

ERK5 kinase activity is dispensable for cellular immune response and proliferation

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Unlike other members of the MAPK family, ERK5 contains a large C-terminal domain with transcriptional activation capability in addition to an N-terminal canonical kinase domain. Genetic deletion of ERK5 is embryonic lethal, and tissue-restricted deletions have profound effects on erythroid development, cardiac function, and neurogenesis. In addition, depletion of ERK5 is antiinflammatory and antitumorigenic. Small molecule inhibition of ERK5 has been shown to have promising activity in cell and animal models of inflammation and oncology. Here we report the synthesis and biological characterization of potent, selective ERK5 inhibitors. In contrast to both genetic depletion/deletion of ERK5 and inhibition with previously reported compounds, inhibition of the kinase with the most selective of the new inhibitors had no antiinflammatory or antiproliferative activity. The source of efficacy in previously reported ERK5 inhibitors is shown to be off-target activity on bromodomains, conserved protein modules involved in recognition of acetyl-lysine residues during transcriptional processes. It is likely that phenotypes reported from genetic deletion or depletion of ERK5 arise from removal of a noncatalytic function of ERK5. The newly reported inhibitors should be useful in determining which of the many reported phenotypes are due to kinase activity and delineate which can be pharmacologically targeted.

ERK5 | bromodomain | inflammation | proliferation | kinase

Extracellular signal-regulated kinase 5 (ERK5, BMK1) is a member of the mitogen-activated protein kinase (MAPK) family, which includes ERK1/2, JNK1/2/3, and p38 α / β / δ / γ (1). However, unlike the other MAPK members, ERK5 contains a unique 400-amino-acid C-terminal domain in addition to the kinase domain. Through the MAPK signaling cascade, mitogen-activated protein kinase kinase 5 (MEK5) activates ERK5 by phosphorylating the TEY motif in the N-terminal activation loop (2). This event unlocks the N- and C-terminal halves, allowing ERK5 to auto-phosphorylate multiple sites in its C-terminal region, which can then regulate nuclear shuttling and gene transcription (3, 4). Noncanonical pathways (including cyclin-dependent kinases during mitosis and ERK1/2 during growth factor stimulation) also exist for phosphorylation of sites in the ERK5 tail (5–7). Although ERK5 has been demonstrated to directly phosphorylate transcription factors (8–10), the noncatalytic C-terminal tail of ERK5 can also interact with transcription factors and influence gene expression (4, 11, 12).

ERK5 can be activated in response to a range of mitogenic stimuli [e.g., growth factors, G protein-coupled receptor (GPCR) agonists, cytokines] and cellular stresses (e.g., hypoxia, shear stress) (13). Like most kinases including MAPK members, ERK5 function is assumed to be driven by its kinase activity. ERK5 deletion is embryonic lethal in mice and a variety of tissue- or development-stage restricted KOs have shown clear phenotypes, suggesting that the catalytic function and/or an aspect of the nonkinase domain(s) have key roles in development and mature organ function (14–18). The availability of the first ERK5 inhibitor XMD8-92 enabled the study of phenotypes resulting from direct kinase inhibition (19, 20). Effects of this inhibitor on cell proliferation were shown to be comparable to overexpression of a

dominant negative ERK5 mutant (20, 21) or to siRNA-mediated ERK5 knockdown (21, 22), implicating a key role of the kinase function. Multiple reports have shown promising and corroborating effects of ERK5 knockdown and pharmacological inhibition in controlling inflammation and tumor growth (20, 22–26).

Given the proposed therapeutic uses for ERK5 inhibitors, we set out to develop improved compounds with high selectivity and potency. With these ERK5 compounds, we show that first-generation ERK5 inhibitors actually derived the bulk of their biological activity from off-target activity on bromodomains (BRDs). Surprisingly, selective inhibition of the kinase activity alone had no effect on cellular immune response or proliferation, in contrast to whole ERK5 protein knockdown. The newly developed ERK selective inhibitors should prove useful in determining which phenotypes derive from catalytic activity and which derive from other functions of this multidomain kinase.

Results and Discussion

Inhibitors of ERK5. We synthesized derivatives of the benzopyrimidodiazepinone XMD8-92, a first-generation ERK5 kinase inhibitor (Fig. 1). Among these compounds were ATP-competitive inhibitors that potently inhibit ERK5 with IC₅₀ values ranging from 8 to 190 nM using the chemoproteomics platform KiNativ, which profiles global kinase inhibition in complex lysates (27, 28). At 1- μ M screening concentration, there was no significant inhibition of any off-target kinases among the greater than 100 kinases

Significance

Whole protein deletion and pharmacological inhibition are frequently used to functionally annotate enzymes. Each has limitations: whole protein deletion removes both enzymatic and nonenzymatic functions, and small molecule inhibitors can have unrecognized off-target activities. When both approaches agree, it's nearly incontrovertible support for protein function. Here we describe a counterexample. ERK5 knockdown and inhibition supported a role for this kinase in a number of biological processes. We show that previously reported ERK5 compounds inhibit bromodomain-containing proteins (BRDs) sufficiently to account for their phenotypic effects. We describe highly specific inhibitors of ERK5 that do not inhibit BRDs. With these, we show that cellular inflammation and proliferation are not dependent on ERK5 catalytic activity, thus making ERK5 unique among the MAP kinases.

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Conflict of interest statement: E.C.K.L., C.M.A., T.K.N., H.W., Y.H., S.S., B.L., J.W.K., and J.S.R., employees of ActivX Biosciences, a wholly owned subsidiary of Kyorin Pharmaceutical Co., Ltd., and J.L., an employee of Kyorin Pharmaceutical Co., Ltd., have commercial interests in the development of ERK5 and BRD inhibitors.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE86577).

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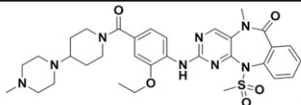
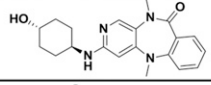
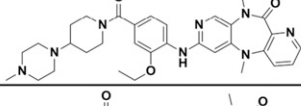
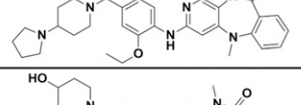
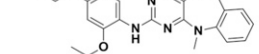
Compound	Structure	Lysate ERK5 IC ₅₀ (nM)	Intra-cellular ERK5 IC ₅₀ (nM)
AX15836		8	9
AX15839		170	430
AX15892		30	110
AX15910		20	17
XMD8-92		190	130

Fig. 1. Compound structures and potencies against ERK5 in cell lysate and in live cells treated with compound.

profiled in a single cellular lysate, indicating excellent specificity (Fig. S1).

The ERK5 inhibitors were also evaluated in an additional KiNativ experimental format wherein compound is incubated with live cells before washing, lysis, and analysis. Such experiments provide an indication of the cellular permeability of the compound and of intracellular target (and off-target) engagement. As seen in Fig. 1, intracellular ERK5 IC₅₀ values were very similar to lysate ERK5 IC₅₀ values, demonstrating that the compounds effectively reached their intracellular target. The most selective ERK5 inhibitor AX15836 was also profiled using live cell KiNativ across several cell types, including peripheral blood mononuclear cells (PBMCs), endothelial cells, and oncogenic cell lines, and verified to maintain its intracellular potency (4–9 nM) across all cells tested.

The compounds were next tested for their ability to inhibit epidermal growth factor (EGF)-mediated auto-phosphorylation of endogenous ERK5 in HeLa cells (4). HeLa cells are commonly used to study ERK5 regulation in part due to the ability to clearly observe ERK5 activation resulting from triggers such as growth factors and mitosis (5, 29, 30). In this assay, activated ERK5 migrates more slowly than unactivated ERK5 on SDS/PAGE by virtue of the protein being phosphorylated. As seen in Fig. 2, when treated at 2 μM, approximately fivefold over the weakest intracellular ERK5 IC₅₀, all of the ERK5 inhibitors substantially blocked the formation of phosphorylated ERK5 on EGF stimulation. Because ERK5 is an intracellular target, this provided additional evidence, together with the KiNativ results, that the compounds were able to effectively engage their intracellular target.

Inhibitor Characterization in Cellular Inflammation Models. ERK5 has been recently studied as a target for mediating inflammation (23, 24, 26). We determined the activity of the ERK5 compounds in cellular assays of inflammatory response. To function in the recruitment of neutrophils and monocytes, the endothelial cell adhesion molecule E-selectin is rapidly synthesized in response to inflammatory stimulation (31). Primary human umbilical vein endothelial cells (HUVECs) were incubated with compounds before stimulation with the Toll-like receptor (TLR1/2) agonist Pam₃CSK₄. Up-regulation of cell-surface E-selectin was quantified by flow cytometry (Table 1). Similar to that reported by Wilhelmssen and colleagues (23), we found the ERK5 inhibitor

XMD8-92 to inhibit up to 38% of the E-selectin expression. Likewise, two other ERK5 inhibitors, AX15839 and AX15910, were effective in reducing E-selectin. Surprisingly, two of the more potent ERK5 inhibitors (AX15836 and AX15892) were inactive in this assay, even at a concentration of compound at least 90-fold higher than the intracellular ERK5 IC₅₀ (Fig. 1).

Given these results, it seemed likely that an additional activity was responsible for the efficacy of AX15839, AX15910, and XMD8-92. We therefore performed a more extensive KiNativ experiment to expand the kinase coverage to more than 200 kinases and furthermore increased the compound screening concentration to 10 μM. However, we still did not find any significant shared inhibition of off-target kinases among these three inhibitors (Fig. S2). XMD8-92 was derived from the polo-like kinase (PLK1) inhibitor BI-2536 (19, 20). Recently, BI-2536 (as well as a number of other kinase inhibitors) has been shown to inhibit the interaction between BRD and acetyl-lysine binding (32–34). BRDs are protein modules that bind to ε-N-acetylated lysine-containing sequences and modulate transcriptional processes. Members of the dual BRD-containing BET (bromo and extra terminal) proteins BRD2, BRD3, BRD4, and BRDT are targets of drugs currently pursued in oncology, neurological diseases, diabetes, atherosclerosis, and inflammation (35, 36). To determine whether XMD8-92 and other ERK5 kinase inhibitors can likewise inhibit BRDs, we screened the compounds against BRD4, the most well-studied and key member of the BET protein family.

Table 2 shows the dissociation constants for compound binding to the first BRD of BRD4 [BRD4(1)]. Using the BROMOScan assay (DiscoverX), two reference BRD inhibitors, JQ1 (37) and I-BET762 (38), exhibited potent BRD4 (1) K_d values. However, analysis of the ERK5 inhibitors using this method revealed a clear split. Compounds that were active in the E-selectin assay, AX15839, AX15910, and XMD8-92, potently interfered with the acetyl-lysine/BRD4(1) interaction. These compounds thus represent dual inhibitors of ERK5 kinase and of BRD. In contrast, compounds that were potent on ERK5 but inactive in the E-selectin assay, AX15836 and AX15892, gave considerably higher BRD4 (1) dissociation constants, indicating loss of off-target BRD inhibition.

Knowing that the dual ERK5/BRD inhibitors were efficacious in the E-selectin HUVEC assay, whereas the ERK5-selective inhibitors had no effect (Table 1), we returned to that assay to measure the activity of the two BRD-selective reference inhibitors. Using 1 μM of I-BET762 and JQ1, we observed E-selectin reductions of 27% and 29%, respectively, confirming the notion that TLR1/2-induced E-selectin expression in endothelial cells

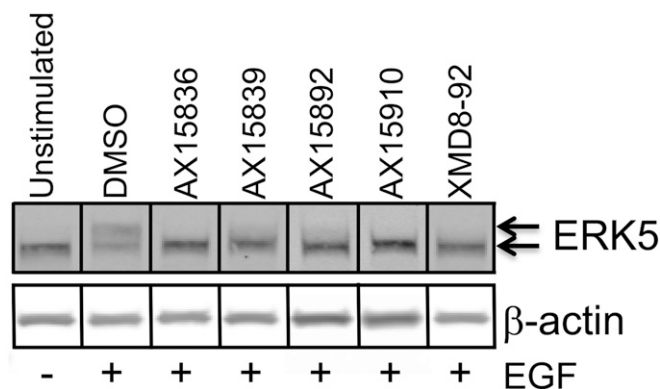


Fig. 2. HeLa cell ERK5 auto-phosphorylation assay. Stimulation of HeLa cells with EGF induced a slower migrating, auto-phosphorylated ERK5 band (upper arrow) separated by SDS/PAGE and detected by Western blot. This p-ERK5 was prevented by pharmacological ERK5 inhibition. Compounds were screened at 2 μM. Figure is a composite of lanes from nonadjacent samples on the same blot.

Table 3. EC₅₀ values of compounds in reducing cytokines IL-6 and IL-8 produced by endothelial cells stimulated with TLR1/2 agonist Pam₃CSK₄

Compound	Inhibitor classification	EC ₅₀ (μM) of cytokine reduction	
		IL-6	IL-8
AX15836	ERK5	>>10	>>10
AX15839	Dual	1.73	1.79
I-BET762	BRD	0.15	0.12

HUVECs were pretreated with compound before stimulation with 10 μg/mL Pam₃CSK₄ for 48 h. Culture supernatants were tested for cytokines by immunoassay. Shown are results from a representative experiment from at least two independent experiments.

We likewise evaluated these inhibitors on the proliferation of the acute myeloid leukemia cell line MV4-11, which expresses the activating internal tandem duplication (ITD) mutation of FLT3 (FLT3-ITD). This driver mutation was reported to constitutively activate ERK5, and inhibition of the upstream kinase MEK5 led to reduced cell proliferation and viability (45). As previously noted in the literature (34), we found reference BRD inhibitors to be effective in this model, with EC₅₀ values of 60 ± 10 and 170 ± 10 nM for JQ1 and I-BET762, respectively (mean ± SD of three experiments). Viability EC₅₀s of the dual ERK5/BRD inhibitors (AX15839, AX15910, and XMD8-92) were less potent and ranged from 1.10 ± 0.25 to 3.28 ± 1.14 μM. Again, however, we observed no effect with the selective ERK5 compounds AX15836 and AX15892 (EC₅₀s > 15 μM). Our studies thus demonstrate that highly specific pharmacological inhibition of ERK5 catalytic activity had no effect on cell growth or viability in cancer cell lines previously characterized to be regulated by this kinase. Although xenograft studies might further delineate a more complex role of ERK5 kinase activity, pharmacokinetic characterization of AX15836 (Table S2) did not indicate it to be optimal for in vivo dosing.

Transcriptome Analysis of Cellular ERK5 Kinase Inhibition. We sought to analyze the effects of selective ERK5 inhibition in comparison to BRD inhibition (either dual ERK/BRD or BRD-only) on genome-wide gene expression. Two cellular models with reported ERK5-regulated signaling were used: Pam₃CSK₄-stimulated HUVECs (23, 24) as a model of inflammation, and EGF-stimulated HeLa cells (5, 20, 29) as an established cell model of ERK5 regulation. Cells were preincubated with DMSO vehicle, AX15836 (ERK5 inhibitor), AX15839 (dual ERK5/BRD inhibitor), or I-BET762 (BRD inhibitor) and then stimulated with agonist. Cellular responses were verified by immunoassays and Western blots using replicate wells in the same experiment.

Table 4. EC₅₀ values of compounds in reducing cytokines IL-6 and IL-8 produced by bronchial epithelial cells stimulated with IL-17A

Compound	Inhibitor classification	EC ₅₀ (μM) of cytokine reduction	
		IL-6	IL-8
AX15836	ERK5	>>10	>>10
AX15839	Dual	1.13	1.07
I-BET762	BRD	0.16	0.07

BEAS-2B cells were pretreated with compound before stimulation with 50 ng/mL IL-17A for 48 h. Culture supernatants were tested for cytokines by immunoassay. Shown are results from a representative experiment from at least two independent experiments.

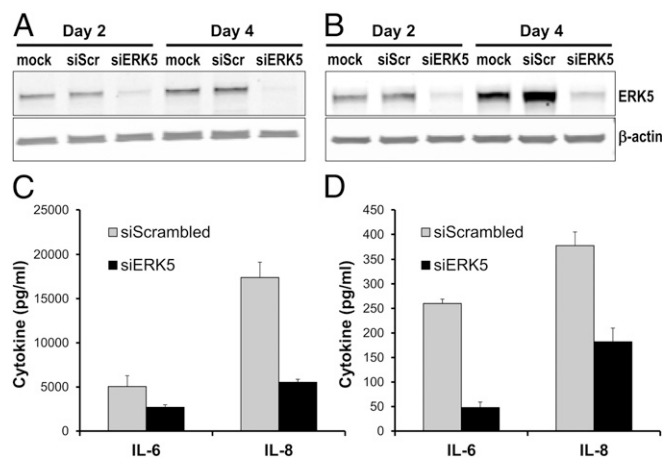


Fig. 3. ERK5 knockdown suppresses inflammatory cytokine response. HUVEC (A) and BEAS-2B (B) cells were transiently transfected with mock treatment, scrambled siRNA control (siScr), or siRNA against ERK5 (siERK5). Expression of ERK5 and β-actin was monitored by Western blot on day 2 (addition of Pam₃CysK₄ or IL-17) and day 4 (collection of culture supernatant for cytokine analyses). Immunoassay of IL-6 and IL-8 from treated HUVEC (C) and BEAS-2B (D) cells indicates significant suppression of inflammatory cytokines when ERK5 is depleted. Figures are representative experiments from two independent experiments.

RNA sequencing (RNA-Seq) of biological triplicates detected at least one transcript for 18,925 genes in HUVEC samples and 17,266 genes in HeLa samples. In both cell types, samples treated with AX15836 showed very few genes to be differentially expressed (Fig. 5A). The total number of genes using the default cutoff (adjusted $P \leq 0.1$) for plotting log-intensity ratios (M-values) versus log-intensity averages (A-values) (MA plot) was seven in HUVEC samples and two in HeLa samples. Moreover, the observed maximal fold-changes in expression compared with the DMSO control samples were modest: below 1.6 and 2 for HUVEC and HeLa samples, respectively. Principal component analysis of all samples further confirmed the lack of differential gene expression in samples treated with the ERK5-only inhibitor AX15836. Conversely, cells treated with the dual ERK5/BRD inhibitor AX15839 and those treated with the BRD inhibitor I-BET762 showed a large number of differentially expressed genes (Fig. 5A). The correlation of fold-changes in expression of those genes is shown in Fig. 5B. The majority of the genes showed comparable expression patterns in that they were expressed at either higher or lower levels in each of the treated

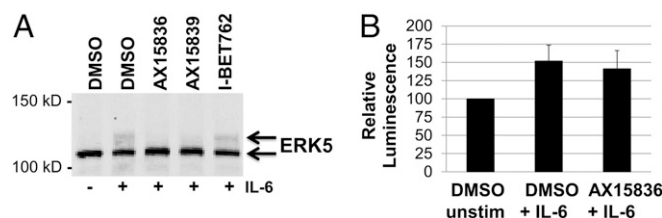


Fig. 4. Multiple myeloma (MM).15 cell growth is not affected by selective ERK5 inhibition. (A) IL-6 activates ERK5 in MM.15 cells, as shown by the appearance of a mobility retarded, auto-phosphorylated ERK5 band (upper arrow) on Western blot. Inhibitors (2 μM) with ERK5 inhibitory activity (AX15836 and AX15839) prevented the induction of p-ERK5. (B) After 3 d of incubation, viable MM.15 cells were quantified in an assay measuring ATP content and expressed as relative luminescence. The ERK5-selective inhibitor AX15836 (1.67 μM) did not significantly reduce IL-6-dependent proliferation relative to the DMSO + IL-6 control. Data graphed in B are the mean ± SD of three independent experiments.

Taken together, these experiments were useful to indicate the range of biological processes that ERK5 could influence. Because ERK5 is a kinase that has been demonstrated to phosphorylate transcription factors, the genetic phenotypes have been interpreted as being the result of removing the catalytic activity. However, the selective ERK5 inhibitors described here lack the antiinflammatory and antiproliferative effects induced by genetic manipulation of ERK5. The simplest explanation for this is that the immune and proliferation effects seen in ERK5 genetic models are due to ablation of nonkinase functions of ERK5. Interestingly, essential noncatalytic functions such as protein scaffolding, allosteric regulation of other enzymes, and DNA binding can be found in all seven major kinase groups (50). Separately, we demonstrate that previously reported ERK5 inhibitors, exemplified by XMD8-92, have off-target activity on an unrelated class of proteins, the BRDs. Our experiments show that BRD inhibition is sufficient to account for the antiinflammatory and antiproliferative cellular responses previously ascribed to ERK5 inhibition via XMD8-92. It appears to be pure happenstance that there is overlap between the phenotypes of BRD inhibition and ERK5 depletion/deletion.

Our findings show that ERK5 is a highly unusual MAP kinase. Like the other MAPK members, ERK5 is involved in a number of central biological pathways. In stark contrast, however, the kinase activity of ERK5 appears to be unnecessary for many of its most widely studied functions.

Materials and Methods

For determination of native kinase engagement, compounds were profiled in cell lysates or live cells using the KiNativ chemoproteomics platform (ActivX Biosciences) (27, 28, 51). For flow cytometry, ERK5 auto-phosphorylation, immunoanalyses, and RNA-Seq, cells were treated for 1 h with compounds before agonist stimulation, followed by standard protocols. Experimental details and compound synthetic schemes are provided in *SI Materials and Methods*. In vivo study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Agilux Laboratories.

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