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Identification of pharmacological inhibitors of the MEK5/ERK5 pathway

Revati J. Tatake [a], Margaret M. O'Neill [a], Charles A. Kennedy [a], Anita L. Wayne [a], Scott Jakes [a,*], Di Wu [b], Stanley Z. Kugler Jr. [b], Mohammed A. Kashem [b], Paul Kaplita [c], Roger J. Snow [b]

[a] Department of Cardiovascular Disease, Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Road, Ridgefield, CT 06877, USA
[b] Department of Medicinal Chemistry, Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Road, Ridgefield, CT 06877, USA
[c] Department of Drug Discovery Support, Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Road, Ridgefield, CT 06877, USA

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ABSTRACT

We have identified two novel MEK5 inhibitors, BIX02188 and BIX02189, which inhibited catalytic function of purified, MEK5 enzyme. The MEK5 inhibitors blocked phosphorylation of ERK5, without affecting phosphorylation of ERK1/2 in sorbitol-stimulated HeLa cells. The compounds also inhibited transcriptional activation of MEF2C, a downstream substrate of the MEK5/ERK5 signaling cascade, in a cellular trans-reporter assay system. These inhibitors offer novel pharmacological tools to better characterize the role of the MEK5/ERK5 pathway in various biological systems.

Mitogen activated protein kinase (MAPK) pathways, involving modular signal transduction for activation, provide an important connection between external stimuli and activation of intracellular signaling. MEK5 is a member of the MAP kinase kinase (MEK) family, which activates a downstream MAPK, ERK5 (also known as BMK1 or MAPK7) [1–4] and is activated by upstream kinases MEKK2 and MEKK3 [5–7]. ERK5 in turn activates various cellular substrates including the myocyte enhancer factor 2 (MEF2) family members [8–10]. The MEK5/ERK5 pathway has been recognized as the new MAPK cascade in addition to ERK1/2, p38, and JNK pathways and has been implicated in cell proliferation and survival as well as in the response to various stressors [11]. The MEK5/ERK5 pathway is activated by a variety of stimuli including osmotic and oxidative stress [12], growth stimuli [13], activation of receptor tyrosine kinases [14], and various other stimuli including fluid shear stress [15–17]. Both MEK5 and ERK5 kinases have large subdomains outside of the kinase domains, which have been suggested to have unique scaffolding functions, sometimes independent of their catalytic function [1–3]. The understanding of the distinct effects of the catalytic and scaffolding functions of MEK5 and ERK5 has been restricted due to the lack of selective inhibitors affecting catalytic activity of these enzymes.

The role of the MEK5/ERK5 pathway has been implicated in survival and progression of tumor cells [18–20], enhanced neuronal survival [21,22], and cardiovascular pathophysiology [23–28]. Both ERK5 and MEK5 null mice are embryonic lethal due to major cardiovascular defects, including impaired myocardial development and integrity of the vasculature [23–25]. Interestingly, in the adult animals, endothelial cell specific inactivation of ERK5 shows a phenotype similar to the conventional MEK5 or ERK5-null animals, while, cardiac-specific inactivation of ERK5 has a normal cardiac phenotype [26], suggesting that the abnormal cardiac phenotype in embryonic MEK5 and ERK5-null animals may be secondary to vascular defects. The MEK5/ERK5 pathway is also implicated in cardiovascular pathology. Activation of the MEK5/ERK5 pathway has been shown to induce eccentric hypertrophy *in vivo* and elongation of cardiomyocytes *in vitro* [16,17,27,28], which is inhibited by dominant negative mutants of MEK5 and ERK5 [16,17]. Although, using genetic tools, significant progress has been made in understanding of the overall role of MEK5/ERK5 pathway in various biological systems; this pathway remains to be further explored due to unavailability of selective inhibitors. In this report, we describe two MEK5 inhibitors, their selectivity profile against a large panel of kinases and their activity in various cellular environments.

Materials and methods

Cells. HeLa and HEK293T cells were grown in RPMI-1640 with 10% heat inactivated FBS, 2 mM glutamine, and 50 μg/ml gentamycin. All reagents for cell culture were purchased from Invitrogen.

Plasmids. The expression constructs pCNDA-MEK5-CA, pCDNA-ERK5, and pFA-MEF2C were generated by PCR amplification of the respective genes and sub cloning into the expression vectors, pCDNA3.1 (Invitrogen) for MEK5 and ERK5 and pFA (Stratagene)

* Corresponding author. Fax: +1 203 791 6196.
E-mail address: scott.jakes@boehringer-ingelheim.com (S. Jakes).

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for MEF2C. Constitutively active MEK5 was generated by site directed mutagenesis of S311 → D and T315 → D using Quickchange mutagenesis kit (Stratagene). All final constructs were checked for accuracy of the sequence. The vector pFR-GAL4-luc was purchased from Stratagene. The cDNA were subcloned into pVL1393 vectors for expression of proteins in baculovirus.

Antibodies. Anti-ERK1/2, anti-phospho-ERK1/2, anti-phospho p38, and anti-phospho JNK antibodies were purchased from Cell Signaling Technology, Anti BMK1/ERK5, anti-phospho-BMK1/ERK5 antibodies were purchased from Upstate Biotechnology.

Catalytic assay. MEK5 and ERK5 proteins isolated from baculovirus expression system were used to measure kinase activity utilizing PKLight ATP Detection Reagent (Cambrex); a homogeneous assay technology using luciferin-luciferase to quantify residual ATP. The assay was performed using 15 nM GST-MEK5 or 20 nM GST-ERK5 and 0.75 μM ATP in assay buffer consisting of 25 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, 0.2% BSA, 0.01% CHAPS, 100 μM Na_3VO_4, 0.5 mM DTT, and 1% DMSO. The kinase reaction mixture was incubated for 90 min at room temperature followed by addition of 10 μL of ATP detection reagent for 15 min. The relative light unit (RLU) signal was measured and the RLU signals were converted to percent of control (POC) values using the formula: POC = 100 * (BCTRL-Signal)/(BCTRL-PCTRL), where signal is the test well signal, BCTRL is the average of background (negative control) well signals on the plate and PCTRL is the average of positive control well signals on the plate.

Western blotting. Briefly, HeLa cells were plated at 5×10^5 cells/ml/well in six-well plates (Costar). The cells were serum starved for 20 h prior to stimulation with sorbitol (Sigma) at a final concentration of 0.4 M for 20 min at 37 °C. When testing MEK5 inhibitors, compounds where added 1.5 h prior to the addition of sorbitol. The cells were harvested and lysed in 50 μl at 4 °C for 5–10 min in RIPA buffer (Pierce) containing Halt protease and phosphate inhibitors (Pierce). The lysates were centrifuged for 10 min at 14,000 rpm and 50 μl lysate was added to 50 μl 2× sample buffer (Novex) and boiled for 4 min at 95 °C. Twenty microliters sample was run on SDS–PAGE 10% Tris–glycine gels and transferred to nitrocellulose. Western blotting was done with appropriate antibodies.

Trans-reporter assay. Exponentially growing HeLa or HEK293T cells were transfected using Effectene (Qiagen). A total of 2 μg DNA mixed as follows (pCDNA-MEK5 CA (0.05 μg) + pCDNA-ERK5 (0.5 μg) + pFA-MEF2C (0.5 μg) + pFR-GAL4-luc (0.5 μg) + pCDNA 3.1 (0.45 μg)) was added to 300 μl DNA-condensation buffer. The complexes were formed by addition of 16 μl enhancer (5 min) followed by 60 μl Effectene (10 min) with a final volume adjusted to 3.0 ml with complete media. Five hours after transfection, the cells were plated into white 96-well culture plates (Packard P12-106-017). The inhibitors were added at various concentrations to the cells 18–24 h prior to determination of the luciferase expression. The luciferase activity was determined using the protocol provided by Steady-Glo (Promega). Compound cytotoxicity was assessed using Alamar Blue (Invitrogen).

Table 1
Selectivity profile of BIX02188 and BIX02189 against closely related kinases

Kinase	IC_{50} [nM]	
	BIX02188	BIX02189
MEK5	4.3	1.5
MEK1	>6300	>6200
MEK2	>6300	>6200
ERK5	810	59
ERK1	>6300	>6200
JNK2	>6300	>6200
TGFβR1	1800	580
EGFR	>6300	>6300
STK16	>6300	>6300

The compounds were tested in a 10 point dose titration starting at 10 μM as the highest concentration. The IC_{50} values were calculated using percent kinase activity relative to control, where control activity is 100% in the absence of inhibitors.

Results and discussion

Selectivity profile of the MEK5 Inhibitors BIX02188 and BIX02189

High-throughput screening of the Boehringer Ingelheim compound collection was carried out against MEK5. Hits were first

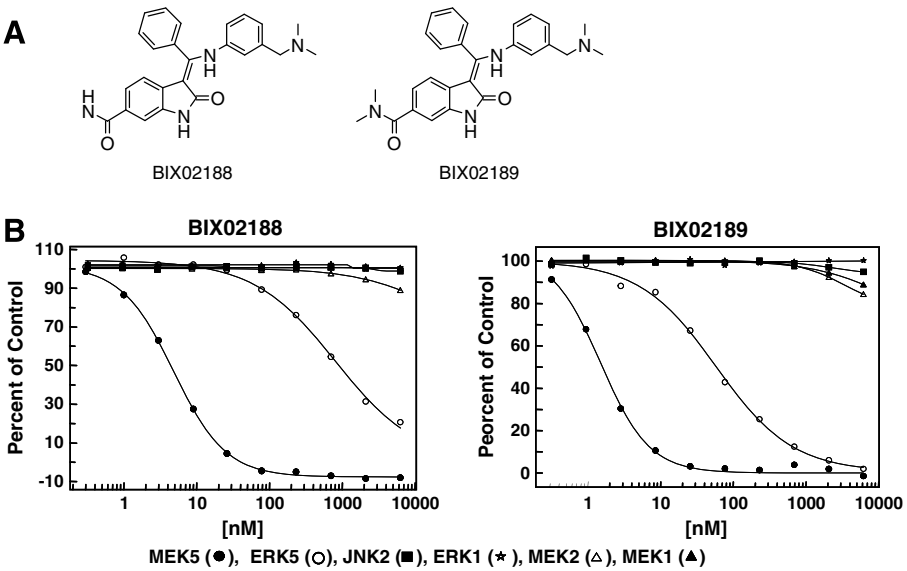


Fig. 1. (A) Chemical structures of BIX02188 and BIX02189; (B) dose titration of BIX02188 and BIX02189 against MEK5, ERK5, MEK1, MEK2, ERK2, ERK5, and JNK2. Catalytic activity of MEK5, ERK5, JNK2, ERK1, MEK2, and MEK1 was determined in the presence of varying concentrations of the MEK5 inhibitors. The results are represented as the percent kinase activity relative to the control measured in the absence of inhibitors (POC). The data representing POC as a function of test compound concentration were fitted to a four-parameter logistic equation of the form: $Y = A + (B - A)/[1 + (x/C)D]$, where A, B, C, and D are fitted parameters (parameter B is fixed at zero POC), and x and y are the independent and dependent variables, respectively. The IC_{50} (50% inhibitory concentration) was determined as the inflection point parameter, C. Each data point represents an average of duplicate observations.

Table 2
Selectivity of BIX02188 and BIX02189 against a kinase panel

Kinase	Test conc. (μM)	BIX02188	BIX02189
ABL	10	***20***	***19***
ACVR1B (ALK4)	10	67	54
AKT1 (PKB alpha)	10	94	87
AMPK	3	97	96
AMPK A1/B1/G1	10	79	74
AuroraB	10	70	57
CAMK-1	3	107	90
CDK2/cyclin A	10	97	87
CHK1	3	61	74
CHK2	3	89	91
CK1	3	92	88
CK2	3	95	113
CSF1R (FMS)	10	***3***	***4***
CSK	3	48	75
deltaPH-PKB beta (S474D)	3	96	91
DYRK1A	3	104	109
EF2K	3	105	103
EGFR (ErbB1)	10	87	81
EPHA2	10	81	77
ERK8	3	95	98
FGFR1	10	74	***24***
FRAP1 (mTOR)	10	103	102
GSK3B	3	117	100
IGF1R	10	94	92
IKBKB (IKK beta)	10	100	100
INSR	10	85	88
JAK3	10	94	***45***
JNK1A1	3	105	91
JNK3 alpha1	3	97	91
KIT	10	***20***	***31***
LCK	10	***8***	***6***
MAP2K1 (MEK1)	10	99	93
MAPK1 (ERK2)	10	93	91
MAPK8 (JNK1)	10	98	97
MAPKAPK1A	3	80	***48***
MAPKAP-K1b	3	92	77
MAPKAPK2	10	102	101
MAPKAP-K3	3	91	100
MARK2	10	92	89
MARK3	3	92	93
MET (cMet)	10	92	94
MKK1	3	113	91
MNK1	3	93	88
MNK2 alpha	3	111	91
MSK1	3	90	87
MST2	3	93	103
MYLK2 (skMLCK)	10	98	98
NEK1	10	98	97
NEK2A	3	98	85
NEK6	3	116	113
NEK7	3	102	91
p38	3	52	69
PAK4	10	117	113
PBK	3	85	81
PDK1	3	97	96
PHKG2	10	96	96
PIM1	10	88	78
PIM2	3	90	83
PKA (rec)	3	94	79
PKBdeltaPH	3	86	84
PKC alpha	10	92	78
PKCA	3	86	81
PKD1	3	91	92
PLK1	10	96	95
PRAK	3	56	61
PRK2	3	96	85
RAF1 (cRAF)	10	97	104
ROCKII	3	86	94
RPS6KA3 (RSK2)	10	***40***	***22***
RPS6KA6 (RSK4)	10	***24***	***6***
S6K1	3	119	112
SGK (SGK1)	10	83	54
smMLCK	3	75	79
Src	3	***3***	***6***
SRPK1	3	96	87
SRPK2	10	101	101
SYK	10	82	85
TBK1	10	92	88
TEK (Tie2)	10	95	100

The kinase selectivity testing was performed at the University of Dundee and Invitrogen Corporation. The compounds were tested at a single concentration of 3 μM or 10 μM. The data are expressed as percent kinase activity in the presence of the inhibitors relative to control, which was 100% in the absence of the inhibitors. The bold and italicized numbers indicate the percent of control values less than 50.

screened for selectivity using MEK1 and MEK2. The selective inhibitors were then screened against the panel of kinases listed in Table 1. Optimization of the hits was then carried out to maximize MEK5 potency and selectivity. This led to the identification of two compounds with favorable selectivity profiles. Boehringer Ingelheim. These are the indolinone-6-carboxamides, BIX02188 and BIX02189 (Fig. 1A), which inhibited MEK5 catalytic activity in a dose dependent manner with IC_{50} 4.3 and 1.5 nM, respectively (Fig. 1B). These belong to the indolinone kinase inhibitor series described earlier [29,30]. They did not inhibit closely related kinases MEK1, MEK2, ERK2, and JNK2. Both compounds inhibited ERK5 catalytic activity, with BIX02189 (IC_{50} = 59 nM) having greater potency than BIX02188 (810 nM). The compounds were further profiled for selectivity against a panel of 79 kinases (Table 2) at a single concentration of either 3 μM or 10 μM. Both compounds were tested in dose response against ten of these kinases as indicated in Table 3. Both inhibitors showed greater than 100-fold selectivity against 85 out of 87 kinases tested.

Inhibition of ERK5 phosphorylation in sorbitol-stimulated HeLa cells by the MEK5 inhibitors

The MEK5/ERK5 pathway was activated by sorbitol treatment of HeLa cells. In order to assess the effects of the MEK5 inhibitors on ERK5 phosphorylation, the cells were treated with the inhibitors for 90 min prior to stimulation. As shown in Fig. 2A and B, both BIX02188 and BIX02189 inhibited ERK5 phosphorylation in a dose dependent manner. When probed with anti-phosphoERK1/2 antibody, we observed that ERK1/2 phosphorylation was not inhibited by treatment of cells with the MEK5 inhibitors BIX02188 and BIX02189. Both inhibitors did not inhibit phosphorylation of p38 and JNK1/2 MAPKs in sorbitol-stimulated HeLa cells (Fig. 2C).

MEK5 inhibitors blocked MEF2C driven reporter gene expression

In this assay, the vectors expressing constitutively active MEK5 (CA-MEK5), ERK5, MEF2C-GAL4 fusion protein and GAL4-Lucifer-

Table 3
Relative potency of BIX02188 and BIX02189 against a selected panel of kinases

Kinase	IC_{50} [nM]	
	BIX02188	BIX02189
ABL1	2100	2400
CSF1R (FMS)	280	46
FGFR1	>10,000	1000
JAK3	7800	440
KIT	550	1100
LCK	390	250
MAPK14 (p38 alpha)	3900	3700
RPS6KA3 (RSK2)	4100	2100
RPS6KA6 (RSK4)	3200	990
SRC	8900	7600

The compounds were tested in a 10 point dose titration. The IC_{50} values were calculated using percent kinase activity relative to control, where control activity is 100% in the absence of inhibitors.

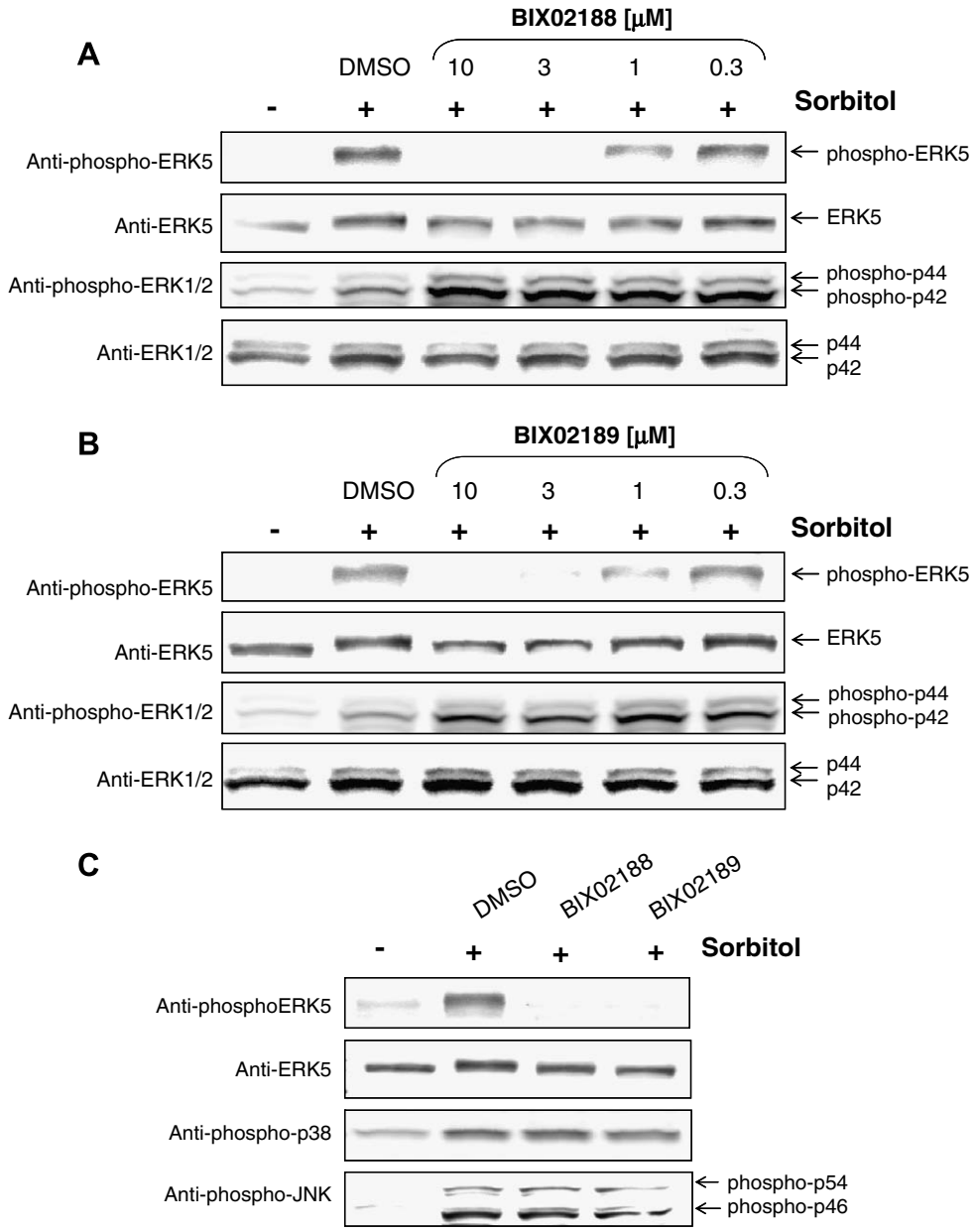
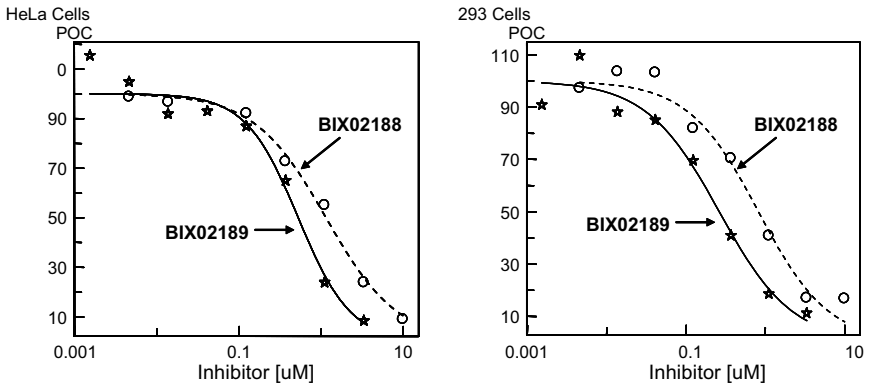


Fig. 2. Inhibition of ERK5 phosphorylated by BIX02188 (A) and BIX02189 (B) in sorbitol-stimulated HeLa cells. The cell lysates of sorbitol-stimulated HeLa cells either in the presence or absence of the inhibitors were immunoblotted using anti-phospho and total ERK5 antibodies or anti-phospho and total ERK1/2 antibodies. (C) The lysates from sorbitol-stimulated HeLa cells that were treated with BIX02188 (10 μM) 02 BIX02189 (10 μM) were immunoblotted using anti-phospho p38 and anti-phospho-JNK antibodies.

ase reporter were transiently transfected in either HeLa or HEK293 cells. When all proteins are expressed, constitutively active MEK5 phosphorylates ERK5 (data not shown), which in turn phosphorylates and activates MEF2C [8,9]. Activated MEF2C-GAL4 fusion protein binds to the GAL4 binding region in the Gal4-Luciferase reporter vector, and as a result, drives expression of the luciferase reporter gene. As shown in fig. 3, BIX02188 and BIX02189 inhibited MEF2 driven luciferase gene expression in a dose dependent manner in two different cell lines. The inhibitors were present for 24 h in the culture and did not show cytotoxic effect as assessed by reduction of Alamar Blue (data not shown).

In summary, we have identified two MEK5 inhibitors, which can be valuable pharmacological tools for unraveling the MEK5/ERK5 pathway. So far the pharmacological-based studies have been complicated by use of pharmacological inhibitors that cross react between ERK1/2 and ERK5 [14,31]. The inhibitors described here are very potent inhibitors of the catalytic activity of MEK5 and are selective against several kinases including closely related kinases, MEK1 and MEK2. Both compounds inhibited phosphorylation of ERK5 in a dose dependent manner, without affecting phosphorylation of ERK1/2 in sorbitol-stimulated HeLa cells. Recently BIX02188 was used for selective inhibition of ERK5 phosphorylation in endothelial cells activated by fluid shear stress [32]. These data are consistent with the selectivity of these inhibitors against the upstream kinases MEK1 and MEK2 in a cell free biochemical assay (Table 1 and Fig. 1B). Both inhibitors did not inhibit phosphorylation of p38 and JNK1/2 MAPKs in sorbitol activated HeLa cells (Fig. 2C) and did not affect catalytic activity of p38 and JNK2 directly (Table 1, Fig. 1B, and Table 3). They inhibited transcriptional activation of MEF2C, a downstream substrate of MEK5/ERK5 signaling cascade, in a trans-reporter system. Activation of the MEK5/ERK5 pathway has been proposed to have a



HeLa Cells		
	IC50 [μM]	Hill
BIX02188	1.15	-0.99
BIX02189	0.53	-1.36

HEK293 Cells		
	IC50 [μM]	Hill
BIX02188	0.82	-0.98
BIX02189	0.26	-0.94

Fig. 3. Inhibition of MEK5/ERK5/MEF2C-driven luciferase expression by BIX02188 and BIX02189 in HeLa and 293T cells. Exponentially growing HeLa or HEK293T cells were transfected with pCDNA-MEK5-CA + pCDNA-ERK5 + pFA-MEF2C + pFR-GAL4-luc using Effectene as described in Experimental procedures. Inhibitors were added to the cells 18–24 h prior to determination of luciferase activity. The results are represented as the percent luciferase activity relative to the control measured in the absence of inhibitors. Each data point represents an average of triplicate observations.

significant role in tumor progression [18–20], neuronal survival [21,22], and cardiovascular pathophysiology, including embryonic development of the heart, endothelial cell function, angiogenesis, and cardiomyopathy [16,17,23–28], primarily using genetic tools. The inhibitors described in this report provide novel pharmacological tools for furthering the understanding of the role of MEK5/ERK5 pathway in different cellular environments.

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