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# Identification of benzimidazole-based inhibitors of the mitogen activated kinase-5 signaling pathway

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ABSTRACT

The MEK-signaling pathways are complex but critical signaling cascades that correlate an extracellular signaling event with internal cell processes. To date at least seven MEK isozymes have been identified. MEK5, in particular, is upregulated in multiple forms of tumors. Analysis of the EGF-induced MEK5 signaling cascade in cultured HEK cells has identified compounds that can inhibit MEK5 phosphorylation of ERK5; observed biological activity is dependent on chemical variation.



The mitogen activated protein kinase (MAPK) pathways are a family of related, and often interconnected, signaling pathways that relay input from extracellular origins and interpret the resultant outcome in the context of the current biological milieu.[1–8] There have been multiple reviews[1,3,9] and several critical advances regarding the categorization,[10,11] the intrinsic biochemistry,[12,13] and the relevance to human disease states[9,14,15] displayed by various members of the MAPK family.

The sequence of signaling typically originates with ligand binding or extracellular stressor followed by one of three transduction mechanisms;[3,16] receptor tyrosine kinase activation, G-protein coupled receptor activation, or hormone receptor transduction. The MAPK pathways exist as a series of phosphorylation events where one kinase (mitogen-activated kinase kinase kinase: MEKK) phosphorylates a second kinase (mitogen-activated kinase kinase: MEK), which then phosphorylates a third kinase (extracellular signal-regulated kinase: ERK). This third kinase, or ERK, is typically translocated to effect modification of cellular function in a specific subcellular compartment.[17] Multiple phosphorylation states of ERKs have been demonstrated and may direct subcellular trafficking and substrate preference for phospho-ERK[15,17] (Fig. 1).

MEKs uniquely display a high degree of substrate specificity. This selectively is derived from multiple interactions of a MEK's D domain with a unique complementary CD domain on the substrate ERK, association with scaffolding proteins,[13,18] association

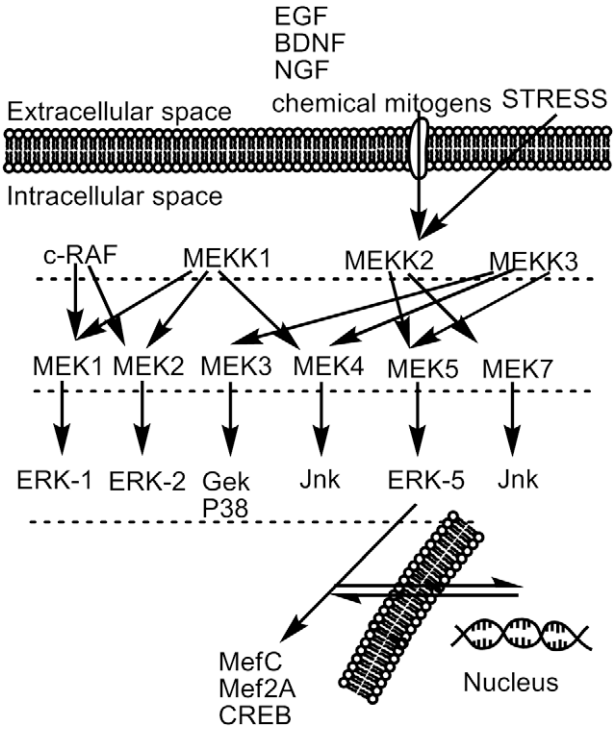


**Figure 1.** The MEK5 signaling cascade.

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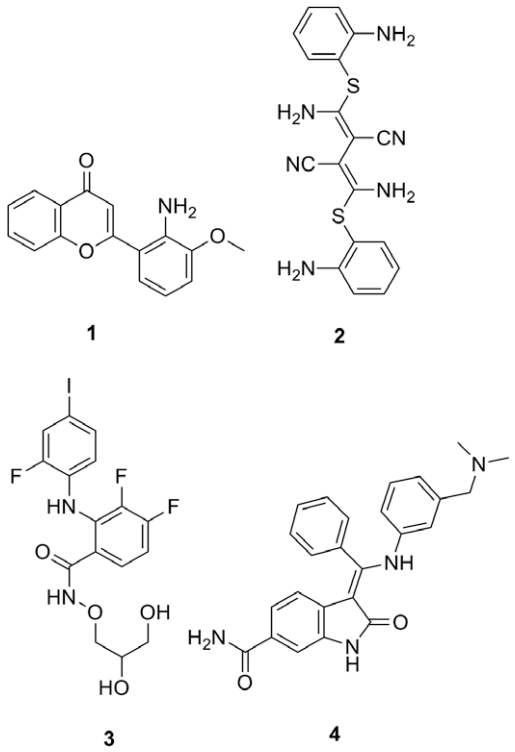



**Figure 2.** Examples of known inhibitors of ERK phosphorylation.

with other kinases, and subcellular localization.[17] MEK5 has been demonstrated to uniquely phosphorylate ERK5.[19]

Activators of signaling cascades utilizing MEK5 mediated phosphorylation of ERK5 include nerve growth factor (NGF), endothelial growth factor (EGF), brain-derived neurotrophic growth factor (BDNF), oxidative stress, ionizing radiation, or the phorbol esters. Substrates for ERK5 include Sap1a, c-Myc, and possibly RSK. ERK5 can also induce MEF2, PPAR$\gamma$1, c-Fos, and c-Jun[15,17] activation. ERK5 has a role during the G1/S cell-cycle transition for EGF-induced cell proliferation through a cAMP response element (CRE) mediated expression of cyclin D1.[20] Additionally, the MEK5/ERK5 pathway has been shown to be significantly upregulated in squamous cell carcinoma,[21] prostrate,[22] and breast[23] cancers. Although MEK5 is clearly involved in maintenance of cardiovascular tissue, has a role in neural plasticity, and displayed a lethal cardiovascular phenotype in MEK5 knock-out mice,[17] three recent studies examining the role of ERK5 in breast cancer,[23] leukemia cells,[24] and unique activation by palytoxin or the phorbol esters[25] has prompted a re-examination of the potential role of MEK5 as an anti-cancer therapeutic target.

The majority of inhibitor studies on the MEKs have focused on MEK1/2 however there is a single paper detailing the identification of a MEK5 inhibitor.[26] Studies examining the ability of compounds to block in vivo EGF-induced ERK1/2 phosphorylation analyzed by western blotting identified flavone **1**, PD98059,[17,19] as a non-competitive ATP site inhibitor of ERK1/2, and 5. The conjugated nitrile **2**, U0126,[27] blocked phorbol-ester stimulated ERK1/2 phosphorylation. Examination of various diphenylanalines resulted in the identification of **3**, PD318088,[28,29] an inhibitor of MEK1/2, and 5. Analysis of an X-ray crystal structure of PD318088, **3**, bound to MEK1 (PDB ID: 1S9J) confirms that **3** binds to an allosteric site on MEK1.[5,9,11] A recent survey of oxindole kinase inhibitors identified BIX02188, **4** as a selective inhibitor of MEK5.[26] We sought to identify new structural classes of inhibitors that could disrupt the MEK-signaling cascade, specifically MEK5 phosphorylation of ERK5 (Fig. 2).

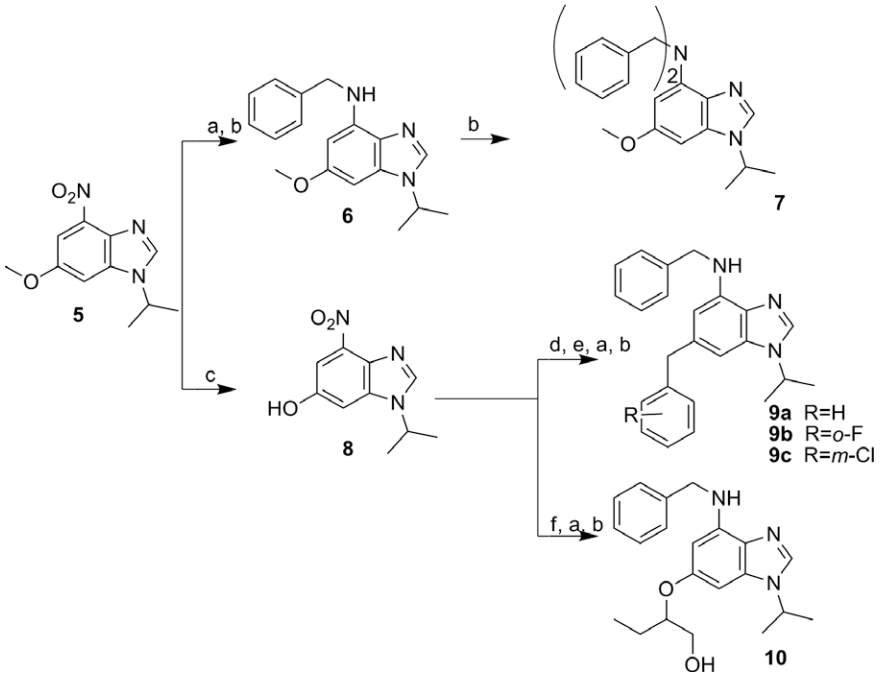
We recently presented a series of novel benzamidazoles designed as CDK5 inhibitors.[30] Given that both CDK5 and MEK5 are serine/threonine kinases, that similarities between the CDK and MEK active sites are mentioned in the literature,[27] and that **2** and **4** explore a chemical scaffold closely associated with CDK2 inhibition, screening a select group of our substituted benzimidazoles for MEK5 inhibitory properties followed logically. Compounds were screened for the ability to block EGF-initiated MEK5 mediated ERK5 phosphorylation in human embryonic kidney cells (HEK293) analyzed by Western blot analysis.

Beginning with the tri-functional benzimidazole scaffolds,[31] **5**, **10**, and **15**, chemical variation at the 1-nitrogen, 4-nitrogen, or 6-carbon permitted generation of hetero or carbon-linked analogs as presented in Schemes 1–3. Variation at the 1-nitrogen was found to be accessible via N-alkylation with benzyl bromide (Scheme 3), but in the case of bulky aliphatic groups, reductive alkylation at the most nucleophilic nitrogen then cyclization was found to be the best strategy.[31] We envisioned the 4-nitro group as a protective group that could be converted to an amine when required. The 6-methoxy group was conveniently converted to the corresponding 6-akoxy group via acidic demethylation then Mitsunobu coupling. The intermediate phenol **7** or **12** could be converted to the triflate[33] then coupled with a benzylic tetrafluoroborate using a Molander–Suzuki coupling.[32] For the initial screening of compounds we elected to examine compounds which possessed properties that were synthetically tractable, capable of additional SAR elaboration, and consistent with prior understanding of kinase inhibitors.[34,35] Additionally we sought to rapidly identify which complementary variations were capable of increasing potency and perhaps selectivity.

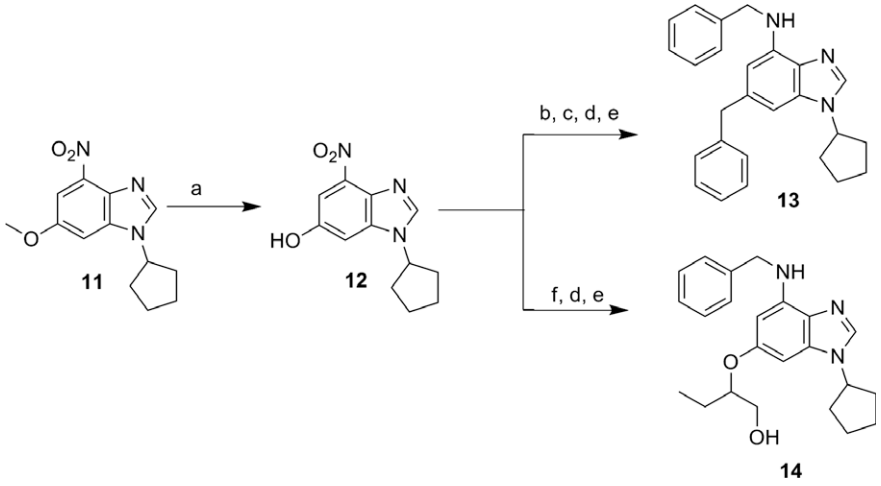
For all compounds examined reduction with Pd/C and hydrogen followed by reductive alkylation gave the desired 4-*N*-benzyl products.[36] Monobenzylation was selectively achieved by careful stoichiometry of benzaldehyde and the avoidance of acid to limit dibenzylation. Demethylation to **8** or **12** required vigorous reaction conditions: 48% HBr heated to 120 °C under microwave irritation. Standard Mitsunobu coupling with the known 1-OTBS butane-1,2-diol[37] gave the desired ethers but required addition of the DIAD generated electrophilic complex in two portions. Alternately, triflate formation then Molander–Suzuki coupling gave the carbon-linked 6-C variants. Regardless of the nature of the 6-substitution, subsequent reduction then reductive alkylation was straight forward.

HEK293 cells were treated with 50 ng/mL of EGF to activate the MEK/ERK pathway for 30 min then each compound was added to a final concentration of 10 μM. Activation of ERK1/2 or MEK5 was examined by western blot analysis for phosphorylated (i.e., activated) ERK5.[38] Of the nine compounds tested, compound **6** significantly inhibited both ERK1/2 and ERK5 phosphorylation induced by EGF treatment (Fig. 3 and Table 1).

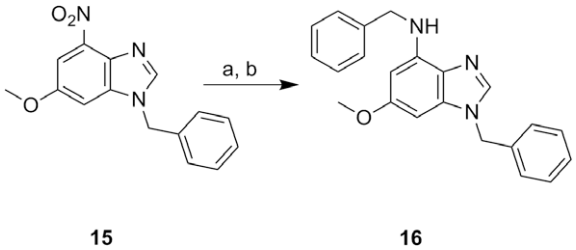
Substitution at the 1-ntirogen included benzyl, isopropyl, and cyclopentyl variations. These groups were selected based on prior observation[39,40] that significant hydrophobic interaction can occur at a common kinase aromatic binding region proximal to the H-bond donor/acceptor pair represented respectively by the 4-nitrogen proton and the 3-*N* benzimidazole nitrogen lone pair. Of these initial variations examined, isopropyl appeared to contribute toward inhibition of ERK5 phosphorylation evidenced by **6**, **9b**, and **9c**. This contribution was dependent on substitution at both the 4-amine, and the 6-carbon. Interestingly, compounds **6** and **14** possessing the hydroxybutyl ether at the 6-carbon were inactive in preventing EGF-induced ERK phosphorylation. This result stands in stark contrast to our observations regarding the role of this group in potentiating CDK5 inhibition for this benzimidazole scaffold.[30] This suggests very different structural requirements for activity between these two enzymes.




**Scheme 1.** Reagents and conditions: (a) 50 psi $H_2$, EtOH, 5% Pd/C, 23 °C, 5 h, 80–95%; (b) benzaldehyde (1.1 equiv), $NaHB(OAc)_3$ (2.0 equiv), DCE, 23 °C, 12 h, 30–60%; (c) 48% HBr, microwave irradiation, 120 °C, 2.5 h, 97%; (d) PNP-OTf, DMF, 23 °C, 2 h; 87%; (e) potassium benzyltrifluoroborate, $CsCO_3$, $PdCl_2(dppf)$, $H_2O$/THF, 110 °C, 2 h, 80–99%; (f) 1-(TBS)butan-2-ol (2.6 equiv), $PPh_3$ (2.6 equiv), DIAD (2.6 equiv), DMF, 0–23 °C, 12 h 65%.




**Scheme 2.** Reagents and conditions: (a) 48% HBr, microwave irradiation, 120 °C, 2.5 h, 97%; (b) PNP-OTf, DMF, 23 °C, 2 h; 86%; (c) potassium benzyltrifluoroborate, $CsCO_3$, $PdCl_2(dppf)$, $H_2O$/THF, 110 °C, 2 h, 17%; (d) 50 psi $H_2$, EtOH, 5% Pd/C, 23 °C, 5 h, 80–95%; (e) benzaldehyde (1.1 equiv), $NaHB(OAc)_3$ (2.0 equiv), DCE, 110 °C, 2 h, 30–60%; (f) 1-(TBS)butan-2-ol (2.6 equiv), $PPh_3$ (2.6 equiv), DIAD (2.6 equiv), DMF, 0–23 °C, 12 h, 50%.




**Scheme 3.** Reagents and conditions: (a) 50 psi $H_2$, EtOH, 5% Pd/C, 5 h, (b) benzaldehyde (1.1 equiv), $NaHB(OAc)_3$ (2.0 equiv), DCE, 60% (two steps).

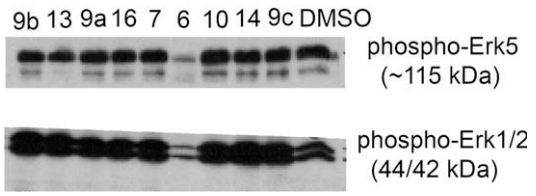



**Figure 3.** Western Blot analysis of EGF-mediated ERK phosphorylation; antibodies utilized detect all phospho-ERK forms, ERK isofoms are separated with SDS–PAGE on the basis of molecular weight.

**Table 1**
Inhibition of EGF-induced ERK phosphorylation in HEK 293 cells

| Compound | *p*-ERK1/2 Relative phsophorylation (%)[a] | *p*-ERK5 Relative phsophorylation (%)[a] |
|---|---|---|
| **6** | 35.98 | 12.35 |
| **7** | 260.57 | 110.05 |
| **9a** | 461.55 | 129.49 |
| **9b** | 309.67 | 70.84 |
| **9c** | 346.25 | 96.27 |
| **10** | 331.95 | 135.22 |
| **13** | 521.18 | 78.00 |
| **14** | 609.86 | 131.53 |
| **16** | 436.18 | 135.59 |
| DMSO | 100 | 100 |

[a] Values are means of duplicate experiments, standard deviation is ±10%.

Of the compounds examined, the relatively simple compound **6** was shown to display inhibition of EGF-induced phosphorylation of ERK. Some modest selectivity was observed for the preferential inhibition of ERK5 phosphorylation relative to ERK1/2 phosphorylation. Interestingly, compound **6** was unique among the compounds examined in that no upregulation of ERK1/2 phosphorylation was observed. This compound was submitted for the NCI 60-cell line screen and was shown to selectively inhibit the growth of MCF-7 cells in the single dose survey. However, compound **6** was not selected for the subsequent log dose–response analysis. It has been noted that the MCF-7 cell line displays unique properties regarding cytosolic effects of ERK5 activity.[17]

Further studies are under way to analyze the biological contribution of and structure variations from compound **6** for inhibition of EGF-mediated ERK5 phosphorylation.

## Acknowledgments

Ms. Darlene Monlish, NIH (NINDS): 1R15NS057772, NIA: AG025848, and NSF equipment grant: NMR: CHE 0614785.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.033.

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36. *1-Isopropyl-6-methoxy-4-nitro-1H-benzo[d]imidazole* (***5***).[31] A well stirred mixture of 5-methoxy-3-nitrobenzene-1,2-diamine (5.0 g, 27.3 mmol), $NaHB(OAc)_3$ (17.36 g, 81.9 mmol), and 110 mL of THF was cooled to an internal temperature of 0 °C. Formic acid (3.77 g, 81.9 mmol) was added with dropwise addition maintaining the internal temperature below 10 °C. After stirring an additional 15 min at 0 °C, acetone (7.93 g, 137 mmol, 5 equiv) was added in one portion. The mixture was stirred overnight. The solvent was removed in vacuo, and the dark red residue was dissolved in formic acid (31 mL, 0.85 mol). Butylated hydroxytoluene, BHT, (20 mg, 0.09 mmol) was added, and the mixture was cooled to 0 °C. Concd HCl (87 mL, 0.95 mol) was added, and the mixture was brought directly to reflux. After maintaining reflux for 15 min, the solvent was removed in vacuo at 80 °C. The residue was adjusted to a pH of 8 with 50% aqueous NaOH then extracted with EA (4 × 50 mL). The combined EA extracts were washed (3 × 10 mL brine) and dried ($Na_2SO_4$). Evaporation of the solvent gave a brown solid, which after silica gel column chromatography (hexanes/EtOAc 1:1) afforded 4.5 g (74%) of the desired product as a yellow solid. $R_f$ 0.32 ($CH_2Cl_2$/MeOH/$NH_4OH$ 100:10:0.1). mp 127.2–128.1 °C. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta$ 8.12 (s, 1H), 7.21 (d, $J$ = 2.3 Hz, 1H), 7.79 (d, $J$ = 2.3 Hz, 1H), 4.58–4.65 (m, 1H), 3.95 (s, 3H), 1.65 (d, $J$ = 6.8 Hz, 6H). Anal. Calcd for $C_{11}H_{13}N_3O_3$: C, 56.16; H, 5.57; N, 17.86. Found: C, 56.45; H, 5.62; N, 17.55.
*1-Isopropyl-6-methoxy-1H-benzo[d]imidazol-4-aminium chloride*. 1-Isopropyl-6-methoxy-4-nitro-1*H*-benzo[*d*]imidazole (833 mg, 3.54 mmol) was dissolved in EtOH (42 mL) and $CHCl_3$ (8 mL) containing 10% Pd/C (150 mg). The hydrogenation flask was evacuated and backfilled with $H_2$ three times, and then shaken under 55 PSI of $H_2$ atmosphere for 12 h at 23 °C. The mixture was filtered through Celite and the Celite pad was washed with 20 mL additional EtOH. Evaporation of the solvent gave 841 mg (98%) of the salt as a light green solid. This material was used directly in the next step.
*N-Benzyl-1-isopropyl-6-methoxy-1H-benzo[d]imidazol-4-amine* (**6**) $NaHB(OAc)_3$ (608 mg, 2.87 mmol, 1 equiv) was added to a solution of 1-isopropyl-6-methoxy-1*H*-benzo[*d*]imidazol-4-aminium chloride (155a, 832 mg, 2.87 mmol) in 1,2-dichloroethane (10 mL) at 23 °C. After stirring for 2 min, benzaldehyde (457 mg, 4.31 mmol) was added. After stirring for 1 min, a second amount of $NaHB(OAc)_3$ (914 mg, 4.31 mmol) was added and stirring was continued for an additional 12 h. Saturated aqueous 8 mL of $NaHCO_{3\ (satd,\ aq)}$ was added. The mixture was stirred until bubbling was stopped and extracted with $Et_2O$ (10 mL × 3). The combined extracts were washed with $NaCl_{(satd,\ aq)}$ and then dried over $Na_2SO_4$. Evaporation of the solvent gave a brown solid, which was subjected to silica gel column chromatography (hexanes/EtOAc 2:1 to 1:2, 0.5% $Et_3N$) to afford 790 mg (93%) of the product as a white solid. $R_f$ 0.58 ($CH_2Cl_2$/MeOH/$NH_4OH$ 100:10:0.1). mp 81.8 – 83.9 °C. $^1H$ NMR (400 MHz, $CDCl_3$): $\delta$ 7.72 (s, 1H), 7.25–7.42 (m, 5H), 6.20 (d, $J$ = 2.1 Hz, 1H), 6.03 (d, $J$ = 2.1 Hz, 1H), 5.29 (t, $J$ = 5.4 Hz, 1H), 4.47–4.53 (m, 1H), 3.80 (s, 3H), 1.59 (d, $J$ = 6.8 Hz, 6H). Anal. Calcd for $C_{18}H_{21}N_3O$: C, 73.19; H, 7.17; N, 14.23. Found: C, 73.33; H, 7.03; N, 14.24.
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38. (a) HEK293 (ATCC, Manassas, VA) cells were grown on 60 mm culture plates (Sarstedt, Newton, NC) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, CA) with 10% heat-inactivated FBS (Atlanta Biological, Lawrenceville, GA), and 0.5% penicillin/streptomycin (Gibco, Carlsbad, CA). Cells were

maintained at 37 °C with 5% $CO_2$. The cells were treated with 50 ng/mL epidermal growth factor (EGF; Sigma) 30 min before treatment with the putative MEK5 inhibitors (10 μM of each). Cells were lysed 30 min following treatment with the inhibitors. The cells were washed with 1x PBS and then lysed in 1% Triton X-100 buffer containing 20 mM Tris (pH 6.8), 137 mM NaCl, 25 mM beta glycerophosphate, 2 mM NaPPi, 2 mM EDTA, 1 mM $Na_3$ $VO_4$, 10% glycerol, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 2 mM benzamidine, 0.5 mM DTT, and 1 mM PMSF. The lysates were then centrifuged at 10,000 rpm for 10 min at 4 °C. Determination of protein in the supernatant was done using a Bradford assay (Biorad, Hercules, CA).; (b) Western Blot Analysis. Equal amounts of protein (60 μg) from each treatment were separated on 10% SDS gels and transferred to PVDF membrane [Millipore, Danvers, MA] for Western blot analysis. Blots were blocked in 5% nonfat milk in 1x TBS/0.1% Tween/0.02% $NaN_3$ for 1 h and then incubated overnight at 4 °C in primary antibody (1:1000 pERK1/2, 1:500 pERK5, Cell Signaling). Blots were washed with 1x TBS/0.1% Tween for 30 min and then incubated with secondary antibody (1:1000, horseradish peroxidase-conjugated goat anti-rabbit, Upstate). Proteins were visualized with enhanced chemiluminescence (Upstate). Films were scanned and quantified using MATLAB, v.7.1 (Mathworks, Natick, MA).

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