Published in final edited form as:

Methods Mol Biol. 2010 ; 661: 107–122. doi:10.1007/978-1-60761-795-2_6.

Use of Inhibitors in the Study of MAP Kinases

Kimberly Burkhard and Paul Shapiro

Abstract

The mitogen-activated protein (MAP) kinases are ubiquitous intracellular signaling proteins that respond to a variety of extracellular signals and regulate most cellular functions including proliferation, apoptosis, migration, differentiation, and secretion. The four major MAP kinase family members, which include the ERK1/2, JNK, p38, and ERK5 proteins, coordinate cellular responses by phosphorylating and regulating the activity of dozens of substrate proteins involved in transcription, translation, and changes in cellular architecture. Uncontrolled activation of the MAP kinases has been implicated in the initiation and progression of a variety of cancers and inflammatory disorders. As such, the ability to manipulate the activity of MAP kinase proteins with specific pharmacological inhibitors has received much attention as research tools for understanding basic mechanisms of cellular functions and for clinical tools to treat diseases. A variety of pharmacological inhibitors have been developed to selectively block MAP kinases directly or indirectly through targeting upstream regulators. This chapter will provide an overview of some of the current inhibitors that target MAP kinase signaling pathways and provide methodology on how to use selective MAP kinase inhibitors and immunoblotting techniques to monitor and quantify phosphorylation of MAP kinase substrates.

Keywords

Mitogen-activated protein kinase; Extracellular signal-regulated kinase; c-Jun N-terminal kinase; p38 MAP kinase; U0126; SB203580

1. Introduction

The mitogen-activated protein (MAP) kinases are ubiquitous regulators of many cellular functions including cell growth, proliferation, differentiation, and inflammatory responses to stress signals (1). The MAP kinase family consists of four major members; the extracellular signal-regulated kinases-1 and 2 (ERK1/2), the c-Jun N-terminal kinases (JNK), p38 MAP kinases, and Big MAP kinase-1 (BMK1) also known as ERK5. Each of the MAP kinases is activated through highly specific interactions with upstream MAP or ERK kinases (MEKs), which phosphorylate threonine and tyrosine residues within the activation loop. Once activated, MAP kinases, in turn, phosphorylate and regulate a variety of substrates including transcription factors, translation regulators, other kinases, structural proteins, and other signaling proteins. Given the prominent role that constitutive activation of the MAP kinases plays in proliferative diseases like cancer, or inflammatory disorders such as rheumatoid arthritis, a number of pharmacological inhibitors have been developed to block MAP kinase signaling (2–4). These inhibitors target multiple proteins in the signaling cascade starting at the plasma membrane receptors all the way to the specific MAP kinase. The ability to manipulate the MAP kinase signaling cascades have been particularly useful for understanding basic biological mechanisms that regulate cell functions and for clinical therapies to treat disease. Table 1 provides a list of some of the major small molecular

weight pharmacological inhibitors and their protein targets within the MAP kinase signaling pathway. Other methods for inhibiting MAP kinase signaling pathways in treating disease include monoclonal antibodies that target extracellular domains or ligands of receptor tyrosine kinases. The use of monoclonal antibodies to block MAP kinase signaling will not be discussed and can be found in other reviews (5).

High throughput screening methods have made it feasible to identify potentially target-specific inhibitor compounds with a desired effect from a large pool of chemical compounds. These types of drug discovery projects first develop the appropriate in vitro and cell-based assays to screen large chemical libraries and assess effects on target kinase activity or a cellular response (6). Once active compounds are identified, chemical modifications and refinement of these lead molecules are made to reach greater inhibition in both the in vitro and cell-based models. Drug development efforts also take advantage of the three-dimensional structures of the MAP kinases that have been solved by X-ray crystallography (7). A detailed understanding of the structure–function relationship for MAP kinases allows the design of inhibitor compounds that bind to specific regions on the MAP kinases including the ATP-binding domain or noncatalytic substrate binding domains (8–10). This approach, in combination with testing in biological assays and high throughput screening, provides an opportunity to identify highly specific compounds with better information on their mechanism of action.

Some of the first high throughput screening of chemical libraries aimed at developing target-selective inhibitors of MAP kinase signaling identified the compound PD98059 to be an allosteric inhibitor of MEK1 (11). Since the MEK1/2 proteins are the only known activators of ERK1/2, MEK-selective compounds are effective inhibitors of ERK1/2 activation. Subsequent studies developed more potent inhibitors of the MEK1/2 proteins including the small molecules U0126 (12), PD184352 and structurally similar PD0325901 (13), and AZD6244 (ARRY-142886) (14). These pharmacological inhibitors of MEK1/2 have been instrumental in understanding basic functions of ERK1/2 signaling and for clinical testing (2, 4). A variety of potent inhibitors of the p38 MAP kinases have also been developed and include SB203580 (15), SB202190 (16), and BIRB-796 (8). Many of the MEK1/2 and p38 MAP kinase pharmacological inhibitors have been shown to be quite specific in their kinase inhibition profiles (17). Other MAP kinase inhibitors such as SP600125 have been shown to inhibit JNK isoforms by competing with the ATP-binding site (18). However, SP600125 has also been shown to inhibit a number of other kinases (19), which must be considered when using this compound for evaluating JNK pathway regulation. Recent studies have identified pharmacological inhibitors that are reasonably selective for MEK5 causing inhibition of the ERK5 pathway without affecting ERK1/2 signaling (20). Moreover, new approaches are identifying new small molecular weight compounds that are designed to block protein–protein interactions between the ERK1/2 MAP kinases and a selective number of substrates (10). This approach may be advantageous for inhibiting some, but not all, of the substrate proteins that are regulated by a particular MAP kinase.

The ultimate goal in identifying selective MAP kinase inhibitors is to use them to treat human diseases. Several pharmacological inhibitors that target MAP kinase signaling pathways have been approved by the US Food and Drug Administration (FDA) for clinical applications (Table 1). These include drugs that inhibit plasma membrane receptor tyrosine kinases (RTK) that activate MAP kinases and are often overactivated in cancer cells (21, 22). The RTK inhibitors include sunitinib (Sutent®), which targets the PDGF, VEGF, and c-Kit receptors and are approved to treat renal cell carcinoma and gastrointestinal stromal tumors. In addition, gefitinib (Iressa®) and erlotinib (Tarceva®) are small molecular weight inhibitors that target the EGF receptor and are approved to treat non-small cell lung cancer and pancreatic cancer. A relatively nonspecific kinase inhibitor, sorafenib (Nexavar®),

targets VEGF receptor and other kinases and is used to treat renal cell and hepatocellular carcinomas. Farnesyl transferase inhibitors (FTIs) were developed to block activation of Ras-G proteins, which are mutated and active in nearly 25% of all human cancers (23). While the FTI class of MAP kinase signaling pathway inhibitors has been less successful in the clinics due to problems with toxicity and lack of efficacy to block Ras, FTIs, such as tipifarnib (ZarnestraTM), are still being tested in clinical trials for treating a variety of solid tumors and hematologic disorders (24).

Additional pharmacological inhibitors that target MAP kinase signaling include drugs that were intended to inhibit nonreceptor tyrosine kinases. The most successful example has been imatinib mesylate (Gleevec[®]), which was developed to inhibit the oncogenic Bcr–Abl fusion protein found in almost every case of chronic myelogenous leukemia (CML) (25). Imatinib mesylate has subsequently been shown to also inhibit other tyrosine kinases such as Src, c-Kit, and PDGF receptors. Several other inhibitors of Bcr–Abl and tyrosine kinases, including dasatinib (Sprycel[®]) and nilotinib (Tasigna[®]), have emerged from the successes of imatinib mesylate and are used to treat imatinib-resistant CML and other hematologic disorders (26).

This chapter will focus on the use of some more common pharmacological inhibitors that target-specific MAP kinases or the direct activation of MAP kinases and the evaluation and quantification of substrate phosphorylation in the context of various stimuli. With the availability of phosphorylation state-specific antibodies, methods for analyzing protein phosphorylation can be readily accomplished by immunoblotting techniques without the need for radioisotopes. Semiquantitative methods using densitometry will be discussed to determine the relative amount of protein phosphorylation following immunoblotting.

2. Materials

2.1. Cell Culture Supplies

1. Dulbecco's Modified Eagles Medium (DMEM) (GIBCO[®] Invitrogen; Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals; Lawrenceville, GA) and 1% (v/v) Penicillin/Streptomycin (PS) (GIBCO[®]).
2. Buffers for washing and passing cells include Hanks' balanced salt solution (HBSS), 0.25% trypsin-EDTA, and phosphate buffered saline (PBS).
3. Six well culture dishes (Becton, Dickinson and Company, BD; Franklin Lake, NJ), teflon cell scrapers (Fisher Scientific; Pittsburgh, PA), and a 5% CO₂ incubator set at 37°C.
4. Tissue lysis buffer (2×) (TLB): 0.2 M Tris–HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate (SDS), 20% (w/v) glycerol, 0.4 M β-mercaptoethanol, and 0.1% bromophenol blue.

2.2. Chemicals and Reagents

1. The p38 MAPK inhibitor, SB203580 (Calbiochem/EMD Chemicals, Inc.; Gibbstown, NJ), is reconstituted in autoclaved water to 20 mM and stored in aliquots at –20°C.
2. The MEK1/2 inhibitor, U0126 (Calbiochem/EMD Chemicals, Inc.), is stored in 100% DMSO at –20°C in 20 µL aliquots.
3. Epidermal growth factor (EGF) (Sigma; St. Louis, MO) is reconstituted in autoclaved water to a concentration of 50 µg/ml, aliquoted, and stored at –20°C.

4. Anisomycin (Sigma) is dissolved in 100% ethanol to 25 mg/ml, aliquoted, and stored at 4°C.
5. Enhance chemiluminescent (ECL) reagents are from GE Healthcare (Piscataway, NJ).
6. General chemicals: methanol, sodium azide (NaN₃).

2.3. SDS-Polyacrylamide Gel Electrophoresis

1. Stock solutions include 3 M Tris-HCl (pH 8.8), 1 M Tris-HCl (pH 6.8), and 10% (w/v) SDS.
2. 30% (w/v) Acrylamide (National Diagnostics; Atlanta, GA) is stored at 4°C. 1% (w/v) Bis-acrylamide (Fisher Scientific) is filtered through a 0.2-μm filter (Millipore; Billerica, MA) and stored at 4°C.
3. *N,N,N,N'*-Tetramethyl-ethylenediamine (TEMED) is stored at room temperature. A 10% ammonium persulfate solution (APS) is made fresh and stored at 4°C for up to 1 week.
4. Running buffer (10×): 0.25 M Tris-HCl, 2 M glycine, and 1% SDS is stored at room temperature.
5. Full-Range Rainbow™ Molecular Weight Marker (GE Healthcare).

2.4. Antibodies

1. The phosphorylation-specific anti-ppERK1/2 monoclonal mouse antibody is stored at -20°C in 20 μL aliquots. See Table 2 for a list of many of the antibodies used to study the phosphorylation status of the major MAP kinases and their substrates.
2. The total p38 MAPK and p-p38 MAPK antibodies are stored at -20°C.
3. ERK2 substrate phosphorylation-specific antibody: phosphorylated p90Rsk-1 (pRsk-1, Thr573) antibody is stored at -20°C.
4. Anti-α-tubulin monoclonal mouse antibody used as a loading control is stored at -20°C in 20 μL aliquots.
5. Secondary antibodies: anti-mouse and anti-rabbit IgG conjugated to HRP (Sigma) are stored at 4°C.

2.5. Immunoblotting for MAP Kinases and Substrate Phosphorylation

1. Polyacrylamide gel electrophoresis apparatus (C.B.S. Scientific Company, Inc.; Del Mar, CA).
2. MagicMark™ western protein standard (Invitrogen; Carlsbad, CA).
3. Electro-blotter semidry transfer system (Ellard Instrumentation Ltd; Monroe, WA) and slot blotter (Schleicher & Schuell BioScience; Keene, New Hampshire) used for phosphorylation quantification.
4. Transfer solutions include 0.25 M Tris base containing 0.4 M Aminocaproic acid, 1.25 M Tris base, and isopropyl alcohol (IPA). Blotting paper (VWR; West Chester, PA).
5. Tris-buffered saline with Tween (TBS-tween) used for western blotting: 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween-20. Blocking buffer; 5% (w/v) nonfat dry milk in TBS-tween.

6. Polyvinylidene difluoride (PVDF) transfer membrane (PerkinElmer; Waltham, MA).
7. Bio-Max ML autoradiography film (Kodak; Rochester, NY).
8. Quantification by densitometry of films was done using the FLOURCHEM® SP imager (Alpha Innotech; San Leandro, CA) and AlphaEase FC™ software (Alpha Innotech).

3. Methods

Pharmacological inhibitors can be used to help determine the relevance of MAP kinase signaling pathways and their biological responses to extracellular signals. The MAP kinases can phosphorylate and regulate dozens of substrate proteins. With the development of specific antibodies that can distinguish a protein's phosphorylation status, the evaluation of MAP kinase activity can be readily achieved by measuring the phosphorylation of MAP kinase substrate proteins. The following protocol describes immunoblotting methods using phosphorylation-specific antibodies for visualizing, quantifying, and analyzing changes in MAP kinase activity and substrate phosphorylation in the presence of pharmacological inhibitors. The methods shown utilize common MAP kinase activators and pharmacological inhibitors in isolated cell cultures. However, the approach can be adapted for use in the context of different agonists or antagonists as well as assessment of MAP kinase signaling in tissue samples. Lastly, the advantages and disadvantages of the quantitative analysis of protein phosphorylation will be discussed along with the use of appropriate analytical controls.

3.1. Preparation of Cultured Cells for Evaluating MAP Kinase Inhibitors

1. HeLa S3 cells (American Type Culture Collection, catalog #CCL-2.2) at ~80% confluence are washed twice with HBSS buffer and trypsin-EDTA (0.25%) is added to the cells and incubated 2–5 min at 37°C or until most of the cells detach from the plate.
2. Complete DMEM media with 10% FBS and 1% PS is added and cells are seeded in 6-well tissue culture plates at 5×10^5 cells per well. The cells are incubated for an additional 24 h at 37°C with 5% CO₂.
3. Cells are pretreated with 1–10 μM U0126 for 15 min (see Note 1). EGF (50 ng/ml) is added to stimulate the ERK pathway, and the cells are incubated for an additional 5 min. Controls include unstimulated and EGF only treated samples.
4. Cells are pretreated for 10 min with 10 μM SB203580 at 37°C and then stimulated with 25 μg/ml anisomycin to activate the p38 MAP kinase pathway for 20 min at 37°C. Controls include unstimulated and anisomycin only stimulated samples.
5. Immediately after incubating with anisomycin or EGF, the cells are placed on ice and washed twice with cold PBS. 300 μl of TLB is added to each well and the cells are removed from the plate using Teflon cell scrapers. The samples are then transferred to 1.5 ml microcentrifuge tubes and heated at 100°C for 5 min before protein separation by gel electrophoresis and detection by immunoblotting.

¹The MEK1/2 inhibitors have been reported to inhibit the MEK5/ERK5 pathway at concentrations of greater than or equal to 10 μM, although concentrations less than 2 μM are sufficient to inhibit MEK1/2 and ERK1/2 activation in cultured cells (27).

3.2. SDS-Polyacrylamide Gel Electrophoresis

1. A 15% gel for an ASG-250 gel apparatus is made by first pouring the separating portion of the gel. The separating gel is made by combining 1.9 ml of 3 M Tris base (pH 8.8), 7.5 ml of 30% acrylamide, 1.3 ml 1% bis-acrylamide, 4.3 ml water, 150 μ l 10% SDS. Add 10 μ l TEMED and 50 μ l APS immediately before pouring gel. Polymerization of the gel is usually complete in 30–45 min.
2. The stacking portion of the gel is made by mixing 625 μ l 1 M Tris base (pH 6.8), 835 μ l 30% acrylamide, 650 μ l 1% bis-acrylamide, 2.8 ml water, and 75 μ l 10% SDS. Immediately before pouring the stacking gel, 5 μ l TEMED and 25 μ l 10% APS is added. The gel is poured, and a comb is inserted avoiding the introduction of air bubbles. Polymerization is usually complete in 15 min.
3. After the gel has polymerized, any unpolymerized acrylamide remaining in the wells can be removed by rinsing with 1 $\times$ running buffer using a syringe. Running buffer (1 $\times$) is added to the top (cathode) and bottom (anode) chambers of the gel apparatus and 5–20 μ l (~50 μ g) of the samples are loaded into each well with a Hamilton syringe. The proteins are separated by applying a constant 35 mA to the gel for 1.5–2 h.

3.3. Immunoblotting for MAP Kinases and Substrate Proteins

1. This method is used for a semidry electro-blotter transfer system. PVDF membrane is first soaked in methanol for 30–60 s and then wash with distilled water. Fifteen pieces of blotting paper are cut to the size of the gel that contains the proteins of interest. Six pieces of blotting paper are soaked with Solution A (12.5 ml of 0.25 M Tris base with 0.4 M aminocaproic acid, 25 ml IPA, and 87.5 ml of water), three pieces are soaked in Solution B (2.5 ml 1.25 M Tris base, 25 ml IPA, and 100 ml water), and six pieces are soaked in Solution C (25 ml 1.25 M Tris base, 25 ml IPA, and 75 ml of water) (see Note 2).
2. The transfer assembly is set up on a plastic tray in the following sequential order; blotting paper soaked in Solution A, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel, PVDF membrane, blotting paper soaked in Solution B, and blotting paper soaked in Solution C. The transfer assembly is flipped with this electro-blotter system such that the current transfers the protein from the gel closest to the cathode (black) to the PVDF membrane closest to the anode (red). The protein transfer is run at 25 V and 90 mA for 1 h (see Note 3).
3. After the completion of the protein transfer, the PVDF membrane is placed in blocking solution and gently agitated on a rocking platform at room temperature for at least 1 h.
4. The blocking buffer is then removed and the membrane is rinsed briefly with TBS-tween followed by incubation with one of the selected primary antibodies diluted 1:250–1,000 in a sterile 15 or 50 ml conical tube using TBS-tween with 1% (w/v) bovine serum albumin (BSA). For antibody dilutions that will be stored at 4°C for extended periods of time and reused, 0.1% (v/v) sodium azide can be added from a 10% (w/v) stock solution as a preservative. Depending on the antibody, incubations

²Solutions A, B, and C can be made up in 500 ml volumes and stored at room temperature until needed. Separate plastic containers can be used to soak blotting paper. Gently allow excess fluid to drain before setting up transfer.

³To ensure that protein transfer is complete, it is important to roll out any bubbles that may be between the PVDF membrane and the gel. This is done twice, once when the PVDF membrane is placed on top of the gel and once after the transfer assembly has been flipped and placed on the electro-blotter transfer system.

on a rocking platform can range from 1 h at room temperature to overnight at 4°C (see Note 4).

5. Following incubation with primary antibody, the primary antibody can be saved and often reused several times; however, the number of times an antibody can be reused must be determined empirically for each antibody. The membrane is washed three times for 10 min with TBS-tween and then incubated with the appropriate secondary antibody of the appropriate species at a dilution of 1:10,000 in TBS-tween with 1% (w/v) BSA. The membrane is gently rocked for 1 h at room temperature and then washed 3–5 times for 15 min with TBS-tween.
6. The TBS-tween is removed and equal amounts (2.5 ml/blot of each) of the ECL reagents are mixed together and immediately poured onto the membrane taking care to ensure that the entire surface of the membrane is exposed to the ECL reagents. After 1 min incubation, the ECL reagents are removed and the membrane is wrapped in plastic wrap.
7. In a dark room, a piece of autoradiography film (Kodak) is placed on top of the membrane with firm and even pressure for 5–300 s depending on the amount of protein of interest and the specificity of the primary antibody. Exposures of greater than 60 s can be done using an autoradiography film cassette (see Note 5).
8. After exposure, the membrane is rinsed briefly with TBS-tween and reprobed with an antibody that can be used as a protein loading control using the protocol above. Common protein loading controls include α -tubulin or β -actin. An example of an immunoblot for activated ERK1/2, the ERK1/2 substrate Rsk-1, or p38 MAP kinases is shown in Fig. 1.

3.4. Quantification of Protein Levels and Phosphorylation by Densitometry

1. The intensity of the proteins detected in the immunoblot can be semiquantified using densitometry. We use a FluorChem SP imaging system (Alpha Innotech; San Leandro, CA) to create a digital image of the autoradiography film of interest. The images collected are manipulated by AlphaEase FC software. However, densitometry can also be performed with a standard desktop scanner and free software such as ImageJ available through the National Institutes of Health (see Note 6).
2. A digital image of the autoradiography film is generated using the desired scanning device.
3. Using the object function within the various software programs, a square or circle can be drawn around the protein band of interest and the average pixel intensity of the area within the region of interest can be determined (see Note 7).

⁴Each antibody needs to be evaluated for specificity. Many antibodies only recognize the intended target protein, whereas other antibodies react nonspecifically with other proteins. If nonspecific interactions are suspected, controls that increase or decrease the expression of the target protein should be used to validate recognition by the antibody.

⁵It is important that the immunoblots be exposed to the autoradiography film for various times so that the protein levels can be accurately quantified by densitometry scanning. Overexposure of autoradiography film can result in very dark bands corresponding to the protein of interest that may exceed the limits of detection for the densitometry scanner and misrepresent the data.

⁶There are a number of gel documentation systems that can perform chemiluminescence analysis and substitute for autoradiography film. We have found that in cases where sensitivity is an issue, as is the case with some phosphorylated proteins; autoradiography film still offers an advantage for protein detection sensitivity. Each investigator will need to determine empirically whether their gel documentation system has the sensitivity to image and quantify protein phosphorylation.

⁷It should be noted that while densitometry is a convenient way to quantify protein expression, it does have limitations. It is recommended that a densitometry standard curve be established for the specific gel documentation system being used to determine the linear range of sensitivity before quantifying protein expression. This can be done by performing serial dilutions of a known protein that has a highly specific antibody.

4. The background for each sample on the autoradiography film must be taken into account. This is usually done by determining the average pixel intensity for region of interest that is of the same size as the region drawn around the protein of interest. The background region is usually in an open area of the autoradiography film just above or below the protein band of interest. The background value is subtracted from the value of the sample to get the net intensity of the protein of interest. However, given the variability of the size of the bands to quantify as indicated in Fig. 1, it is often advantageous to have the region of interest be of constant size for each of the samples. This can be accomplished by using a spot or dot blotter as described in the following section.

3.5. Quantification of Phosphorylation by Densitometry Using a Spot Blotter

1. If the primary antibody is specific for the target protein, spot/dot blot systems can be used. Described here is a protocol for the Minifold® I spot blot system (Schleicher & Schuell BioScience; Keene, NH), which allows for a more rapid estimate of protein expression as it eliminates the gel electrophoresis and protein transfer steps (see Note 8).
2. The filter support plate is placed on the vacuum manifold. Two pieces of pre-wet blotting paper are then placed on the filter support plate. The PVDF membrane soaked in methanol, rinsed with TBS-tween, and placed directly on top of the blotting paper. The sample well plate is carefully placed on top of the membrane and the clamps are securely fastened.
3. Cell lysate samples are then applied to the membrane, taking care not to create air bubbles (see Note 9).
4. Applying a vacuum to the system will aspirate the samples onto the PVDF membrane in 3–5 min.
5. Once the samples have been aspirated through the PVDF membrane, the system is dismantled and the membrane is immunoblotted as described in Subheading 3.3.
6. The spot blotter can provide a more consistent size of signal for each protein of interest in the samples for quantification as described in Subheading 3.4. An example of the use of the slot blotter to quantify ERK1/2 pathway activation following stimulation with EGF is shown in Fig. 2.

Acknowledgments

The authors would like to thank Kimberly Still for technical assistance. This work was supported by the National Institutes of Health (CA120215).

References

1. Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. *Adv Can Res.* 1998; 74:49–139.

⁸It is important to note that this method of quantification can only be applied to target proteins that are specifically recognized by their respective antibodies. Nonspecific antibodies interactions should be determined by gel electrophoresis and immunoblotting prior to using the slot blotter.

⁹The amount of sample that will be added is dependent upon the number of cells or size of tissue and amount of lysis buffer used. If the number of cells in each condition is approximately equal, then serial dilutions of the positive and negative control samples can be done, if they are available. The dilution that gives the highest signal (positive control) to background (negative control) should be used for the other samples in the experiment. In our hands, the optimum signal to background ratio for several phosphorylation-specific antibodies correspond to ~1–2 µg of total protein loaded per sample. However, the optimal amount of protein loaded should be determined in each laboratory setting.

2. McCubrey JA, Milella M, Tafuri A, Martelli AM, Lunghi P, Bonati A, Cervello M, Lee JT, Steelman LS. Targeting the Raf/MEK/ERK pathway with small-molecule inhibitors. *Curr Opin Investig Drugs*. 2008; 9:614–630.
3. Cohen P. Targeting protein kinases for the development of anti-inflammatory drugs. *Curr Opin Cell Biol*. 2009; 21:317–324. [PubMed: 19217767]
4. Friday BB, Adjei AA. Advances in targeting the Ras/Raf/MEK/Erk mitogen-activated protein kinase cascade with MEK inhibitors for cancer therapy. *Clin Cancer Res*. 2008; 14:342–346. [PubMed: 18223206]
5. Kalyn R. Overview of targeted therapies in oncology. *J Oncol Pharm Pract*. 2007; 13:199–205. [PubMed: 18045779]
6. von Ahlsen O, Bomer U. High-throughput screening for kinase inhibitors. *Chembiochem*. 2005; 6:481–490. [PubMed: 15742384]
7. Wang Z, Canagarajah BJ, Boehm JC, Kassisa S, Cobb MH, Young PR, Abdel-Meguid S, Adams JL, Goldsmith EJ. Structural basis of inhibitor selectivity in MAP kinases. *Structure*. 1998; 6:1117–1128. [PubMed: 9753691]
8. Pargellis C, Tong L, Churchill L, Cirillo PF, Gilmore T, Graham AG, Grob PM, Hickey ER, Moss N, Pav S, Regan J. Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nat Struct Biol*. 2002; 9:268–272. [PubMed: 11896401]
9. Regan J, Breitfelder S, Cirillo P, Gilmore T, Graham AG, Hickey E, Klaus B, Madwed J, Moriak M, Moss N, Pargellis C, Pav S, Proto A, Swinamer A, Tong L, Torcellini C. Pyrazole urea-based inhibitors of p38 MAP kinase: from lead compound to clinical candidate. *J Med Chem*. 2002; 45:2994–3008. [PubMed: 12086485]
10. Hancock CN, Macias A, Lee EK, Yu SY, Mackerell AD Jr, Shapiro P. Identification of novel extracellular signal-regulated kinase docking domain inhibitors. *J Med Chem*. 2005; 48:4586–4595. [PubMed: 15999996]
11. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci U S A*. 1995; 92:7686–7689. [PubMed: 7644477]
12. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem*. 1998; 273:18623–18632. [PubMed: 9660836]
13. Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, Tecle H, Barrett SD, Bridges A, Przybranowski S, Leopold WR, Saltiel AR. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat Med*. 1999; 5:810–816. [PubMed: 10395327]
14. Kohno M, Pouyssegur J. Targeting the ERK signaling pathway in cancer therapy. *Ann Med*. 2006; 38:200–211. [PubMed: 16720434]
15. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett*. 1995; 364:229–233. [PubMed: 7750577]
16. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*. 1994; 372:739–746. [PubMed: 7997261]
17. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J*. 2000; 351:95–105. [PubMed: 10998351]
18. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci U S A*. 2001; 98:13681–13686. [PubMed: 11717429]
19. Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. *Biochem J*. 2003; 371:199–204. [PubMed: 12534346]

20. Tataka RJ, O'Neill MM, Kennedy CA, Wayne AL, Jakes S, Wu D, Kugler SZ Jr, Kashem MA, Kaplita P, Snow RJ. Identification of pharmacological inhibitors of the MEK5/ERK5 pathway. *Biochem Biophys Res Commun.* 2008; 377:120–125. [PubMed: 18834865]
21. Arora A, Scholar EM. Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther.* 2005; 315(3):971–979. [PubMed: 16002463]
22. Mendelsohn J, Baselga J. Epidermal growth factor receptor targeting in cancer. *Semin Oncol.* 2006; 33:369–385. [PubMed: 16890793]
23. Hahn SM, Bernhard E, McKenna WG. Farnesyltransferase inhibitors. *Semin Oncol.* 2001; 28:86–93. [PubMed: 11706400]
24. Mesa RA. Tipifarnib: farnesyl transferase inhibition at a crossroads. *Expert Rev Anticancer Ther.* 2006; 6:313–319. [PubMed: 16503848]
25. Wong S, Witte ON. The BCR-ABL story: bench to bedside and back. *Annu Rev Immunol.* 2004; 22:247–306. [PubMed: 15032571]
26. Bocchia M, Forconi F, Lauria F. Emerging drugs in chronic myelogenous leukaemia. *Expert Opin Emerg Drugs.* 2006; 11:651–664. [PubMed: 17064224]
27. Mody N, Leitch J, Armstrong C, Dixon J, Cohen P. Effects of MAP kinase cascade inhibitors on the MKK5/ERK5 pathway. *FEBS Lett.* 2001; 502:21–24. [PubMed: 11478941]

Fig. 1.

(a) Immunoblots of active ERK1/2 (ppERK1/2) and phosphorylated p90Rsk-1 (pRsk-1) in HeLa cell lysates following treatment with EGF for 5 min in the absence or presence of the MEK1/2 inhibitor U0126. The expression of α -tubulin was used as a protein loading control. (b) Immunoblots of active p38 MAP kinase (p-p38) in HeLa cell lysates following treatment with anisomycin in the absence or presence of SB203580. Total p38 MAP kinase expression was used as a protein loading control.

Fig. 2.

Quantification of ERK1/2 activation and substrate phosphorylation by spot blotter. The untreated and EGF-treated samples from Fig. 1. were immunoblotted for active ERK1/2 (ppERK1/2), phosphorylated Rsk-1 (pRsk-1), and α -tubulin as a loading control using a slot blotter (*inset*). The graph shows an approximately 40-fold and 8-fold increase in active ERK1/2 and phosphorylated Rsk-1, respectively, when the spot blotter data was quantified by densitometry. These relative changes in protein phosphorylation correlates well with the immunoblots shown in Fig. 1.

Table 1

Pharmacological inhibitors of MAP kinases and proteins that regulate MAP kinase signaling pathways

Target family	Specific target	Inhibitor	Vendor (catalog#)
Receptor tyrosine kinase	EGFR	Gefitinib (Iressa [®])	American Custom Chemicals (ACC) Corporation (184475-35-2)
	VEGFR	Erlotinib (Tarceva [®])	ACC (183321-74-6)
		Lapatinib (Tykerb [®])	Axon Medchem (1128)
			SELLECK (S1028)
	PDGFR		ACC (231277-92-2)
		Sunitinib (Sutent [®])	ACC (557795-19-4)
			Axon Medchem (1398)
		Sorafenib (Nexavar [®])	ACC (284461-73-8)
			Axon Medchem (1397)
Non-receptor and receptor tyrosine kinases	Bcr-Abl,	Nilotinib (Tasigna [®])	SELLECK (S1033)
			ACC (64157-10-0)
	Bcr-Abl, c-Src	Dasatinib (Sprycel [®])	ACC (302962-49-8)
			Axon Medchem (1392)
	Bcr-Abl, c-SCT, c-Kit, PDGFR	Imatinib (Gleevec [®])	ACC (220127-57-1)
			Axon Medchem (1394)
G-proteins	Ras	Tipifarnib (Zarnestra [™])	Onicon Pharmachemie (192185-7201)
MAPKKK	Raf	Sorafenib (Nexavar [®])	ACC(284461-73-8)
			Axon Medchem (1397)
MAPKK	MEK1/2	U0126	EMD Biosciences (662005)
			SELLECK (S1102)
		PD184352	Axon Medchem (1368)
			SELLECK (S1020)
	MEK5	AZD6244	SELLECK (S1008)
		BIX02188, BIX02189	Boehringer Ingelheim (<i>not commercially available</i>)
MAPK	p38	SB203580	EMD Biosciences (55389)
			Axon Medchem (1364)
		SB202190	EMD Biosciences (559388)
			Axon Medchem (1363)
		BIRB-796	Axon Medchem (1353)

The MAP kinases (MAPK) are regulated sequentially through receptor and non-receptor tyrosine kinases, G-proteins, MAP kinase kinase kinases (MAPKKK), and MAP kinase kinases (MAPKK)

Table 2

List of antibodies for the phosphorylated and phosphorylation-independent (total) forms of MAP kinases and phosphorylated forms of MAP kinase substrate proteins

Protein targets	Antibody (phosphorylation site recognized)	Vendor (catalog#)
ERK1/2	Activated ERK1/2 (Thr185/Tyr187)	Sigma-Aldrich (M9692)
		Santa Cruz Biotechnology (sc-16982)
		EMD Biosciences (442706)
		Epitomics (148101)
	Total ERK2	Epitomics (1586-1)
		Santa Cruz Biotechnology (sc-154)
p38 α , β , γ	Activated p38 MAP kinase (Thr180/Tyr182)	EMD Bioscience (442685)
		Santa Cruz Biotechnology (sc-94)
		Cell Signaling Technology (CST) (9211)
		Epitomics (1229-1)
	Total p38 α , β , γ MAP kinase	CST (9212)
		EMD Biosciences (506123)
JNK1/2	Activated JNK1/2 MAP kinase (Thr183/Tyr185)	Epitomics (1544-1)
		Santa Cruz Biotechnology (sc-6254)
		CST (9251)
		Santa Cruz Biotechnology (sc-1648)
	Total JNK1	Santa Cruz Biotechnology (sc-572)
	Total JNK2	
ERK5	Activated pERK5 MAP kinase (Thr218/Tyr220)	Santa Cruz Biotechnology (sc-572)
		CST (2271)
		Epitomics (1719-1)
		Millipore (07-039)
Phosphorylated ERK1/2 substrates	p90RSK-1 (Thr573)	Sigma-Aldrich (E1523)
		CST (9346)
		BD Biosciences (610225)
		ELK-1 (S383)
		CST (9186)
		c-Myc (T58/S62)
		CST (9401)
		MNK-1 (T197/T202)
		CST (2111)
		PPAR- γ (S112)
		Millipore (05-816)
		Connexin-43 (S255)
		Santa Cruz Biotechnology (sc-12899-R)
		Tyrosine Hydroxylase (S31)
		Millipore (AB5423)
		Estrogen receptor- α (S118)
		Epitomics (1091-1)
		Tau (S199/S202)
		Biosource (44-768G)
		Epitomics (1242-1)
		eEF2 (T56/T58)
		Epitomics (1853-1)
		eIF-2 α (S51)
		Epitomics (1090-1)
		eIF-4B (S504)
		Epitomics (2260-1)
		eIF-4E (S209)
		Epitomics (2227-1)

Protein targets	Antibody (phosphorylation site recognized)	Vendor (catalog#)
Phosphorylated p38 substrates	ATF-2 (T71)	CST (9221)
		Epitomics (1268-1)
	MAPKAPK-2 (T334)	CST (3041)
	MNK-1 (T197/T202)	CST (2111)
	Stat-1 (S727)	CST (9177)
Phosphorylated JNK substrates	MSK-1 (S369/S376)	CST (9591)
	c-Jun (S63)	CST (9261)
		Epitomics (1527-1)
	c-Jun (S73)	CST (9264)
		Epitomics (1107-1)
	c-Jun (S63/S73)	Santa Cruz Biotechnology (sc-16312-R)
	p53 (T81)	CST (2676)

Note that not all antibodies against each MAP kinase isoform are listed