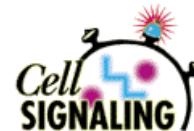


U0126: A Novel, Selective and Potent Inhibitor of MAP Kinase Kinase (MEK)



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Mitogen-activated protein kinases (MAPKs) are involved in a number of cell signaling pathways. These enzymes are activated by dual phosphorylation of threonine and tyrosine residues in response to a multitude of extracellular stimuli. Activation of MAPKs are mediated by another set of enzymes referred to as MAPK kinases or MEKs. In this article, we introduce MEK Inhibitor U0126, a compound that exhibits potent MEK inhibitor activity both in vitro and in vivo.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) have been implicated in a variety of cellular functions, including mitogen-induced cell cycle progression through the G1 phase, regulation of embryonic development and cell and neuronal differentiation (1). These enzymes are proline-directed serine/threonine kinases that are activated by dual phosphorylation of threonine and tyrosine residues in response to diverse extracellular stimuli. There are at least three distinct MAP kinase signal transduction pathways in mammalian cells, each leading to activation of either the extracellular-regulated kinases (ERKs), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) or p38 (Figure 1). Since the budding yeast is known to have more than three distinct MAPK pathways, it is logical to expect that there might be additional MAPK pathways in mammalian cells (1-3).

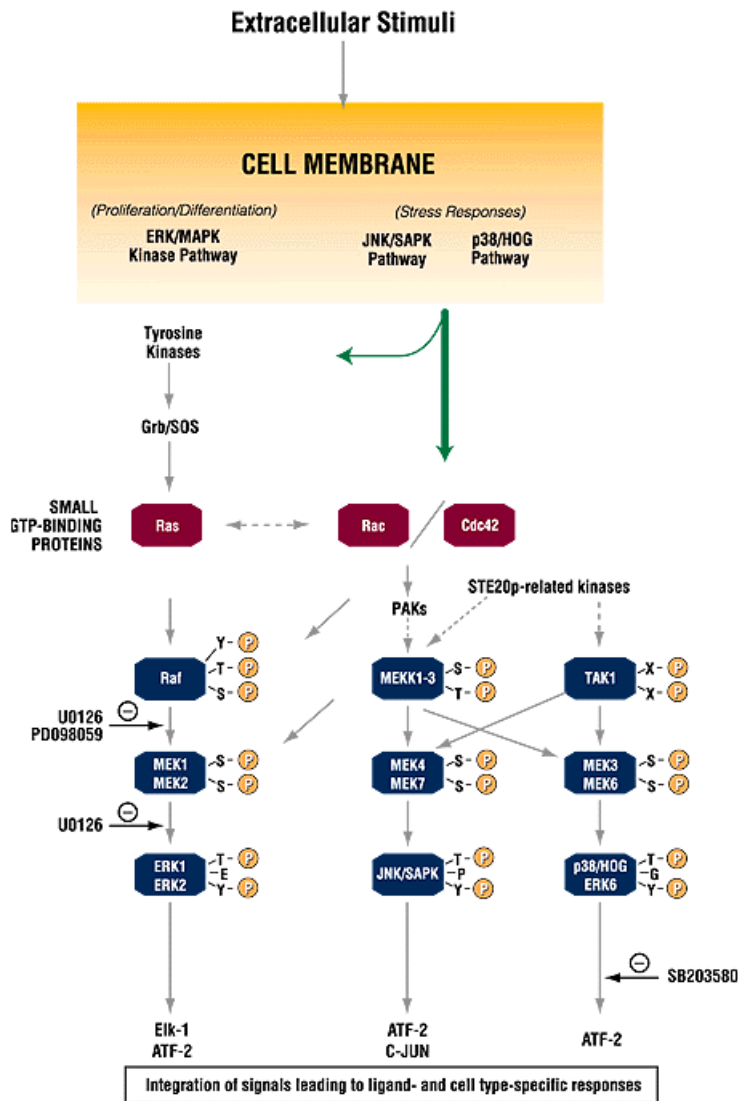


Figure 1. Activation of different ERK/MAPK signaling cascades by different extracellular stimuli. PD098059 acts by inhibiting the activation of MEK1/2 by Raf kinase, while U0126 acts directly upon MEK1/2. Another inhibitor, SB203580, acts by inhibiting the kinase activity of p38.

The three mammalian MAPKs identified to date--ERK, JNK and p38--are phosphorylated on both threonine and tyrosine by dual-specificity MAP kinase kinases (MAPKKs or MEKs), which in turn are activated by serine/threonine phosphorylation by MAP kinase kinase kinase (MEKK). Mammalian ERK1 and ERK2, and their upstream activators MEK1 and MEK2, are acutely stimulated by growth and differentiating factors (e.g., epidermal growth factor, platelet-derived growth factor and nerve growth factor [NGF]) by receptor tyrosine kinases, heterotrimeric G protein-coupled receptors, or cytokine receptors (1,2). The mammalian JNKs and p38 are implicated in responses to cellular stress, inflammation and apoptosis. They are activated by lipopolysaccharides, IL-1, TNF-alpha, ionizing or ultraviolet radiation, the translation inhibitors cycloheximide and anisomycin, tumor promoters, heat shock or hyperosmotic stress.

INHIBITORS AND THE MAP KINASE PATHWAYS

Specific and selective inhibitors have been used to distinguish among the three main MAP kinase pathways and to understand the role of various MAP kinases in cellular regulation by extracellular stimuli. The availability of SB203580, a specific inhibitor of p38, has made it possible to identify effects mediated by or requiring active p38 protein kinase (4). Similarly, the availability of another inhibitor, PD098059, which inhibits MEK1 and MEK2 activation, has been used to study ERK-mediated responses (5). It follows that the addition of the appropriate dose of both SB203580 and PD098059 to inhibit p38 and ERK, respectively, while not perturbing the activity of JNK could be a useful strategy in investigating the MAP kinases.

Recently it was reported that arsenite blocks growth factor-induced activation of the ERK cascades by preventing the guanine nucleotide exchange factor mSos from converting Ras to its active GTP-bound state (6). Such specific and selective inhibitors provide excellent tools for the study of complex signaling pathways.

A NOVEL MEK INHIBITOR

Promega introduces a novel and potent MEK Inhibitor U0126, that inhibits activation of MAPK by MEK *in vitro* and *in vivo* (7,8). U0126 is an organic compound (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) that has been identified as an inhibitor of AP-1 transactivation in cell-based reporter assays (7). It specifically inhibits MEK1 and MEK2 (7) and thus inhibits activation of ERK1 and ERK2. Inhibition is noncompetitive with respect to ERK and ATP substrates (7).

Although PD09859 and U0126 appear to exhibit similar mechanisms for inhibiting MEK1/2, U0126 exhibits a mode of inhibition distinct from that of PD098059. It inhibits the constitutively active MEK mutant deltaN3-S218E/S222D at significantly lower concentrations than PD098059 (IC₅₀ of 0.072μM versus 10μM) (7). However, the inhibition of the wild-type-activated MEK requires greater concentrations for both U0126 and PD098059 (IC₅₀ of 0.53μM and >100μM, respectively). This is due to the large differences in affinities of both compounds for these two activated forms of MEK; the affinity differences between the MEK/ERK complex and free enzyme are 44-fold greater for U0126 and 357-fold greater for PD098059 (7). The affinity of PD098059 for MEK is so weak that concentrations over 400μM are required to inhibit 50% of the wild-type MEK1 activated through Raf-mediated phosphorylation. This indicates that the inhibitory effects of PD098059 are due mainly to its binding directly to the inactive (nonphosphorylated) form of MEK and blocking its activation by Raf-mediated phosphorylation (5,7). MEK Inhibitor U0126, on the other hand, does not affect MEK phosphorylation *in vivo* at concentrations sufficient to inhibit downstream ERK phosphorylation (7). From this, it appears that U0126 inhibits MEK directly by inhibiting the catalytic activity of the active enzyme.

ACTIVITY OF MEK INHIBITOR U0126

To establish the effective concentration of MEK Inhibitor U0126 for inhibiting 50% of ERK2 phosphorylation by MEK1, we performed *in vitro* phosphorylation of an ERK2:GST-fusion protein in the presence of various concentrations of U0126 and detected the dually phosphorylated (active) ERK2 on a Western blot by probing with Promega's newly formulated Anti-ACTIVE™ MAPK pAb (Cat.# V8031). (See the article "New Anti-ACTIVE™ MAPK & 'pan ERK' Antibodies for Western Analysis," which begins on page 9.) When MEK1 was not included in the assay, no phosphorylated ERK2 was detected. However, when MEK1 was added to the reaction with nonactivated ERK2 at 25°C for 30 minutes, the phosphorylated ERK2 fusion protein gave an intense band at 62kDa. The inhibitor U0126 was then tested at various concentrations to determine the IC₅₀. As shown in Figure 2, a concentration of less than 10μM was required to inhibit the wild-type MEK1 phosphorylation of ERK2. This is consistent with data published by Favata *et al.* (7). When we tested PD098059 for comparison with U0126, we found that significantly higher concentrations of PD098059 (400μM) were required to inhibit 50% of wild-type MEK1 activity. These data also support findings by Favata *et al.* (7), which show that the affinity of PD098059 for free MEK1 is significantly less than that for U0126.

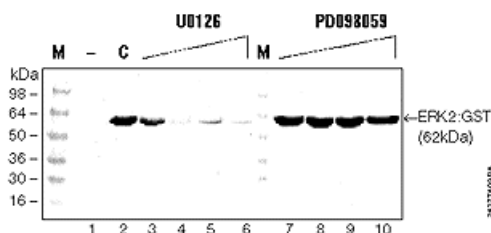


Figure 2. Inhibitory effect of U0126 and PD098059 on *in vitro* activation of ERK2 by MEK1. Various concentrations of each compound in DMSO were incubated with a reaction mixture containing active MEK1, nonactivated ERK2 and buffer components for 3 minutes. The reactions were started with the addition of ATP and incubated for 30 minutes at 25°C then terminated with 50mM EDTA. Samples were analyzed by SDS-PAGE and Western blotting using Anti-ACTIVE™ MAPK pAb (Cat.# V8031). Detection of active ERK1/2 bands was carried out using TROPIX chemiluminescence kit. Lanes M, molecular weight markers; lane 1, no MEK1; lane 2, no MEK Inhibitor U0126; lanes 3-6 contain 10, 25, 50 and 100μM of U0126; lanes 7-10 contain 25, 50, 100 and 400μM of PD098059.

In vivo studies of U0126 inhibition of MEK activity were performed using nerve growth factor-stimulated PC12 cells. Cells were grown in RPMI medium and serum starved for 16 hours. The inhibitor U0126 was then added to the medium at a concentration of 10μM for 15 minutes, followed by the addition of NGF (50ng/ml) for either 5 or 30 minutes. Cells were harvested, lysed and analyzed by SDS-PAGE and Western blot using Anti-ACTIVE™ MAPK pAb and chemiluminescent detection. The results in Figure 3 show activation of ERK1/2 in cells treated with NGF for 5 minutes and diminished activation after 30 minutes. U0126 inhibited the activation of ERK1 and ERK2 at both 5 and 30 minutes, where no signal of activated ERK1/2 was observed. The amount of total ERK1/2 remains the same regardless of treatment, as shown by the use of Promega's Anti-ERK 1/2 pAb (Cat.# V1141), which recognizes total ERK1 and ERK2. This confirms that U0126 is an effective inhibitor of ERK activation by MEK *in vivo*, as shown previously with other cell types (7,8).

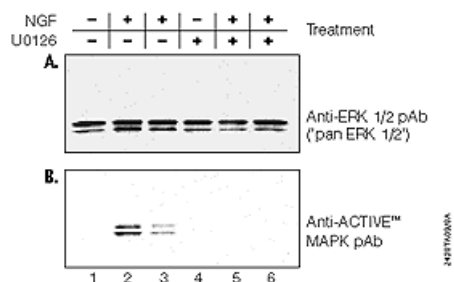


Figure 3. Inhibitory effect of U0126 on ERK1/2 signaling cascade in the presence of NGF. PC12 cells were grown in serum-free RPMI medium for 16 hours. The inhibitor U0126 was added to the medium at 10 μ M in 0.2% DMSO for 15 minutes. Nerve growth factor (NGF) was then added at a final concentration of 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-ACTIVETM MAPK pAb (Cat.# V8031) using the TROPIX chemiluminescence detection system. **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-ACTIVETM MAPK pAb, which recognizes only the active ERK1/2, it is shown that U0126 completely inhibits 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 + NGF for 5 minutes; lane 6, U0126 + NGF for 30 minutes.

CONCLUSIONS

In summary, MEK Inhibitor U0126 is a selective and potent inhibitor of MEK1 activity *in vitro*, as well as activation of ERK1/2 by MEK1 and 2 *in vivo*. The compound appears to inhibit MEK1 directly but with higher potency towards the constitutive mutant than the wild-type enzyme. In contrast, PD098059 inhibits MEK1/2 activation by binding to the inactive enzyme preventing its activation by Raf kinase, indirectly inhibiting activation of ERK1/2. Because of this, PD098059 may not be useful for inhibiting endogenously active MEK1/2, while U0126 is capable of directly inhibiting activated MEK1 and preventing endogenously active MEK1/2 from phosphorylating and activating ERK1/2. Therefore, the compound U0126 acts one step further downstream of PD098059 and blocks MEK1/2 activity. This inhibitor will serve as a useful tool to investigate the effect of various signals on the activation of MAPK in cells and further delineation of the MAPK pathways in cellular regulation.

ACKNOWLEDGEMENTS

The authors greatly appreciate the generous support of Dr. James M. Trzaskos of Du Pont Pharmaceuticals during all phases of this project.

REFERENCES

1. Lewis, T.S., Shapiro, P.S. and Ahn, N.G. (1998) *Adv. Cancer Res.* **74**, 49.
2. Robinson, M.J. and Cobb, M.H. (1997) *Curr. Opin. Cell Biol.* **9**, 180.
3. Ip, Y.T. and Davis, R.J. (1998) *Curr. Opin. Cell Biol.* **10**, 205.
4. Cuenda, A. *et al.* (1995) *FEBS Lett.* **364**, 229.
5. Dudley, D.T. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* **92**, 7686.
6. Doza, Y.N., Hall-Jackson, C.A. and Cohen, P. (1998) *Oncogene* **17**, 19.
7. Favata, M. *et al.* (1998) *J. Biol. Chem.* **273**, 18623.
8. DeSilva, D.R. *et al.* (1998) *J. Immunol.* **160**, 4175.

Ordering Information

Product	Size	Cat.#
MEK Inhibitor U0126	5mg	V1121
Anti-ACTIVE TM MAPK pAb, Rabbit (pTEpY)	40 μ l	V8031
Anti-ERK 1/2 pAb	40 μ l	V1141

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