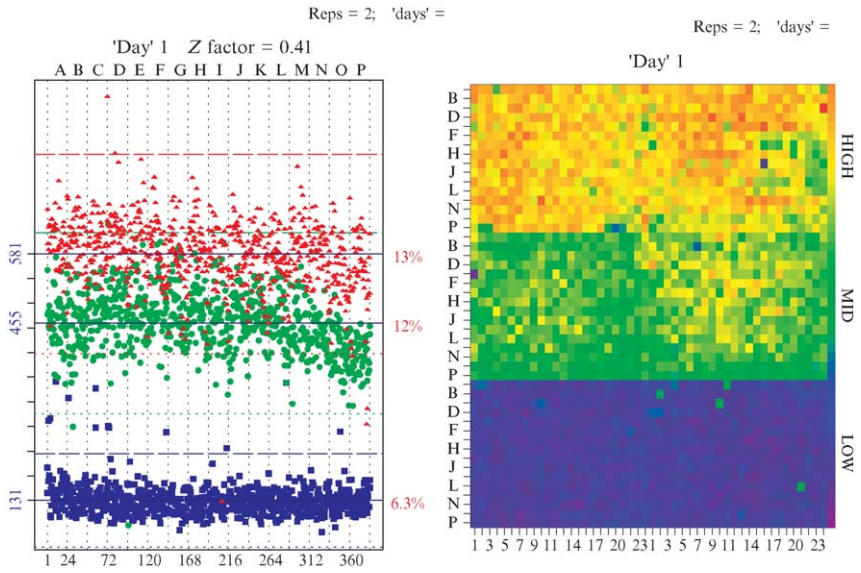


FIG. 6. Three hundred eighty-four-well MK2-EGFP translocation assay: Signal window and variability assessment. HeLa-MK2-EGFP A4 cells (2.5×10^3) were seeded in 384-well Matrical glass plates in EMEM + 10% FBS and were incubated overnight at 37° and 5% CO_2. Two full 384-well plates were treated for 40 min under the following conditions: media alone (red triangles), 200 ng/ml anisomycin (blue squares), and the p38 inhibitor SB203580 + 200 ng/ml anisomycin (green circles). Plates were fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, and fluorescent images were collected on the ArrayScan . The cytoplasm-to-nuclear translocation algorithm was used to analyze the images and quantify the anisomycin induction and/or SB203580 inhibition of the translocation response. The Z factor was calculated according to the method of Zhang *et al.* (1999). In addition to the population scatter plots of the calculated Cytonuc differences, plate heat map views of the six plates are also presented.

condition: media alone (red triangles), 200 ng/ml anisomycin (blue squares), and p38 inhibitor + 200 ng/ml anisomycin (green circles) (Fig. 7). In addition to the population scatter plots, plate heat map views of the six plates are also presented (Fig. 7). The average Cytonuc difference for the two media control plates was 581, and the average Cytonuc difference for the two anisomycin-treated control plates was 131, producing an average assay signal to background window of 4.3-fold. A comparison of media control plate variability data to anisomycin-treated plate variability data produced a Z factor of 0.41, indicating that the assay was compatible with HTS (Zhang *et al.*, 1999).

Standardized Operation Procedure for the MK2-EGFP Translocation Assay

1. Wash 384-well Matrical glass plates once with isopropanol and rinse with water on an MRD8 Titertek plus plate washer to remove a cytotoxic residue due to the plate adhesive.

2. Detach 70 to 80% confluent HeLa-MK2-EGFP-A4 monolayers from tissue culture flasks with trypsin-versene and pellet by centrifugation for 5 min 800*g*. Cells are resuspended in complete media (EMEM + 10% FBS + 2 m*M* L-glutamine + penicillin/streptomycin +800 μg/ml G418) to a cell density to 6.25×10^4 cells/ml.

3. Seed cells into 384-well Matrical glass plates using the Multidrop (Titertek) at 40 μl/well (2500 cells/well). Incubate plates at 37°, 5% CO_2, and 95% humidity for 18 to 24 h.

4. Transfer 20 μl of prewarmed compounds and plate controls to the wells of assay plates using a 384-well transfer head on the Multimek (Beckman) and incubate plates at 37°, 5% CO_2, and 95% humidity for 40 min. For inhibitor screens, add anisomycin (200 n*M* final concentration) simultaneously with the compounds.

5. After the 40-min incubation, fix plates by adding 20 μl of prewarmed (37°) formaldehyde (1.0% final) + 2 μg/μl Hoechst 33342 stain using the Multidrop (Titertek) and incubate at room temperature in a fume hood for 12 min.

6. Aspirate the fixation solution, add 50 μl of phosphate-buffered saline (PBS) to wells to wash the monolayers, aspirate the PBS wash solution, add another 50 μl of PBS to the wells, and seal the plates. Perform this aspiration and wash process on a MAP C2 plate handler (Titertek).

7. Acquire images on the ArrayScan platform and analyze for the translocation of MK2-EGFP from nucleus to cytoplasm using the nuclear translocation algorithm.

8. ArrayScan 3.1 settings: Select a dual BGIp filter set for excitation and emission of the Hoechst and EGFP fluorescence. Use the XF100-filter-Hoechst filter set for the Hoechst 33342 nuclear stain collected in channel 1, and select the XF100-FITC filter set for the EGFP fluorescence collected in channel 2. Perform scans sequentially, and set up the ArrayScan to collect a minimum of 100 valid objects per well, or a maximum of 4 fields of view per well, whichever comes first.

MK2-EGFP Translocation HTS Assay for p38 Inhibitors

Two compound cassettes are selected for screening in the MK2-EGFP translocation assay to identify p38 inhibitors: a library of 854 pharmacologically active compounds obtained from RBI and a 32,000 compound

TABLE I
MK2-EGFP TRANSLOCATION SCREEN SUMMARY DATA

MK2GFP ArrayScan			% of total
Rapid MTS	# Screened	32891	100
	# >50%	483	1.47
5-point IC 50's	# tested	448	100
	# Confirmed	234	52.53
10-point IC 50's	# tested	241	100
	# Confirmed	217	90.04

TABLE II
ARRAYSCAN 3.1 INSTRUMENT PERFORMANCE DATA[a]

HTS phase	Criteria	Performance
Rapid	# Plates	110
	Total scan time	98.6 h (4 days)
	Mean scan time/384-well plate	45.4 min
	Mean scan time/well	7.1 s
	Average fields/well	1.18
	Average valid objects/well	135
5-point IC 50's	# Plates	8
	Total scan time	9.5 h
	Mean scan time/384-well plate	71.3 min
	Mean scan time/well	11.1 s
	Average fields/well	1.44
10-point IC 50's	# Plates	9
	Total scan time	8.7 h
	Mean scan time/384-well plate	57.9 min
	Mean scan time/well	9.05 s
	Average fields/well	1.41

[a] Instrument settings: 100 objects or 4 fields/well; autoexposure isodata threshold 10%.

kinase-biased cassette. Based on the assay signal window and *Z*-factor data (Fig. 6), the active criterion for the primary screen was set at 50% inhibition of the anisomycin-induced MK2-EGFP translocation.

In the course of the MK2-EGFP screen, a total of 32,891 compounds were tested at a final concentration of 50 μM (0.5% DMSO) for their ability to inhibit anisomycin-induced MK2-EGFP translocation. Of the 32,891 compounds tested, 483 (1.47%) met the active criterion and produced 50% inhibition of anisomycin-induced MK2-EGFP translocation (Table I).

The primary MK2-translocation HTS assay was performed over a period of 5 days of operations, screening at a rate of 20, 25, 25, 25, and 15 384-well plates per day. The total scan time for the primary screen on the ArrayScan was 98.6 h (4 days), the mean scan time/384-well plate was 45.4 min, the mean scan time/well was 7.1 s, and the average number of frames/well was 1.18 (Table II).

The primary screen performed very well and of the 474 actives identified in the primary screen (Table I), only 448 were available from the compound library for testing in the five-point IC_{50} confirmation assays. Compounds were tested in five-point IC_{50} curves, threefold dilution series, starting at a maximum concentration of 50 μM. Of the 448 compounds tested, 234 (52.2%) were confirmed as dose-dependent inhibitors of anisomycin-induced MK2-EGFP translocation with IC_{50} values <50 μM (Table I). The five-point IC_{50} confirmation assays were performed in 1 day of screening operations, and the total scan time on the ArrayScan was 9.5 h, the mean scan time/384-well plate was 71.3 min, the mean time/well was 11.1 s, and the average number of fields/well was 1.44 (Table II).

Two hundred forty-one compounds were ordered for 10-point IC_{50} curves and were tested in a threefold dilution series, starting at a maximum concentration of 50 μM. Of the 241 compounds tested, 217 (90.04%) were confirmed as inhibitors of anisomycin-induced MK2-EGFP translocation with IC_{50} values <50 μM (Table I). The 10-point IC_{50} confirmation assays were performed in 1 day of screening operations, and the total scan time on the ArrayScan was 8.7 h, the mean scan time/384-well plate was 57.9 min, the mean time/well was 9.05 s, and the average number of fields/well was 1.41 (Table II). Of the 217 compounds confirmed with IC_{50} values <50 μM, 18 produced IC_{50} values <1.0 μM, 55 produced IC_{50} values in the 1 to 10 μM range, and the remaining 144 had IC_{50} values in the 10 to 50 μM range (Table III). A total of 73 compounds were identified that reproducibly inhibited anisomycin-induced MK2-EGFP translocation with IC_{50} values <10 μM (Table III).

TABLE III
MK2-EGFP TRANSLOCATION 10-POINT IC_{50} RANGES

	ArrayScan	%
# tested	241	100
# Confirmed	217	90.04
# <1.0	18	7.47
# 1–10 μM	55	22.82
# 10–50 μM	144	59.75
>50 μM	24	9.96

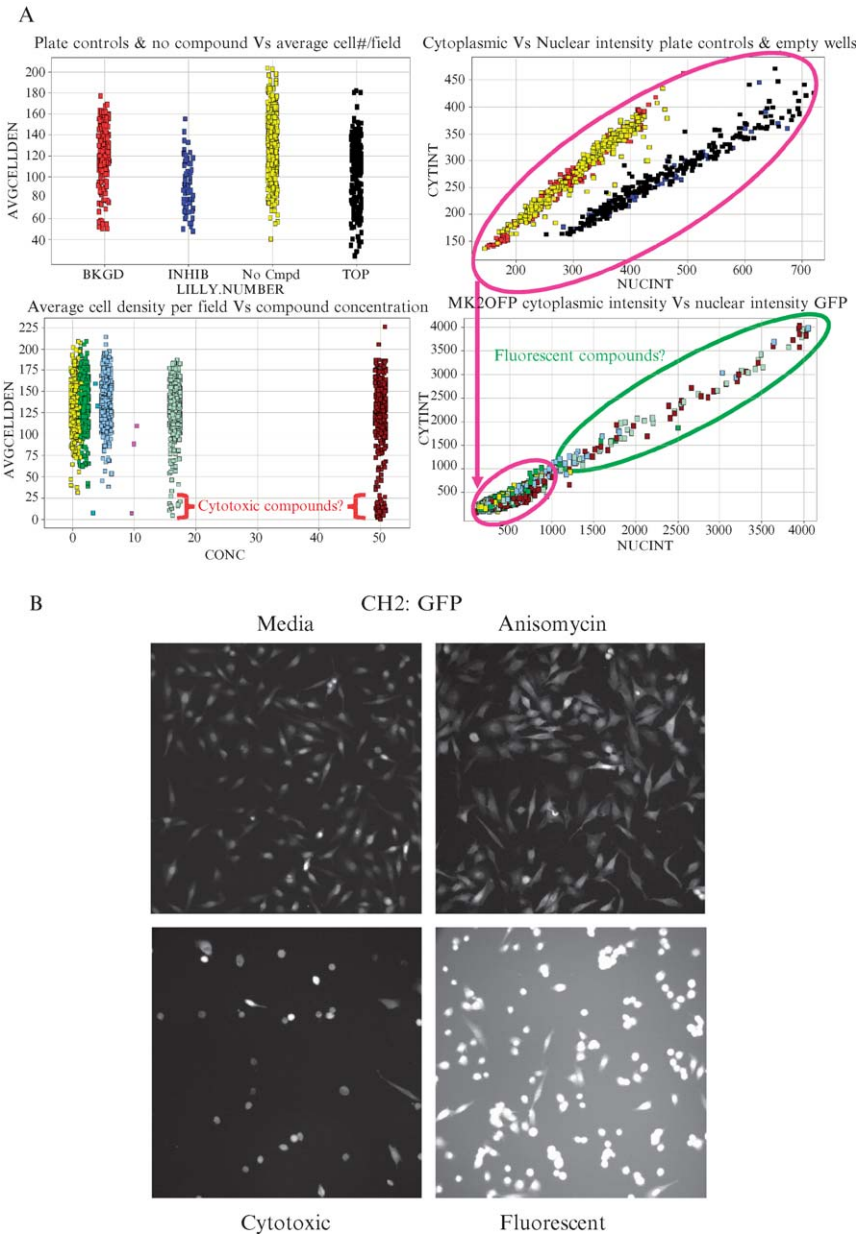


FIG. 7. Nuclear translocation algorithm secondary analysis for cytotoxicity and fluorescence. The cytoplasm-to-nuclear translocation algorithm provides data on a number of parameters that can be used to identify potential interference due to compound fluorescence

The cytoplasm-to-nuclear translocation algorithm provides data on a number of parameters that can be used to identify potential interference due to compound fluorescence or compound effects such as cytotoxicity or disruption of cell adherence. These data can be visualized with the ArrayScan view software in a variety of data plots or can be exported to data visualization software such as Spotfire (Fig. 7). A comparison of the images and secondary analysis parameters from the plate controls and empty wells versus the compound wells indicated that some of these parameters could be useful to discriminate compound effects or fluorescence interference (Fig. 7). The average cell number per field parameter indicates the number of nuclei found to pass the intensity and size filters selected in the algorithm. The ArrayScan was set up to capture a minimum of 100 cells per well or a maximum of 4 image fields per well, whichever came first. Wells with significantly <40 cells/well were likely treated with compounds that are either cytotoxic or significantly disrupt cell adherence (Fig. 7). When the average cell number per field parameter was plotted for the different plate controls, very few if any of the wells had fewer than 40 cells per well (Fig. 7A). In contrast, a plot of the average cell number per field parameter versus compound concentration indicated that a number of wells had significantly less than 25 cells per well (Fig. 7A). This was especially apparent at the two highest concentrations of the dose response, 50 and 16.67 μM. Upon checking the images from these wells (Fig. 7B), it is apparent that a number of compounds had reduced the number of cells per well, due to either a dose-dependent cytotoxicity or a dose-dependent reduction in cell adherence.

By comparing data from plate controls and empty wells in a scatter plot of the average cytoplasmic intensity/well versus average nuclear intensity/well, it was apparent there are two distinct populations apparent in data corresponding to the anisomycin-treated controls in one population and the untreated or maximally inhibited controls in the other (Fig. 7A). These two control populations do not contain any wells

or off-target compound effects such as cytotoxicity or disruption of cell adherence. These data were exported from the ArrayScan analysis software to Spotfire and visualized in a variety of scatter plots (A). The average number of cells per field parameter was plotted for the different plate controls or versus compound concentration to assess cytotoxicity or a reduction in cell adherence. A scatter plot of the average cytoplasmic intensity/well versus average nuclear intensity/well in the target channel (EGFP) was used to identify fluorescent compounds. Representative images from control wells and compound wells that exhibited either a dose-dependent cytotoxicity/reduction in cell adherence or a dose-dependent fluorescence are presented (B).

that exceed thresholds of cytoplasmic intensity >500 and/or nuclear intensity >750. However, when the compound wells are included in the analysis, even though the majority of wells fall below these thresholds, there were a significant number of wells that exceed the thresholds of cytoplasmic intensity >1000 and/or nuclear intensity >1000. This was especially apparent at two of the highest concentrations of the dose response, 50 and 16.67 μM. Upon checking the images from these wells (Fig. 7B), it is apparent that a number of compounds were likely fluorescent.

Of the 217 actives confirmed in the 10-point IC_{50} assays, 59 (27%) exhibited average cell numbers/field data with significantly <40 cells/well, indicating that they were likely cytotoxic or significantly disrupted cell adherence (Table IV). Thirteen (6.0%) of the compounds exceeded thresholds of cytoplamic intensity >1000 and/or nuclear intensity >1000, indicating that they were likely fluorescent (Table IV).

Seventy-three of the confirmed actives produced IC_{50} values <10.0 μM in the MK2-EGFP translocation assay (Table III). A cross target query of the Lilly-RTP HTS database for the confirmed active compounds in the MK2-EGFP translocation assay revealed that the most prevalent targets with confirmed IC_{50} values in the database were *in vitro* kinase targets, including p38a. Around 40 compounds were profiled in cell-based imaging assays for MAPK signaling pathway selectivity; MK2-EGFP translocation, cJun activation, and ERK activation (see Nickischer *et al.*, 2006). Compounds were also structurally confirmed by LCMS analysis (data not shown). In sum, upon completion of the hit assessment for the p38 inhibitors identified in the MK2-EGFP translocation assay, a new structural class of p38a inhibitor had been identified (data not shown) and three of the hits were selected as potential p38a inhibitor hit-to-lead scaffolds.

TABLE IV
MK2-EGFP TRANSLOCATION 10-POINT IC_{50} SECONDARY ANALYSIS HIT ASSESSMENT

Secondary analysis	ArrayScan	
Total # tested	241	%
>50 μM	24	9.96
<50 μM	217	90.04
Cytox	59	27.19
Fluorescent	13	5.99
<50 μM	145	66.82

Discussion

This chapter described the conversion and assay development of a 96-well MK2-EGFP translocation assay developed on the ArrayScan imaging platform into a higher-density 384-well format HCS assay (Figs. 1–6). This chapter also presented a case history of the performance of the MK2-EGFP translocation assay run on the ArrayScan imager as a ''high-content'' screen of a 32K kinase-biased library to identify p38 inhibitors (Fig. 7, Tables I–IV). As described in this chapter, the MK2-EGFP translocation assay performed very well on the ArrayScan platform and a number of p38 inhibitor hits were selected for hit-to-lead follow-up SAR.

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. Although many imaging applications perform well on wide-field HCS imagers, there is a perception that confocal capability is desirable, perhaps because many HCS assays have their genesis on stand-alone confocal microscope platforms. Confocal scanning systems work by illuminating the specimen in one or more small regions (spots or lines) and building up an image by scanning the illumination through the specimen while measuring the emission in synchrony with the scanning. Confocal HCS systems can be further divided based on illumination scan design, with systems available that use point scanning, line scanning, and multipoint scanning (e.g., spinning disk). Confocal imaging systems have a definite advantage in rejecting background fluorescence from material outside the plane of focus, either due to the specimen being significantly thicker than the depth of field or due to some fluorescent component in the well plate or surrounding media, such as excess label. For assays with a high solution background or with thick, multilayer cell preparations, confocal imaging will certainly be an advantage. However, confocal HCS imagers are more complex to build and therefore are typically more expensive than wide-field HCS systems such as the ArrayScan, INCell 1000, or Image Express 5000. The same assay plates that were described in this chapter were also read on the Incell 3000 confocal line-scanning platform (GE-Healthcare) with equivalent results, except that the scan times were on average ~2-fold shorter (data not shown). When comparing throughputs on different imaging platforms it is good practice to run as many distinct biologies and combinations of fluorophores as possible. The nature and quality of the sample preparations will have a significant impact on the performance of any imaging platform.

p38 has been a major target for drug discovery by the pharmaceutical industry, as indicated by the numerous patent applications and small molecule inhibitors that have been developed (English and Cobb, 2002; Fabbro *et al.*, 2002; Noble *et al.*, 2004; Regan *et al.*, 2002). Many of these p38 inhibitors have exhibited significant efficacy in cellular assays and animal disease models, and several have progressed into human clinical trails for the treatment of inflammation and cancer (English and Cobb, 2002; Fabbro *et al.*, 2002; Noble *et al.*, 2004; Regan *et al.*, 2002). For example, SB203580 and BIRB 796 and their analogs have been shown to be potent and selective inhibitors of p38 MAPK (English and Cobb, 2002; Fabbro *et al.*, 2002; Noble *et al.*, 2004; Regan *et al.*, 2002). SB203580 and BIRB 796 produced IC_{50} values of 60 and 18 n*M*, respectively in a THP-1 tumor necrosis factor-α production cell model (Regan *et al.*, 2002). In the MK2-EGFP translocation assay described here, SB203580 produced an average IC_{50} of 103 ± 4 n*M* and RWJ 68543 produced on average an IC_{50} of 108 ± 28 nm. As an indicator of selectivity the JNK1/2 inhibitor SP 600125 failed to inhibit the anisomycin-induced MK2-EGFP translocation response. In a similar MK2-GFP redistribution assay developed in a BHK-1 cell background and quantified on the Incell 3000 imaging platform, SB203580 produced an IC_{50} of 3.4 μM for anisomycin-induced redistribution (Almholt *et al.*, 2004).

A total of 32,891 compounds, including a 32K kinase-biased library, were tested at a final concentration of 50 μM (0.5% DMSO) for their ability to inhibit anisomycin-induced MK2-EGFP translocation on the ArrayScan platform. Of the 32,891 compounds tested, 483 (1.47%) met the active criterion and produced $\geq$50% inhibition of anisomycin-induced MK2-EGFP translocation (Table I). Of the compounds available from the compound library, 448 were tested in five-point IC_{50} curves, and 234 (52.23%) were confirmed as dose-dependent inhibitors of anisomycin-induced MK2-EGFP translocation with IC_{50} values <50 μM (Table I). Two hundred forty-one compounds were tested in 10-point IC_{50} curves, and 217 (90.04%) were confirmed as inhibitors of anisomycin-induced MK2-EGFP translocation with IC_{50} values <50 μM (Table I). Through use of multiparameter data provided by the cytoplasm-to-nuclear translocation algorithm and by checking images, a number of compounds were identified that were potential artifacts due to interference with the imaging format. These included fluorescent compounds, or compounds that dramatically reduced cell numbers due to cytotoxicity or by disrupting cell adherence. A total of 145 compounds that were not fluorescent or cytotoxic produced IC_{50} values <50.0 μM in the MK2-EGFP translocation assay (Table V). Of the confirmed actives, 73 (30%) produced IC_{50} values

<10.0 μM in the MK2-EGFP translocation assay (Table III), and a cross target query of the Lilly-RTP HTS database confirmed their inhibitory activity against *in vitro* kinase targets, including p38a (data not shown). Compounds were confirmed structurally by LCMS analysis and profiled in cell-based imaging assays for MAPK signaling pathway selectivity: MK2-EGFP translocation, cJun activation, and ERK activation (see Nickischer *et al.*, 2006). The hit assessment of the p38 inhibitors identified in the MK2-EGFP HCS run on the ArrayScan identified a new structural class of p38a inhibitor (data not shown), and three of the hit scaffolds were selected for p38a inhibitor hit-to-lead chemistry SAR.

The previously described BHK-1 MK2-GFP redistribution assay (Almholt *et al.*, 2004) was utilized for an HTS of 183,375 compounds that were screened at 10 μM for inhibitors of MK2-GFP redistribution. A throughput of 19,000 compounds per day was achieved, and 1960 (1.1%) inhibited redistribution >35%. The secondary analysis parameters of the Incell 3000 nuclear trafficking module were used to eliminate cytotoxic and fluorescent compounds, dropping the hit rate to 1109 compounds (0.6%) (Almholt *et al.*, 2004). Six hundred thirty-four hits were selected for retesting, and 206 (0.11%) were confirmed at exhibiting >25% inhibition of MK2-GFP redistribution without causing cell rounding or producing fluorescence (Almholt *et al.*, 2004). Hits from the primary screen were categorized via a number of secondary assays, and two main classes of compounds were identified: direct inhibitors of the MK2 nuclear export process and inhibitors of the upstream p38 MAPK pathway (Almholt *et al.*, 2004). One of the confirmed actives was shown to resemble a p38 kinase inhibitor structurally and functionally (Almholt *et al.*, 2004).

The MK2-EGFP screen described here differed from the previously described redistribution screen in three main ways: the MK2-EGFP assay was ~30-fold more sensitive to inhibition by the p38 inhibitor SB 203580 than the redistribution assay, a 32K kinase-biased library was screened rather than a 185K random library, and compounds were screened at 50 μM rather than at 10 μM. Nevertheless, both assays successfully identified p38 inhibitors and demonstrated the utility of HCS assays for screening compounds that interfere with therapeutically important signaling pathways.

There are some 518 protein kinases encoded in the human genome that share a catalytic domain, the ATP-binding site, conserved in sequence and structure (English and Cobb, 2002; Noble *et al.*, 2004). The vast majority of known kinase inhibitors were found to be competitive with ATP, and thus are believed to interact within the ATP-binding site (English and Cobb, 2002; Noble *et al.*, 2004). The conservation of the ATP-binding site within the kinase

family, the fact that a significant number of the other enzymes in cells use ATP, and the millimolar intracellular concentration of ATP raise significant concerns about inhibitor potency in cellular assays, kinase selectivity, and adverse off-target effects (English and Cobb, 2002; Noble *et al.*, 2004). There have been some examples of kinase inhibitors that appear to bind preferentially to the inactive (unphosphorylated) form of the enzyme, thereby blocking activation of the kinase, or bind to an allosteric site spatially distinct from the ATP pocket that causes a shift in the conformation of the kinase that is incompatible with ATP binding (English and Cobb, 2002; Noble *et al.*, 2004; Regan *et al.*, 2002). While it may be technically challenging to design an *in vitro* kinase assay to measure inactive enzyme or allosteric kinase inhibitors, cellular assays may be configured to test both these and ATP competitive kinase inhibitors. Image-based cellular assays provide multiparameter quantitative and qualitative information beyond the single parameter target data typical of most other assay formats, and thus are termed ''high content.'' HCS may be configured for simultaneous multiple target readouts (multiplexing) and can provide information on cellular morphology, population distributions, and subcellular localizations and relationships. By selection of the appropriate probes (antibodies, fluorescent protein fusion partners, biosensors, and stains), HCS can be designed to measure and discriminate between on-target and off-target effects of lead compounds and can provide cell-based models that have the potential to improve the conversion rate of drug candidates to successful drugs.

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