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Identification of a selective ERK inhibitor and structural determination of the inhibitor–ERK2 complex

Makoto Ohori [b,*], Takayoshi Kinoshita [c], Mitsuru Okubo [a], Kentaro Sato [a], Akiko Yamazaki [a], Hiroyuki Arakawa [a], Shintaro Nishimura [a], Noriaki Inamura [a], Hidenori Nakajima [a], Masahiro Neya [a], Hiroshi Miyake [a], Takashi Fujii [a]

[a] *Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Tokodai 5-2-3, Tsukuba, Ibaraki 300-2698, Japan*
[b] *Lead Discovery Research Laboratories, Astellas Pharma Inc., Miyukigaoka 21, Tsukuba, Ibaraki 305-8585, Japan*
[c] *Department of Biological Science, Graduate School of Science, Osaka Prefecture University, Gakuencho 1-1, Sakai, Osaka 599-8531, Japan*

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Abstract

Selective inhibition of extracellular signal-regulated kinase (ERK) represents a potential approach for the treatment of cancer and other diseases; however, no selective inhibitors are currently available. Here, we describe an ERK-selective inhibitor, FR180204, and determine the structural basis of its selectivity. FR180204 inhibited the kinase activity of ERK1 and ERK2, with K_i values 0.31 and 0.14 μM, respectively. Lineweaver–Burk analysis of the binding interaction revealed that FR180204 acted as competitive inhibitor of ATP. In mink lung epithelial Mv1Lu cells, FR180204 inhibited TGFβ-induced luciferase-expression. X-ray crystal structure analysis of the human ERK2/FR180204 complex revealed that Q105, D106, L156, and C166, which form the ATP-binding pocket on ERK, play important roles in the drug/protein interaction. These results suggest that FR180204 is an ERK-selective and cell-permeable inhibitor, and could be useful for elucidating the roles of ERK as well as for drug development.

Keywords: ERK; MAPK; ERK inhibitor; Selective kinase inhibitor; Transforming growth factor β; AP-1

To respond to extracellular stimuli, cells possess intracellular signaling cascades such as mitogen-activated protein kinase (MAPK) pathways [1,2]. The extracellular signal-regulated kinase (ERK) signaling pathway, one of the MAPK pathways, consists of MAPK kinase kinases (e.g., c-Raf), the MAPK kinases (MEK1 and MEK2), and the MAPKs (ERK1 and ERK2), and is an important mediator of a number of cellular fates, including growth, proliferation, and survival, in mammalian cells. Activated ERK1/2 can phosphorylate various substrates including nuclear substrates, cytoskeletal proteins, and MAPK-activated protein kinases [1].

Selective inhibition of ERK would be potential approach for treatment of cancer or other diseases; however, no selective ERK inhibitors are currently available [1,3,4]. In many tumor cells, the mutation of Ras or Raf causes the hyperactivation of ERK followed by unregulated cell proliferation [1], suggesting that inhibition of ERK represents a potential approach for the treatment of cancer. Mice lacking ERK1 are viable and fertile, but have deficient thymocyte maturation [5], enhanced long-term memory [6,7], decreased adiposity, and fewer adipocytes than wild-type animals [8], indicating that the interference with ERK activity presents an attractive opportunity for the treatment of inflammatory diseases, memory impairments, and diabetes. The proinflammatory cytokine, transforming growth factor β (TGFβ), activates the transcription factor AP-1 via the ERK pathway, and this activation plays important roles in many cellular responses, such as collagen production and metalloproteinase expression [2], suggesting that

* Corresponding author. Fax: +81 29 847 8313.
E-mail address: makoto.ohori@jp.astellas.com (M. Ohori).

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ERK inhibition may improve disease states involving TGFβ, such as collagen diseases.

Despite the physiological importance of ERK signaling, no selective ERK inhibitors are currently available [1,3,4]. For example, the ERK inhibitors, olomoucine and SB220025, are not selective for ERK, and only inhibit ERK at high concentrations [9,10]. Fox et al. [9] have revealed that the single residue change in ERK2 (Q105A), which is designed to mimic the ATP-pocket of p38 MAPK, enhances the binding of SB202190; however, it cannot inhibit wild-type ERK. In addition, there was no report about the crystal structure of human wild-type ERK2 and the complex with selective ERK inhibitors. Here, we report the identification of a selective ERK inhibitor, FR180204, and the structural analysis of the interactions between human wild-type ERK2 and FR180204. We also demonstrate that ERK plays an important role in TGFβ-signaling using this selective inhibitor.

Materials and methods

Materials. Dephosphorylated bovine myelin basic protein (MBP), human recombinant ERK2, and anti-phosphorylated MBP antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Goat anti-mouse IgG (H + L) polyclonal antibody conjugated with horseradish peroxidase was obtained from Zymed Laboratories (San Francisco, CA). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (St. Louis, MO). U0126 and phRL-TK were purchased from Promega (Madison, WI). The luciferase reporter plasmid pAP-1luc was obtained from Stratagene (La Jolla, CA). Mv1Lu cells were obtained from American Type Culture Collection (Manassas, VA). Opti-MEM I was purchased from Invitrogen (Carlsbad, CA). TGFβ was purchased from PeproTech (London, UK). PreScission protease, glutathione–Sepharose 4B, and pGEX-6P-1 were purchased from Amersham Biosciences (Piscataway, NJ). SuperSignal chemiluminescent substrate was purchased from Pierce Biotechnology (Rockford, IL). The chemical library was composed of chemical compounds synthesized by Fujisawa Pharmaceutical (Osaka, Japan).

ERK assay. Nunc-Immuno MaxiSorp plates (Nalge Nunc International, Rochester, NY) were coated with 20 μg/ml MBP solution in phosphate-buffered saline (PBS). After washing with PBS containing 0.05% Tween 20 (T-PBS), blocking buffer (T-PBS containing 3% BSA) was added to each well and the plates were incubated for 10 min at room temperature. After washing with T-PBS, chemical compounds, ATP and recombinant ERK2 (Upstate Biotechnology) diluted in assay dilution buffer (20 mM Mops, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and 50 μg/ml BSA) and were added to each well. Vehicle groups (containing 0.1% DMSO) and kinase-withdrawal groups were used for the control and basal determinations. After incubation for 1 h at room temperature, plates were washed twice with T-PBS. Anti-phospho MBP antibody (0.2 μg/ml) was added to each well, and the plates were incubated for 1 h at room temperature. After washing, anti-mouse HRP-conjugated polyclonal antibodies were added and the plates were incubated for 30 min. SuperSignal chemiluminescent substrate was used for the measurement of HRP activity according to the manufacturer's instructions. Prism 4.0 software (GraphPad Software, San Diego, CA) was used for the Lineweaver–Burk plot analysis, IC_{50} and K_i determinations.

Cell culture. Mink Mv1Lu cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS; Dainippon, Osaka, Japan), 50 μg/ml penicillin/streptomycin, 100 μM of nonessential amino acids, and 1 mM of sodium pyruvate. Human U937 cells were maintained in RPMI1640 medium supplemented with 10% FBS and 50 μg/ml penicillin/streptomycin.

Plasmid construction. Human total RNA was isolated from U937 cells (TRIzol; Invitrogen). Human *ERK2* cDNA (GenBank Accession No. M84489) was amplified from the RNA by using a SuperScript One-step RT-PCR kit (Stratagene) using the following primer pairs: 5′-CAATT GATGGCGGCGGCGGCGGCGGGC-3′ (sense) and 5′-TTAA GATCTGTATCCTGGCTGGAATCTAGCAGTCTCTTCAAAAAT-3′ (antisense). The amplified fragment was subcloned into pCRII-topo vector (Invitrogen) according to the manufacturer's instructions. The *Mfe*I–*Not*I fragment from this plasmid was inserted into pGEX-6P-1 at the *Eco*RI and *Not*I sites.

Human ERK2 protein purification. *Escherichia coli* DH5α cells were transformed with pGEXERK2. The cells were cultured in 100 ml LB media containing 100 μg/ml ampicillin at 37 °C on a shaker for 12 h, then transferred to two liters of LB media and incubated for 4 h at 37 °C. The protein was induced by 0.75 mM of isopropyl-1-thio-β-D-galactopyranoside at 22 °C for 4 h. The extracted supernatant was loaded onto a glutathione–Sepharose 4B column and mixed for 1 h. The column was washed by cleavage buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 0.1% Tween 20). The GST-tagged ERK2 protein was digested by 8% of PreScission protease in cleavage buffer for 4 h at 4 °C. ERK2 without the tag was eluted by PBS containing 5 mM dithiothreitol. ERK2 was further purified using a MonoQ 5/50 column (Amersham Biosciences) with a linear salt gradient of 0–1 M NaCl in 30 column volumes of MonoQ buffer (25 mM Tris–HCl, pH 8.0, 5 mM dithiothreitol), and size exclusion chromatography was performed on a Superdex HR200 column (Amersham Biosciences) in Superdex buffer (25 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 5 mM dithiothreitol) at 4 °C using an AKTA explorer system (Amersham Biosciences).

X-ray crystallography. Rod-like crystals of FR180204/ERK2 were obtained at 277 K using a reservoir solution of 30–35% polyethylene glycol 5000, 5 mM dithiothreitol, 100 mM Bis-Tris–HCl buffer, pH 6.5, and 150 mM ammonium sulfate. Diffraction data were collected using an RAXIS-V image plate detector (RIGAKU) at SPring8 beamline 32B2.

Table 1
Crystallographic data collection and refinement statistics

Spacegroup	$P2_1$
Unit cell dimensions (Å)	
a	48.86
b	69.99
c	63.30
β (°)	116.5
Data collection statistics	
Resolution (Å)	44.03–2.50
No. of observations	52486
No. of unique reflections	12794
R_{merge}	0.061 (overall) 0.229 (2.71–2.50 Å shell)
Completeness (%)	96.0 (overall) 94.8 (2.71–2.50 Å shell)
Final refinement parameters	
No. of non-hydrogen atoms	3034
Resolution (Å)	44.03–2.50
Working *R* value	0.263
No. of reflections, working set	12794
Free *R* value	0.272
No. of reflections, test set	656
rms deviation from ideal geometry of final model	
Bond lengths (Å)	0.007
Bond angles (°)	1.6

R value $= \sum|F_{obs} - F_{calc}|/\sum|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. R_{free} is the cross-validation R factor computed for the test set of reflections (5% of total reflections were used), which are omitted during the refinement process.

The program Crystal Clear (RIGAKU) was used for integration and scaling of the intensities. The structure of the complex was solved and refined using the programs AMoRe [11] and CNX (Accelrys, San Diego, CA), and the protein model 2ERK from the Protein Data Bank. Coordinates and structure factors have been deposited in the Protein Data Bank (Accession No. 1TVO). Details of the data collection and refinement are shown in Table 1.

Dual luciferase assay. Mv1Lu cells were suspended with 400 μl opti-MEM I containing pAP-1luc (40 μg) and phRL-TK (1 μg), and transferred into an electroporation-cuvette of 0.4 cm-gap (Equibio, Monchelsea, UK). The cuvette was shocked with an electrical discharge of 270 V/cm and 1200 μF in an Easyject Plus electroporator (Equibio). The transfected Mv1Lu cells were plated on 96-well plates at a density of 1×10^5 cells/well. After treatment with TGFβ or medium, the plates were incubated for 18 h at 37 °C with inhibitors or the vehicle. Luciferase activity was then measured with a dual-luciferase assay system (Promega) according to the manufacturer's instructions.

Results

FR180204 is a selective inhibitor of ERK

We performed a high-throughput phosphorylation assay to identify compounds that inhibited ERK-mediated phosphorylation of MBP and identified FR180204 (Fig. 1). FR180204 inhibited ERK1 and ERK2 with an IC_{50} value of 0.51 μM ($K_i = 0.31$ μM) and 0.33 μM ($K_i = 0.14$ μM), respectively (Fig. 2). FR180289, which has a structure similar to that of FR180204 (Fig. 1), did not show inhibitory activity for ERK1 and ERK2, indicating that the 3′-position amino group plays an important role in the interaction. Furthermore, FR180289 is useful as a negative control compound. FR180204 was 30-fold less potent against the related kinase p38α, with an IC_{50} value of 10 μM (Table 2). When it was tested against other kinases (human recombinant MEK1, MKK4, IKKα, PKCα, Src, Syc, and PDGFα), FR180204 failed to inhibit any kinases at less than 30 μM (Table 2). Kinase inhibitors are commonly classified as being ATP-competitive or non-competitive, depending on the results of a Lineweaver–Burk analysis. FR180204 is classified as an ATP-competitive inhibitor because the convergence points of the regressed lines are on the *y*-axis (Fig. 2B).

The K_m and V_{max} in the absence of inhibitor are 5.9 ± 0.8 μM and $7.0 \pm 1.3 \times 10^5$ RLU, in the presence of 2.5 μM FR180204 are 46 ± 18 μM and $7.6 \pm 0.3 \times 10^{-5}$ RLU. The change in K_m value without affecting the V_{max} also indicates FR180204 is an ATP-competitive inhibitor.

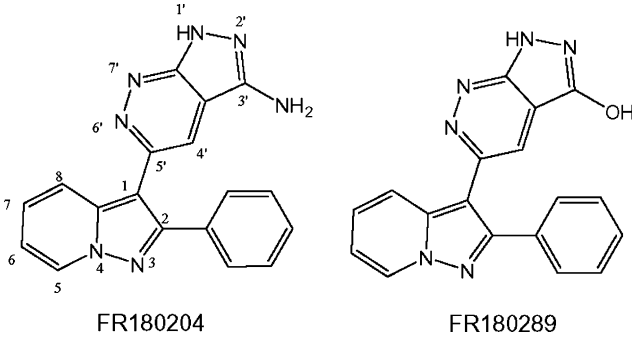

Fig. 1. The structure of the selective ERK inhibitor, FR180204. FR180289, which has a structure similar to that of FR180204, lacks the inhibitory activity for ERK.

X-ray crystal structure reveals binding features

The X-ray crystal structure of FR180204/ERK2 was determined at 2.5 Å resolution (Figs. 3A and B). This is the first report of the crystal structure of human wild-type ERK2. FR180204 was bound to the ATP-pocket, which consists of two lobes, the activation loop and glycine-rich loop. Hydrogen bonds were observed between (1) the 2′-position nitrogen atom of the pyrazolopyridazine ring and the main chain amide group of Met 108, (2) the 3′-position amino group of the pyrazolopyridazine ring and both the carbonyl groups of Gln 105 and Asp 106, and (3) the 3-position nitrogen atom of pyrazolopyridine ring and the amino group of Lys 54. The overall protein structure of the complex was similar to the previously reported ERK2 structures ligated with the lower-potency compounds, SB220025 ($IC_{50} = 18$ μM) and olomoucine ($IC_{50} = 27$ μM) [10,12]. There were, however, three major differences compared to the previous molecular structures. The first is the interaction with lysine 54 of the ATP pocket, an essential for binding to the phosphate part of ATP [12]. In the SB220025/ERK2 complex, as well as the olomoucine/ERK2 complex, however, Lys 54 did not participate in inhibitor interactions. However, high-potency p38α inhibitors, including SB203580, also interact with the corresponding lysine residue in p38 [10], suggesting that these lysine residues in ATP pockets are important interaction sites for high-potency inhibitors. Second, two novel hydrophobic interactions were observed in the FR180204/ERK complex. The pyrazolopyridazine ring had a CH–π type interaction with Leu 156, and the pyrazolopyridine ring had an SH–π type interaction with Cys 166. And third, the glycine-rich loop of the FR180204/ERK2 complex had a different conformation from those of the other complexes previously reported [12,13]. These novel interaction patterns provide the basis for the selectivity and potency of FR180204.

FR180204 inhibits TGFβ-induced AP-1 activation

Based on the biochemical and structural analysis, we assessed the activity of FR180204 in a cell-based functional assay. TGFβ is known to activate AP-1-dependent transcription in Mv1Lu mink lung epithelial cells [14,15]. TGFβ 1 treatment induced an 11-fold increase in luciferase activity in AP-1-transfected cells (Fig. 4, vehicle groups). FR180204 dose-dependently inhibited AP-1 transactivation, with an IC_{50} of 3.1 μM. These inhibitory effects were not caused by cytotoxicity, because the inhibitors did not affect the expression of the internal control gene, *Renilla* luciferase, which was regulated by the constitutively active promoter of the thymidine kinase gene (data not shown). The IC_{50} value of the negative control compound, FR180289, was more than 10 μM.

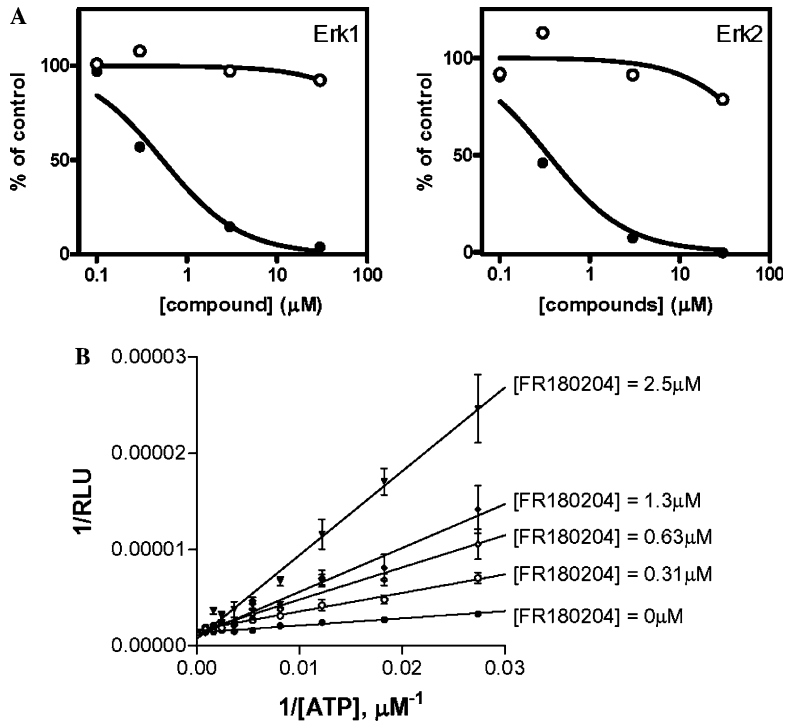

Fig. 2. Biochemical analysis of the effects of FR180204 on phosphorylation by ERK1 and ERK2. (A) Dose–response curve of the effects of FR180204 and FR180289 on MBP phosphorylation by ERK1 or ERK2. MBP phosphorylation by ERK1 (left panel) or ERK2 (right panel) was measured in the presence of FR180204 (●) or FR180289 (○) using an ERK assay. For the control of MBP phosphorylation, vehicle (0.1% DMSO) treatment was used. For the basal control, a kinase-withdrawal sample was used. The results shown are the average ± SEM of three experiments. (B) Lineweaver–Burk analysis of the effects of FR180204 on the ERK activity. ERK assays were performed at serial diluted concentrations of ATP and the reciprocal values are plotted. ERK2 activity was determined using SuperSignal chemiluminescent reagent either in the absence (●) or in the presence of 0.31 (○), 0.63 (◊), 1.3 (♦), or 2.5 (▲) mM FR180204. The results shown are the average ± SEM of four experiments.

Table 2
The inhibition of Ser/Thr kinases and protein tyrosine kinases by FR180204

	p38α	MEK1	MKK4	IKKα	PKCα	Src	Syk	PDGFα
IC_{50} (μM)	10	>30	>30	>30	>30	>30	>30	>30

Discussion

We have here reported the discovery of a selective ERK inhibitor, FR180204, and its unique molecular interactions with ERK. FR180204 inhibited ERK1 and ERK2 with 30-fold greater selectivity against p38α, and more than 100-fold greater selectivity against other kinases (Table 2). The crystal structure and the sequence alignments provide a basis for the selective interaction: although ERK and p38 are structurally similar, FR180204 binds in a novel way to the unique residues of ERK1/2 (Fig. 3C; Gln 105, Asp 106, Leu 156, and Cys 166 of ERK2; green arrows), which are different from the corresponding residues of p38 (Fig. 3C; Thr 106, His107, Ala 157, and Leu 167, respectively). In contrast, FR180204 does not show selectivity between ERK1 and ERK2, putatively, because of the conservation of these same residues in ERK1 and ERK2 (Fig. 3C; black and green arrows). These structural studies should facilitate the generation of more potent and selective ERK inhibitors.

FR180204 inhibited TGFβ-induced AP-1 activation in Mv1Lu cells (Fig. 4). These data indicate that FR180204 is cell-permeable and can inhibit the ERK-signaling cascade. It is also suggested that ERK plays an important role for the TGFβ signaling. FR180289 would be a good negative control for FR180204, because it does not show inhibitory activity in vitro or in the cell assay, in spite a similar chemical structure (Figs. 1, 2, and 4). The fact that FR180289 differs from FR180204 only by a single amino group suggests that the loss of inhibitory activity results from the lack of interaction with Gln 105 or Asp 106 of ERK2 (Figs. 1 and 3). Kinase inhibitors often exhibit unexpected inhibition of other enzymes, so a negative control compound such as FR180289 would also be useful for inhibitor experiments.

The crystal structure of FR180204 is also useful for the generation of ERK1 or ERK2 isoform-selective inhibitors. Recent genetic ablation studies have revealed that neither ERK1 nor ERK2 is able to compensate for all of the functions mediated by the other [7,16,17]. As mentioned in the

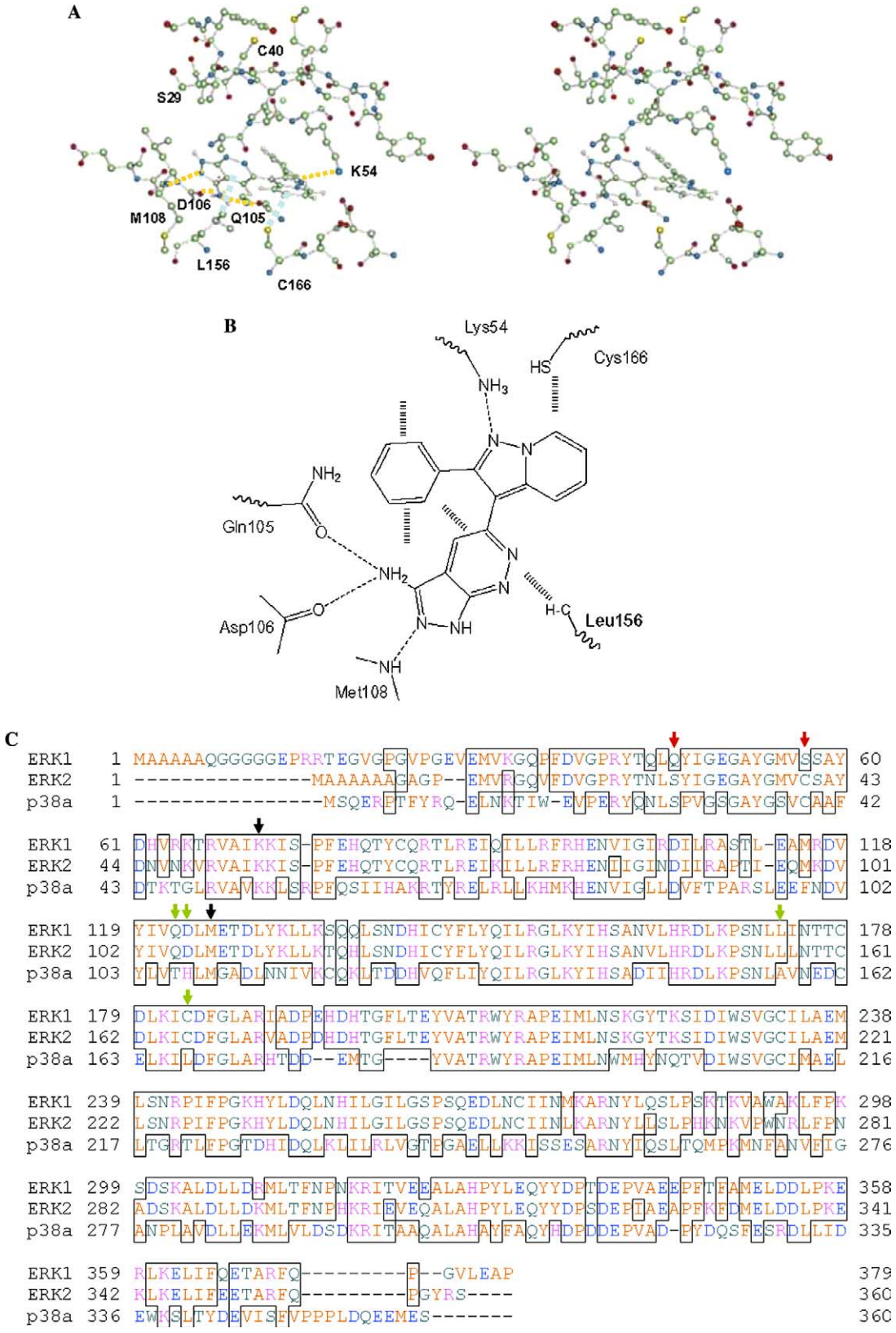

Fig. 3. Interaction of FR180204 with ERK2 in the crystal structure. (A) Stereo drawing of the FR180204/ERK2 active site. Carbon atoms are colored light green, oxygen red, sulfur yellow, and nitrogen blue. Hydrogen bonds are indicated by yellow dashed lines. CH–π and SH–π interactions are indicated by light blue dashed lines. (B) Illustration of the binding. (C) The sequence alignment was performed using the program GENETYX ver. 6. ERK1: human ERK1 protein (Swiss-Prot Accession No. P27361); ERK2: human ERK2 protein (P28482); p38a: human p38α protein (Q16539). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

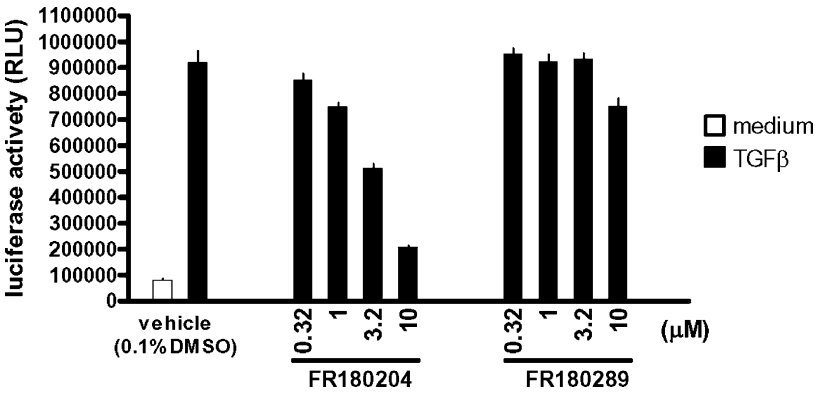

Fig. 4. Effects of FR180204 on AP-1-dependent gene expression induced by TGFβ. The reporter plasmid pAP-1luc, which is the firefly luciferase expression plasmid regulated by a seven-tandem AP-1 responsive element, and phRL-TK, which is the *Renilla* luciferase expression plasmid regulated by the thymidine kinase promoter, were transfected into Mv1Lu cells by electroporation. Cells were then treated with 10 ng/ml TGFβ or medium. To calculate the IC_{50} values, the RLU values of the TGFβ-untreated or -treated cells were used as the basal or activation control values, respectively. The results shown are the average ± SEM of four experiments.

Introduction, mice lacking ERK1 have a defect of thymocyte maturation in spite of the expression of ERK2 [5], and mice lacking ERK2 die during embryogenesis due to a defect in trophoblast development [17,18]. This lack of compensation suggests that each isoform has specific functions in certain cellular events. Although ERK1 and ERK2 isoform-selective inhibitors have not yet been generated, such inhibitors would help to elucidate the isoform-specific roles. The differences in corresponding positions between ERK1 and ERK2 are important to design an isoform-selective inhibitor, For example, Ser 29 and Cys 40 of ERK2 are different from the corresponding residues of ERK1 (Gln 46 and Ser 57) (Fig. 3C; red arrows), which are located at both ends of the flexible glycine-rich loop and near to the docking groove of FR180204 (Fig. 3A). The addition of various substituents to FR180204 to interact with these residues may generate different binding affinities for each isoform.

The wide role of ERK in physiopathology suggests that FR180204 might have broad therapeutic potential [1,2,19]. TGFβ plays important roles in many cellular responses, such as collagen production and metalloproteinase expression. FR180204 inhibits TGFβ-induced AP-1 activation, so that collagen diseases provoked by TGFβ are a potential therapeutic application. The therapeutic applications of MEK inhibitors, such as anti-cancer or immunosuppressive, may also be applicable to FR180204. MEK inhibitors suppress tumor growth, T-cell activations, and ischemic brain injury in animal models via inhibition of the ERK pathway. A novel MEK inhibitor, PD0325901, has recently entered clinical development as an anti-cancer agent [3]. ERK1-knockout mice revealed that the loss of ERK1 suppresses adiposity and provides resistance to high-fat diet-induced obesity [8]. The application of FR180204 for obesity and insulin resistance would also be worth testing. Although further studies will be needed, FR180204 may be useful as a therapeutic agent for the treatment of cancer, immune diseases, ischemic brain injury, and obesity.

In conclusion, we have identified FR180204 as a selective inhibitor of ERK. Our biological analysis reveals that FR180204 is a selective and ATP-competitive ERK inhibitor. The crystal structure of the FR180204/ERK2 complex provides the structural information that will be helpful in designing inhibitors with higher potency and selectivity. The effectiveness of FR180204 in a cell-functional assay suggested that it would be useful as a biological tool to elucidate the involvement of ERK.

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