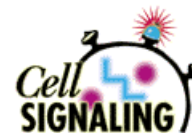


U0126: An Inhibitor of MKK/ERK Signal Transduction in Mammalian Cells



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Editor's Note: Multiple names are in use for many of the proteins in the kinase signaling pathways. For clarity in this report, the authors have chosen to use the term "MKK," for mitogen-activated protein kinase kinase. The other term in common use for this enzyme is MEK (MAP/ERK kinase). ERK is the acronym for extracellular signal-regulated protein kinase, which is synonymous with MAPK.

The MAPK signaling pathway remains the focus of intensive research because it plays a pivotal role in mediation of cellular responses to a variety of signaling molecules. Due to the complex nature of this pathway and the multiplicity of the various enzymes that undergo simultaneous alteration in their structure or functions, it is important to develop biochemical reagents that are highly specific and highly selective to individual enzymes in the signaling pathway. MEK Inhibitor U0126 is such a reagent since it specifically inhibits MKK1/2, in both the inactive and active forms. In contrast, PD098059 only inhibits the activation of MKK1/2. U0126, which inhibits a human MKK1-G1C mutant enzyme with an IC_{50} of less than 200nM as well as the activation of ERK1/2 in cells at 10 μ M, is at least 50-fold more potent than PD098059. Thus, U0126 can serve as a powerful tool to investigate the activation of ERK1/2 by MKK1/2.

INTRODUCTION

Among the key signaling pathways regulating mammalian cell growth and differentiation is the MKK/ERK pathway, comprised of MAP kinases, ERK1/2, and MAP kinase kinases, MKK1/2 (1). ERK1/2 and MKK1/2 are acutely stimulated by growth and differentiation factors in pathways mediated by receptor tyrosine kinases, heterotrimeric G protein-coupled receptors or cytokine receptors, primarily through p21Ras-coupled mechanisms. These enzymes are ubiquitous and are generally expressed at micromolar levels in mammalian cells (2), although some variation in expression between different tissues has been noted (3,4).

Enhancement of MKK or ERK activity in response to cell stimulation involves phosphorylation at residues located within the activation lip of each kinase. In the case of MKK, phosphorylation at two serine residues (Ser²¹⁸/Ser²²² in human MKK1; Ser²²²/Ser²²⁶ in human MKK2) by upstream protein kinases, Raf-1, c-Mos or MEKK (MAPK kinase kinase), leads to maximal enzyme activation. Subsequently, MKK1/2 activates ERK1/2 by phosphorylating regulatory threonine and tyrosine residues (Thr²⁰²/Tyr²⁰⁴ in hERK1; Thr¹⁸⁵/Tyr¹⁸⁷ in hERK2). Thus, MKKs fall within a relatively rare class of protein kinases with dual specificity toward Ser/Thr and Tyr residues on exogenous substrates.

In vitro, MKK1 and MKK2 show comparable activity toward ERK1 and ERK2 (5,6). A few examples exist where ERK1 and ERK2 are activated with distinct magnitudes or kinetics following growth factor (e.g., nerve growth factor) stimulation on intact cells (7,8); however, in most instances, both enzymes are robustly activated upon cell stimulation.

The MKK/ERK pathway is necessary for cell cycle progression through G1 in most metazoan cells. This is demonstrated by elevated DNA synthesis or cell growth under conditions of ERK activation by expressing constitutively active mutant Raf-1 or MKK1, and inhibition of mitogen-induced DNA synthesis with antisense RNA or dominant-negative mutant kinases. Sustained ERK activation also accounts for many features of transformation observed with oncogenic Ras, Raf and Src. In contrast to the role of the MKK/ERK cascade in regulating cell growth, the MKK/ERK pathway also plays an important role in regulating embryonic development and cell differentiation. For example, this pathway has been shown to promote differentiation of mammalian cell lines along neuronal or blood cell lineages and prevent commitment to adipocyte cell fates. Thus, different cell types utilize the MKK/ERK pathway to modulate responses as varied as cell proliferation, cell growth arrest and lineage-specific gene expression.

DOWNSTREAM TARGETS OF THE MKK/ERK PATHWAY

In vitro targets for ERKs include nuclear transcription factors, metabolic enzymes, cytoskeletal proteins and other signaling components (1). Many of these appear to be physiological targets, based on peptide map comparisons between in vitro and in vivo phosphorylation sites.

Demonstrating physiological regulation requires reagents that enable further correlation between kinase activation and substrate

phosphorylation of targets in response to extracellular stimuli. For example, inhibition of signaling by transfecting dominant-negative mutant pathway components into cells can demonstrate that certain pathways are required for regulating specific targets of interest. However, cell transfection strategies are not always suitable for attenuating signal transduction, because transient transfection often fails to produce high expression efficiency, and stable transfection can be variable due to cells that are resistant to stable expression (e.g., primary cultures) or 'leaky' transcription from so-called inducible promoters.

In many situations, cell permeable inhibitors are the most viable approach for testing the requirement of specific signaling pathways for given cell responses. Using these reagents, the involvement of ERKs in various cellular responses can be established by correlating dose-response curves for signaling inhibition with those of kinase inhibition. PD098059 is a selective inhibitor of MKK1 and blocks MKK/ERK activation in intact cells at concentrations ranging from 10 μ M to its solubility limit of 100 μ M (9,10). Although PD098059 inhibits the active mutant deltaN4/S218E/S222D or "G1C" (11), it has little effect in vitro on MKK1 activated by phosphorylation, leading to the hypothesis that its effect in intact cells occurs through inhibition of MKK1/2 phosphorylation by upstream kinases (10). To date, several hundred studies have been published using this compound, illustrating the advantage of identifying pathway regulatory mechanisms using pharmacological strategies.

U0126, A NOVEL INHIBITOR OF MKK1/2

U0126 (1,4-diamino-2,3-dicyano-1,4 bis[2-aminophenylthio]butadiene) was recently described as a novel inhibitor of MKK1 and MKK2 (12). The compound, identified in a screen for inhibitors of AP-1 transactivation in a cell-based reporter assay, inhibited phorbol 12-myristate 13-acetate (PMA)-induction of genes controlled by the 12-O-tetradecanoyl-phorbol 13-acetate (TPA) response element (TRE), at a concentration of 12 μ M.

U0126 inhibits both MKK1 and MKK2 at submicromolar concentrations in vitro, and appears to be more effective toward constitutively active MKK1/2 mutants than MKK activated by phosphorylation (12). In our hands, the IC₅₀ was <200nM for inhibiting the constitutively active mutant MKK1-G1C (11) (Figure 1, Panel A) and was 10 μ M for inhibiting wildtype MKK1 activated by phosphorylation (Figure 1, Panel B). Significantly higher concentrations of U0126 are needed to inhibit MKK3, MKK4, MKK6, ERK1, ERK2, p38 MAPK, JNK, protein kinase C, c-Abl, cdk2 and cdk4 (12), supporting the utility of this compound in selectively blocking the ERK pathway.

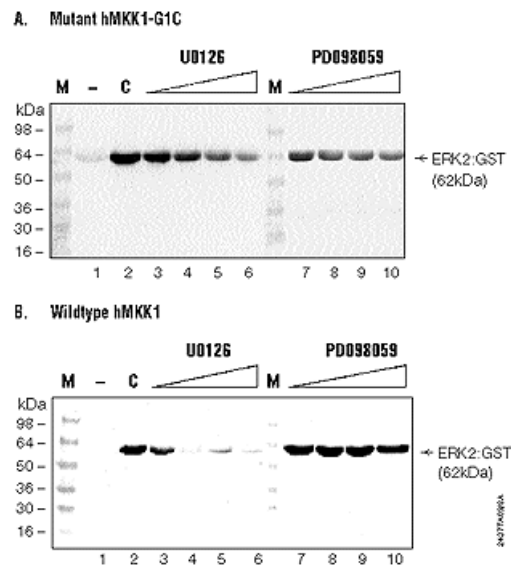


Figure 1. MKK1 inhibition by U0126 or PD098059, measured in vitro. Panel A: Dose response of inhibition of constitutively active mutant hMKK1-G1C (11) by MKK1 inhibitors. Lanes 36: 0.1, 0.2, 0.5 and 1.0 μ M U0126; lanes 710: 2, 5, 10 and 50 μ M PD098059. **Panel B:** Inhibition of Raf-1 kinase-activated wildtype hMKK1 by MKK1 inhibitors. Lanes 36: 10, 25, 50 and 100 μ M U0126; lanes 710: 25, 50, 100 and 400 μ M PD098059. Lanes () are negative controls and lanes C are protein plus no inhibitor in both panels. Assays were performed by measuring phosphate incorporation into ERK2:GST after 30 minutes. Reactions were quenched, resolved by SDS-PAGE and immunoblotted with Anti-ACTIVE[®] MAPK pAb (Cat.# V8031).

By comparison, in vitro studies show that PD98059 inhibits mutant MKK1-G1C and phosphorylated MKK1 with IC₅₀ = 10 μ M and 400 μ M, respectively (by densitometric scanning of the gel images in Figure 1). U0126 and PD098059 have been reported to bind to MKK1 at overlapping sites, both compounds acting as noncompetitive inhibitors of MKK1 (11). Thus, the hypothesized mode of interaction may occur outside of the substrate or ATP binding sites on the kinase. Comparison of binding affinity (K_i) values suggests that the affinity of U0126 for MKK1 exceeds that of PD098059 by at least 20 to 40-fold (11).

INHIBITION OF ERK SIGNALING IN INTACT CELLS

We examined MKK and ERK signaling by transiently transfecting NIH3T3 cells with hemagglutinin-tagged wildtype hMKK1. After overnight starvation, cells were challenged with a combination of 5% fetal calf serum and 50nM PMA for 2 hours, harvested and analyzed by Western blotting. The effect of U0126 on signaling was tested by pre-incubating cells with the inhibitor for 30 minutes prior to addition of serum plus PMA. The activity state of ERK1/2 was examined by probing Western blots with Anti-ACTIVE[®] MAPK pAb (Cat.# V8031), which preferentially reacts with activated ERK1/2 at its regulatory phosphorylation sites ([Figure 2, Panel A](#)). These experiments show that, at 20 μ M U0126, inhibition of ERK phosphorylation in response to serum plus PMA is nearly complete.

Likewise, the activity state of MKK1/2 modified at regulatory phosphorylation sites was examined by probing blots with anti-phospho-MEK1/2 (New England Biolabs), to detect mono- or diphosphorylated MKK1 ([Figure 2, Panel B](#)). Monophosphorylated MKK1 has measurable activity that is approximately 25% that of dually phosphorylated enzyme (13); therefore, this antibody distinguishes between active and inactive forms of MKK. In contrast to the observed inhibition of ERK, U0126 did not inhibit MKK1/2 phosphorylation in response to serum plus PMA, indicating that the compound does not interfere with MKK activation by upstream kinases. In fact, inhibition of ERK by U0126 appears to enhance the activation of overexpressed or endogenous MKK. A similar effect has also been observed upon inhibiting ERK by overexpressing MAP kinase phosphatase-1 (14). The results indicate that U0126 inhibits MKK1/2 in intact cells without interfering with its activation.

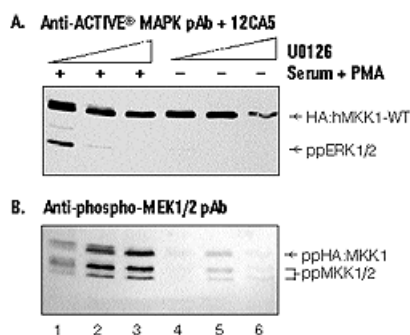


Figure 2. U0126 inhibits ERK1/2 but not MKK1/2 phosphorylation. NIH3T3 cells were transiently transfected with hemagglutinin (HA)-tagged hMKK1-WT for 24 hours, then starved for 18 hours in DMEM without FCS. Starved cells were pre-incubated with 0, 20 or 100 μ M U0126 for 30 minutes, then treated with 5% serum plus 50nM PMA for 2 hours (lanes 13) or left untreated (lanes 46). U0126 was dissolved in DMSO to a stock concentration of 10mM, then diluted directly into culture medium. Extracts were prepared and aliquots examined by Western blotting using primary antibodies. **Panel A:** A mixture of 12CA5 (anti-hemagglutinin) and Anti-ACTIVE[®] MAPK pAb; **Panel B:** Anti-phospho-MEK1/2 antibody (New England Biolabs). Mobility of expressed MKK1, endogenous MKK1/2 and endogenous, dually phosphorylated (pp) ERK1/2 are indicated.

In parallel experiments, NIH3T3 cells were transfected with the constitutively active mutant MKK1-G1C. After overnight starvation, the activity state of ERK was high due to constitutive MKK signaling, and was not increased by challenge with serum plus PMA ([Figure 3](#), lanes 1 and 2). Pretreatment with U0126 for 30 minutes led to ERK dephosphorylation, whether or not cells were stimulated ([Figure 3](#), lanes 3 and 4). The results show that U0126 blocks signaling from the active mutant MKK1 in 30 minutes, consistent with its effects on MKK1 in vitro (Figure 1).

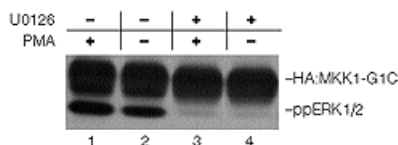


Figure 3. U0126 inhibits constitutively active mutant MKK1 in intact cells. NIH3T3 cells were transiently transfected with hemagglutinin (HA)-tagged hMKK1-G1C (delta N4/S218E/S222D) for 24 hours, then starved for 18 hours. Starved cells were pre-incubated with 0 or 20 μ M U0126 for 30 minutes, then treated with 5% serum plus 50nM PMA for 2 hours (lanes 1 and 3) or left untreated (lanes 2 and 4). Cell extracts were separated by SDS-PAGE and immunoblotted with a mixture of Anti-ACTIVE[®] MAPK pAb (Cat.# V8031) and anti-phospho MEK1/2 pAb (New England Biolabs). Mobility of expressed MKK1 and endogenous dually phosphorylated ERK1/2 are indicated.

The pheochromocytoma (PC12) line is a widely used model for neuronal differentiation, in which activation of the MKK/ERK pathway by nerve growth factor (NGF) or basic fibroblast growth factor (bFGF) causes cell cycle arrest in G1, cytoskeletal rearrangements leading to neurite outgrowth and expression of neuronal markers. Addition of 10 μ M U0126 to PC12 cells for 15 minutes suppresses MKK activity and ERK activation in response to NGF. Under these conditions, activation of ERK1/2 is blocked completely ([Figure 4](#)), similar to the effects of PD098059 previously reported (15). This suggests that U0126 is capable of inhibiting nerve growth factor (NGF)-induced signaling through the MKK/ERK pathway in PC12 cells.

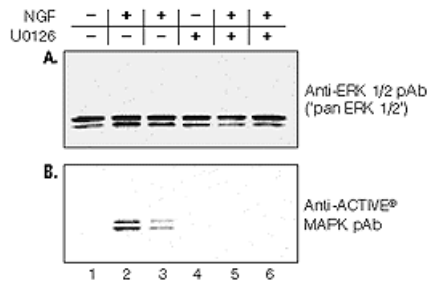


Figure 4. U0126 inhibits NGF-induced activation of ERK1/2 in PC12 cells. PC12 cells were grown in serum-free RPMI medium for 16 hours. The inhibitor U0126 was added to the medium at 10 μ M in 0.2% DMSO for 15 minutes. Nerve growth factor (NGF) was then added at a final concentration of 50ng/ml, and the cells were incubated for either 5 or 30 minutes before harvesting and lysing. Protein samples (20 μ g) were analyzed by SDS-PAGE and Western blot. Detection was performed with Anti-ERK 1/2 pAb (Cat.# V1141) and Anti-ACTIVE[®] MAPK pAb (Cat.# V8031) using a chemiluminescence detection system (Tropix). **Panel A:** The amount of total ERK1/2 shows little change with the various treatments, as detected by the Anti-ERK 1/2 pAb. **Panel B:** Using Anti-ACTIVE[®] MAPK pAb, which recognizes only the active ERK1/2, U0126 completely inhibited the activation of ERK1/2 in NGF-treated PC12 cells at both 5 and 30 minutes. Lane 1, no NGF; lane 2, NGF for 5 minutes; lane 3, NGF for 30 minutes; lane 4, U0126; lane 5, U0126 plus NGF for 5 minutes; lane 6, U0126 plus NGF for 30 minutes.

In response to cell stimulation, a significant fraction of ERK is redistributed to nuclei and retained there for several hours (16-18). This enables transmission of signaling to the nucleus, where an important end result is transcriptional control. Nuclear uptake is strongly correlated with ERK-dependent regulation of DNA synthesis in fibroblasts and neuronal differentiation in PC12 cells (19, 20), and appears to require positive signaling through the MKK/ERK pathway and phosphorylation of ERK (18, 21). The effect of U0126 on nuclear localization of active ERK can be visualized in single cells by indirect immunofluorescence detection with Anti-ACTIVE[®] MAPK pAb. **Figure 5**, Panel A shows enhanced ERK phosphorylation and nuclear uptake in response to stimulation with serum plus PMA. Pretreatment of cells with 20 μ M U0126 30 minutes prior to stimulation blocks ERK activation and nuclear uptake completely (**Figure 5**, Panel C).

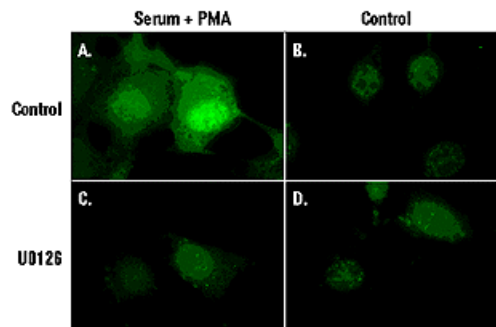


Figure 5. U0126 inhibits nuclear localization of ERK in response to mitogenic stimulation. NIH3T3 cells were grown on coverslips to 50% confluence, then starved for 18 hours, pre-incubated with 0 or 20 μ M U0126 for 30 minutes, then treated with 5% serum plus 50nM PMA for 2 hours or left untreated as indicated. Cells were fixed with 0.1% glutaraldehyde, 2% formaldehyde and permeabilized with methanol prior to incubation with Anti-ACTIVE[®] MAPK pAb* (1:100 dilution) and Texas Red[®]-conjugated donkey anti-rabbit secondary antibody (0.8 μ g/ml). Fluorescence images were viewed and photographed using an AxioPlan[™] (Zeiss[®]) fluorescence microscope with a SenSys[™] (Photometrics) digital CCD camera system.

*Immunostaining was performed using the discontinued Anti-ACTIVE[®] MAPK pAb (Cat.# V6671). Promega's new Anti-ACTIVE[®] MAPK pAb is Cat.# V8031.

Differentiation of blood cells along platelet lineages has also been shown to require MKK/ERK signaling. In several leukemia cell lines, activation of ERK by transfection with active MKK mutants leads to inhibition of proliferation, cell attachment and morphological change to a fibroblast-like shape, and induction of megakaryocyte-specific genes, including integrin $\alpha_{IIb}\beta_3$, a highly selective marker expressed only in megakaryocyte and platelets in humans (2224). The requirement for ERK activation in the differentiation response to PMA (in K562 and CMK cells) or thrombopoietin (in UT7 cells) has been shown by suppressing megakaryocyte morphology and $\alpha_{IIb}\beta_3$ induction by PD098059. U0126 at 20 μ M blocked PMA-induced megakaryocyte differentiation in K562 cells, assayed by its inhibition of cell attachment and spreading (**Figure 6**). This demonstrates the ability of U0126 to interfere with ERK-mediated responses of blood cell differentiation.

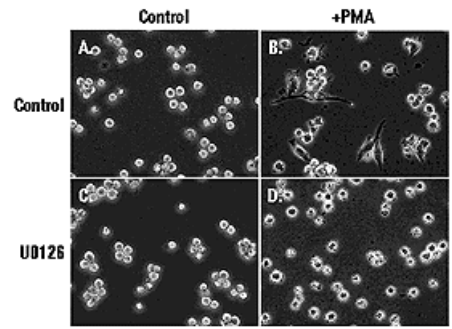


Figure 6. U0126 inhibits PMA-induced megakaryocyte differentiation in K562 cells. K562 cells grown to 5×10^5 cells/ml in 10% FCS/RPMI were pre-incubated with 0 or $20 \mu\text{M}$ U0126 for 30 minutes, then left untreated or treated with 50nM PMA as indicated for 48 hours. Cells were viewed and photographed by phase-contrast microscopy. Characteristic attachment and spreading of cells that accompanies megakaryocyte differentiation in response to PMA (upper right) is blocked by U0126 (lower right).

SUMMARY

Signal transduction through the MKK/ERK pathway regulates a large number of cellular responses; however, the mechanisms by which these enzymes modulate downstream targets are still rather poorly understood. Selective cell-permeable kinase inhibitors provide a useful tool to dissect these mechanisms and distinguish their regulation by different signaling pathways. MEK Inhibitor U0126 has properties in common with the widely used PD098059, sharing the ability to inhibit the MKK/ERK pathway in response to mitogenic stimulation. Unlike PD098059, U0126 exhibits similar potency for both MKK1 and MKK2, higher affinity for MKK binding and enhanced solubility in aqueous solution. In intact cells, U0126 blocks ERK activation at one-tenth the concentration of PD098059, and inhibits MKK activity without interfering with phosphorylation and activation of MKK. The available information comparing inhibition of several protein kinases suggests selectivity for MKK1 and MKK2. However, as with all inhibitors, verification of specificity will require further testing with more enzymes under a wide range of conditions.

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Product	Size	Cat.#
MEK Inhibitor U0126	5mg	V1121

Anti-ACTIVE [®] MAPK pAb, Rabbit, (pTEpY)	40µl	V8031
Anti-ERK 1/2 pAb, Rabbit	40µl	V1141

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