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LCB 03-0110, a Novel Pan-Discoidin Domain Receptor/c-Src Family Tyrosine Kinase Inhibitor, Suppresses Scar Formation by Inhibiting Fibroblast and Macrophage Activation[S]

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ABSTRACT

Wound healing generally induces an inflammatory response associated with tissue fibrosis in which activated macrophage and myofibroblast cells are primarily involved. Although this is known to be the underlying mechanism for scarring and various fibrotic pathologies, no effective intervention is currently available. We identified (3-(2-(3-(morpholinomethyl)phenyl)thieno[3,2-b]pyridin-7-ylamino)phenol (LCB 03-0110), a thienopyridine derivative, as a potent inhibitor of discoidin domain receptor family tyrosine kinases and discovered that this compound strongly inhibits several tyrosine kinases, including the c-Src family, spleen tyrosine kinase, Bruton's tyrosine kinase, and vascular endothelial growth factor receptor 2, which are important for immune cell signaling and inflammatory reactions. LCB 03-0110 suppressed the proliferation and migration of primary dermal fibroblasts induced by transforming growth factor β1 and type I collagen, and this result correlated with the inhibition ability of the compound against enhanced expression of α-smooth muscle actin and activation of Akt1 and focal adhesion kinase. In J774A.1 macrophage cells activated by lipopolysaccharide LCB 03-0110 inhibited cell migration and nitric oxide, inducible nitric-oxide synthase, cyclooxygenase 2, and tumor necrosis factor-α synthesis. LCB 03-0110 applied topically to full excisional wounds on rabbit ears suppressed the accumulation of myofibroblast and macrophage cells in the healing wound and reduced hypertrophic scar formation after wound closing, without delaying the wound closing process. Taken together, the pharmacological activities of LCB 03-0110 suggest that it could be an effective agent for suppressing fibroinflammation by simultaneously targeting activated fibroblasts and macrophages.

Introduction

The biological mechanism of wound healing is remarkably similar in almost all tissues (Gurtner et al., 2008). For example, the sequence of events during wound healing after a skin injury caused by cutting or burn are similar to that concerning injuries of the liver, lung, kidney, brain, and other organs despite the differences in the type of insult and the organ involved. The wound healing process starts with inflammatory responses followed by the generation of new tissue. Inflammatory cells such as macrophages and neutrophils infiltrate the wound area and become activated because they are necessary for the defense against infection by invading microbes. Furthermore, these inflammatory cells secrete chemicals and cytokines such as nitric oxide (NO), PDGF, TGF-β, and VEGF, which stimulate the growth of granulation tissue and the recruitment and growth of endothelial cells for angiogenesis. Studies have suggested that the activation of macrophage cells is responsible for tissue scarring and fibrosis rather than for the regeneration of damaged

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ABBREVIATIONS: NO, nitric oxide; iNOS, inducible nitric-oxide synthase; DDR, discoidin domain receptor; FAK, focal adhesion kinase; LPS, lipopolysaccharide; TNF, tumor necrosis factor; COX, cyclooxygenase; PDGF, platelet-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; Btk, Bruton's tyrosine kinase; Syk, spleen tyrosine kinase; FLT, fms-like tyrosine kinase; Hck, hematopoietic cell kinase; Lck, lymphocyte-specific protein tyrosine kinase; ECM, extracellular matrix; DMEM, Dulbecco's modified Eagle's media; HEK, human embryonic kidney; FBS, fetal bovine serum; TBS, Tris-buffered saline; HPF, high-power field; RTK, receptor tyrosine kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; IκB, inhibitor of nuclear factor-κB; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Eph, ephrin; LCB 03-0110, (3-(2-(3-(morpholinomethyl)phenyl)thieno[3,2-b]pyridin-7-ylamino)phenol.

tissue (Martin et al., 2003; Duffield et al., 2005; Martin and Leibovich, 2005). Macrophage cells produce several cytokines during wound healing (Hübner et al., 1996), including PDGF, which was suggested to promote transformation of fibroblasts into proliferative and more contractile myofibroblast-type cells (Mori et al., 2008). The transformed myofibroblast cells secrete excessive extracellular matrix (ECM) proteins, including fibrous collagen, that cause scar formation and organ fibrosis. This fibrosis-associated inflammation, called fibroinflammation, contributes to fibrotic pathologies in various tissues, for example, liver cirrhosis, kidney and lung fibrosis, and atherosclerosis, as well as skin disorders such as hypertrophic scars, keloids, and psoriasis (Nickoloff et al., 2006; Gurtner et al., 2008). Because a synergistic overactivation of macrophage and fibrotic cells is a hallmark for fibroinflammation, the simultaneous suppression of the activation of both cell types could be an effective way to inhibit fibroinflammation.

Discoidin domain receptors (DDRs) are members of the receptor tyrosine kinase (RTK) family, with two closely related types, DDR1 and DDR2, that share 89% homology in their tyrosine kinase domain (Vogel et al., 2006). They bind to native forms of various collagens for their activating ligands (Shrivastava et al., 1997; Vogel et al., 1997). DDR1 promotes the accumulation of macrophages in atherosclerotic sites (Franco et al., 2009). DDR1 null mice showed a reduced accumulation of macrophage cells with a decreased inflammatory response and a suppression of fibrosis in mouse models of atherosclerosis (Franco et al., 2008), renal disorder (Flamant et al., 2006), and lung fibrosis (Avivi-Green et al., 2006). Activation of DDR2 in fibroblast cells induced cell proliferation, migration, and remodeling of the ECM with the induction of matrix metalloproteases (Olaso et al., 2002), and its increased expression was associated with the activation and proliferation of hepatic stellate cells in liver cirrhosis (Olaso et al., 2001), chondrocytes in osteoarthritis (Xu et al., 2005), synovial fibroblast cells in rheumatoid arthritis (Wang et al., 2002), and vascular smooth muscle cells in atherosclerosis (Ferri et al., 2004). The activity of DDR2 was suggested to be necessary for epithelial-mesenchymal transition of HK-2 renal epithelial cells (Walsh et al., 2011). Chemical proteomics approaches and an inhibition study identified imatinib, nilotinib, and dasatinib as potent small-molecule inhibitors against the discoidin domain receptor tyrosine kinase family (Bantscheff et al., 2007; Rix et al., 2007; Day et al., 2008). These three chemicals were originally developed for the inhibitors against Bcr-Abl tyrosine kinase, and they are now being used for patients with chronic myeloid leukemia. Of them, imatinib and nilotinib are more or less selective by inhibiting a few tyrosine kinases, whereas dasatinib is known to inhibit more than a dozen tyrosine kinases.

c-Src family is a non-RTK family that consists of eight kinases: Fgr, Fyn, Src, Yes, B-cell lymphocyte kinase, Hck, Lck, and Lyn (Robinson et al., 2000). c-Src family proteins are multifunctional: they are involved in various cellular signaling pathways such as cell proliferation, migration, survival, and adhesion to the ECM (Gauld and Cambier, 2004). In addition to their well established roles in tumorigenesis, they perform a proinflammatory function by stimulating intracellular signal transduction, leading to acute inflammatory responses. For example, Hck, Fgr, and Lyn are the predominant c-Src family tyrosine kinases that induce production of multiple cytokines and chemokines such as TNF-α, interleukin-1, and interleukin-6 in macrophages upon activation by lipopolysaccharide (LPS) (Cohen, 2002). Hck and Fgr are also involved in integrin-mediated cell signaling to promote macrophage migration and attachment to the sites of inflammation (Suen et al., 1999). Fgr and Lyn are associated with integrin-dependent activation and adhesion of neutrophils (Berton et al., 1994; Yan et al., 1995), and adhesion-dependent degranulation of neutrophils requires both Fgr and Hck (Mócsai et al., 1999). The Tec and Syk tyrosine kinase family also belong to the non-RTK family of proteins. Syk was reported to play a key role in the downstream signaling of the B-cell receptor in B cells (Hutchcroft et al., 1991; Yamada et al., 1993), FCεIR receptor in mast cells, and FCγRIIA receptor in macrophages, monocytes, and platelets (Turner et al., 2000). Btk, one of the five Tec family members, is expressed in B cells, mast cells, and macrophages and is involved in the signaling of these cells by being activated downstream of the c-Src and Syk families (Conley et al., 2009). For these reasons, c-Src, Btk, and Syk family kinases have been suggested as promising targets in the development of anti-inflammatory agents (Bradshaw, 2010).

In this work, we identified (3-(2-(3-(morpholinomethyl)phenyl)thieno[3,2-b]pyridin-7-ylamino)phenol (LCB 03-0110), a potent small-molecule inhibitor against the DDR family and c-Src tyrosine kinase family as well as other tyrosine kinases such as Btk and Syk. Because DDR and c-Src family tyrosine kinases are reported as important mediators for the activation of fibroblasts and macrophages, respectively, we evaluated LCB 03-0110 as a novel antifibroinflammatory agent both at the cellular level and in an animal model of skin wound healing.

Materials and Methods

Materials

***N*-(3-(4-methoxybenzyloxy)phenyl)-2-(3-(morpholinomethyl) phenyl)thieno[3,2-b]pyridin-7-amine.** A solution consisting of 3-(7-(3-(4-methoxybenzyloxy)phenylamino)thieno[3,2-*b*]pyridin-2-yl)benzaldehyde (compound A; Fig. 1) (30 mg, 0.06 mmol) and morpholine (64 μl, 0.61 mmol) in dichloromethane (1 ml) was stirred at room temperature for 20 min. The reaction was treated sequentially with sodium acetate (16 mg, 0.19 mmol) and $NaBH(OAc)_3$ (82 mg, 0.38 mmol) and stirred at room temperature for an additional 5 h. The reaction was diluted with dichloromethane (30 ml) and saturated ammonium chloride solution (30 ml). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated un-

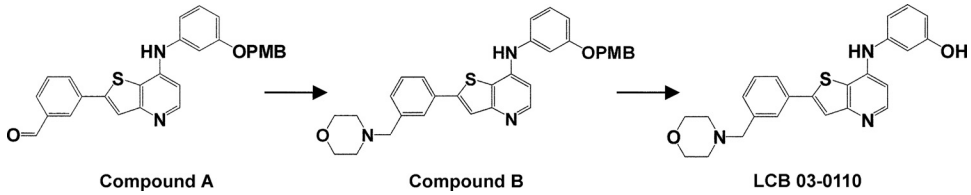

Fig. 1. Synthesis and chemical structure of LCB 03-0110. Left, compound A. Center, compound B. Right, LCB 03-0110..

der vacuum. The residue was purified by flash column chromatography (dichloromethane/methanol 25:1) to give *N*-(3-(4-methoxybenzyloxy)phenyl)-2-(3-(*N*-morpholinomethyl)phenyl)thieno[3,2-*b*]pyridin-7-amine (17 mg, 48%) (compound B; Fig. 1). ^{1}H-NMR (400 MHz, $CDCl_3$); δ 8.20 (d, J = 5.6 Hz, 1H), 7.79 (s, 1H), 7.69 (s, 1H), 7.57 (s, 1H), 7.37 to 7.28 (m, 4H), 6.94 to 6.83 (m, 6H), 6.19 (s, 1H), 5.01 (s, 2H), 3.80 (s, 3H), 3.74 to 3.73 (m, 3H), 3.52 to 3.50 (m, 4H), 2.49 to 2.48 (m, 3H); liquid chromatography/mass spectrometry: 538 (MH+).

LCB 03-0110. A solution of compound B (15 mg, 0.03 mmol) in dry dichloromethane (1 ml) under a nitrogen atmosphere at room temperature was treated with 4 N HCl in dioxane (1 ml). After stirring for 15 h at room temperature, the reaction was concentrated. The residue was redissolved in a mixture of dichloromethane-methanol-ether. Concentration and drying under vacuum gave (3-(2-(3-(morpholinomethyl)phenyl)thieno[3,2-b]pyridin-7-ylamino) phenol (14 mg, 99%) as a bright yellow solid. ^{1}H-NMR (600 MHz, acetone-d6); δ 8.37 (d, J = 6.0 Hz, 1H), 8.08 (brs, 1H), 8.02 (s, 1H), 7.77 to 7.73 (m, 2H), 7.57 t, J = 7.8 Hz, 1H), 7.33 (t, J = 8.4 Hz, 1H), 7.29 to 7.26 (m, 1H), 7.02 to 7.01 (m, 1H), 6.93 to 6.91 (m, 3H), 4.52 (s, 2H), 4.07 to 4.03 (m, 4H), 3.59 to 3.55 (m, 4H); liquid chromatography/mass spectrometry: 418 (MH+). More detailed description for the synthesis of LCB 03-0110 was published previously (Yang et al., 2010).

Antibodies against Akt1, phospho-Akt (pSer473), p38, phospho-p38 (pThr180/pTyr182), IκB-α, phospho-IκB-α (pSer32), stress-activated protein kinase/JNK, phospho-stress-activated protein kinase/JNK (pThr183/pTyr185), p44/p42 MAPK, phospho-p44/p42 MAPK (pThr202/pTyr204), and GAPDH were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against iNOS, DDR1, and β-actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies of COX-2 and DDR2 were from Millipore Corporation (Billerica, MA) and R&D Systems (Minneapolis, MN), respectively. Antibodies against FAK and its phosphor-specific antibodies (pTyr397, pTyr407, pTyr576, pTyr577, and pTyr861) were from Biosource International (Camarillo, CA). Antibodies of α-smooth muscle actin and F4/80 were from Sigma (St. Louis, MO) and Abcam plc (Cambridge, UK), respectively.

In Vitro Inhibition Assay against Kinases

In vitro kinase inhibition assay against recombinant activated DDR2 tyrosine kinase and nonactivated DDR2 kinase was performed as described previously (Yang et al., 2005). In brief, the inhibition assays were performed by using 20 μl of a reaction mixture containing 1 μl of each inhibitor in DMSO, 4 μg of histone H2B as a peptide substrate, and 10 to 20 ng of the purified form of either activated or nonactivated recombinant DDR2 tyrosine kinase domain protein in 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.01 mM ATP, and 0.1 μCi of [^{32}P]ATP. After 15 min of incubation at 30°C, the reaction was stopped by adding a half volume of 30% phosphoric acid. The reaction mixture was spotted on P81 cellulose paper (Millipore Corporation) and washed five times using 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 0.1 M NaCl. The radioactivity in each spot was quantitated with BAS ^{32}P-image analyzer (Fuji Film, Tokyo, Japan). Other kinase inhibition assays were carried out by the Reaction Biology Corporation (Malvern, PA) using the HotSpot assay platform at 10 μM ATP concentration.

Cell Cultures and Treatments

All cells were cultured using DMEM (Invitrogen, Carlsbad, CA) with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen) at 37°C in a 5% CO_2 humidified incubator. Human embryonic kidney (HEK) 293 and J774A.1 murine macrophage cell lines were obtained from the American Type Culture Collection (Manassas, VA). HEK293 cells stably expressing either human DDR1 or DDR2 and the autophosphorylation of the receptors induced by type I collagen treatment were described previously (Siddiqui et al., 2009). One million J774A.1 cells in 12-well plates were treated with LCB 03-0110 in DMEM containing 1% FBS 30 min before stimulation with LPS (100 ng/ml). To prepare primary dermal fibroblasts from 8-week-old BALB/c mice (Taconic Farms, Germantown, NY), a dorsal section was cut into small pieces and washed with sterile phosphate-buffered saline before being treated with 650 U/ml collagenase in DMEM for 2 h at 37°C. The undigested tissue was removed by filtering, and filtered cells were precipitated by centrifugation for 5 min at 1500 rpm and cultured. A half-million dermal fibroblasts were cultured to a confluent monolayer overnight in a six-well dish with or without a coating with 200 μg/ml rat tail type I collagen (Sigma). Then, the medium was switched to DMEM containing 0.5% FBS before treatment with LCB 03-0110 in the presence or absence of 5 ng/ml TGF-β1 (R&D Systems).

Western Blot Analysis

Equal amounts of the total cell lysates were subjected to 7 or 10% SDS-polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride membranes (Millipore Corporation). The membrane was blocked with 5% skim milk in TBS buffer before being incubated with primary antibodies dissolved in 5% skim milk solution in TBS overnight at 4°C. The resulting membrane was washed five times with TBS and applied with horseradish peroxidase-conjugated secondary antibody. A chemiluminescence detection kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was used for signal detection.

Measurement of TNF-α and NO

The amount of TNF-α in the cell culture medium was analyzed with an enzyme-linked immunosorbent assay kit purchased from R&D Systems and used according to the manufacturer's recommendations. Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by Griess reaction (Green et al., 1982). In brief, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (Sigma) and incubated at room temperature for 15 min. Absorbance at 540 nm was determined, and nitrite concentration was calculated from a sodium nitrite standard curve.

Cell Migration and Invasion Assay

Migration assay of mouse dermal fibroblast was carried out by using the wound healing method on the confluent cell monolayer. Twelve-well plates were coated or not with 200 μg/ml type I collagen (Sigma) and added with 200,000 mouse dermal fibroblast cells. After overnight incubation, a scratch wound was made on the cell monolayer, and the medium was replaced with DMEM containing 0.5% FBS. Cells were treated with LCB 03-0110 and incubated for an additional 24 h before being fixed with formalin. J774A.1 macrophage invasion assay was conducted by using Transwell cell culture chambers (24 wells, 8-mm pore size; Corning Life Sciences, Lowell, MA). In brief, 100,000 J774A.1 cells in 100 μl of DMEM containing 1% FBS were plated into the upper chamber, which was precoated with 50 μl of 50 μg/ml collagen IV in sterile water (Sigma); then, cells were treated by LPS (Sigma) with or without LCB 03-0110. One milliliter of serum-free DMEM containing 20 ng/ml monocyte chemotactic protein-1 (R&D Systems) was added to the lower chamber. After 24 h of incubation at 37°C in the cell culture incubator, noninvaded cells on the upper side of the membrane were removed with a cotton swab, and invaded cells were fixed and stained with 1% crystal violet.

Rabbit Ear Wound Healing Model and Estimation of Hypertrophic Scar Formation

New Zealand white female rabbits (Taconic Farms) weighing 2.5 to 3.5 kg were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg), and four full-thickness wounds of 6-mm diameter were made over the ventral surface down to the bare cartilage in each ear by using a 6-mm biopsy punch (Stiefel, Wachtersbach, Germany) and a

microsurgical technique. One hundred fifty microliters of 0.1% LCB 03-0110 dissolved in 0.9% saline containing 10% polyethylene glycol 400 and 10% ethanol were applied topically to the wound sites every 3 days, whereas the carrier solution only was used for the nontreated control group. All wounds were covered by occlusive DuoDERM polyurethane dressing (ConvaTec, Stillmann, NJ) after treatment. Twenty-one days after wounding, the animals were sacrificed, and the scar tissue was excised by using an 8-mm punch biopsy followed by fixation in 10% formalin to obtain paraffin-embedded tissue. Slices (6 μm) were deparaffinized with xylene and stained with hematoxylin and eosin. Digital images were taken at 100× amplitude under a light microscope. Hypertrophic scar formation was evaluated by histomorphometric analysis following a previously described procedure (Morris et al., 1997). Each digital image was processed with Image-Pro Plus (Adobe Photoshop; Adobe Systems, San Jose, CA) to measure the total area of the new scar, which included the original tissue area before wounding and the elevated scar region over it. The original tissue area before wounding was estimated by measuring the thickness of adjacent unwounded dermis. The scar elevation index for each hypertrophic scar was derived by calculating the ratio of the total area of the new scar to the estimated original tissue area. The animal experiment was approved by the Institutional Ethics Committee for Animal Care of Korea Institution of Science and Technology and followed the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) as adopted and promulgated by the National Institutes of Health.

Immunohistochemistry

For immunohistological evaluation of dermal tissue, 5-μm-thick slices of tissue section were deparaffinized with xylene and rehydrated, treated with 3% H_2O_2 for 5 min, and blocked with 1% normal sera. The slides were then incubated with antibodies of α-smooth muscle actin (1:400 dilution) or F4/80 (1:100 dilution), followed by incubation with goat anti-mouse antibody conjugated with horseradish peroxidase (1:200 dilution; GenDEPOT, Baker, TX). The signals on the tissues were developed with DAB+ Substrate-Chromogen (Dako, Carpinteria, CA) according to the manufacturer's instructions. Thereafter, counterstaining was performed with hematoxylin, and all sections were photographed under a microscope.

Statistical Analysis

Data were analyzed and plotted on graphs by using SigmaPlot software (Systat Software Inc., San Jose, CA). Statistical analysis for comparisons between two groups was performed by using an unpaired Student's *t* test. Statistical analysis for estimating a significant difference in the wound closing rate between wounds of the LCB 03-0110-treated group and nontreated control group was performed by using repeated-measures analysis of variance. A p value of <0.05 was considered statistically significant.

Results

LCB 03-0110. We aimed to discover a small-molecule inhibitor against DDR tyrosine kinase 2. Using the activated form of DDR2 cytosolic kinase domain protein, we searched our in-house compound library for the inhibitor. Optimization of a hit compound by medicinal chemistry efforts produced a novel compound, LCB 03-0110 (Fig. 1). This compound showed a potent inhibition against the activated tyrosine kinase activity of DDR2 with IC_{50} values (95% confidence interval) of 6 nM (4–9 nM), whereas it gave IC_{50} values (95% confidence interval) of 145 nM (128–164 nM) against the nonactivated form of DDR2 tyrosine kinase, indicating that the compound is more inhibitory against the activated form of DDR2 tyrosine kinase (Fig. 2A). The inhibition kinetics experiment of LCB 03-0110 against the activated DDR2 tyrosine kinase indicated that inhibition occurs in an ATP-competitive manner (Fig. 2B). Using HEK293 cells engineered to overexpress either DDR1b (HEK293-DDR1b) or DDR2 (HEK293-DDR2), we analyzed the inhibition of collagen-induced DDR1b or DDR2 receptor autophosphorylation by LCB 03-0110. LCB 03-0110 could suppress the induced autophosphorylation of DDR1 and DDR2 similarly with an IC_{50} of approximately 164 and 171 nM, respectively (Fig. 2, C and D). These results suggest that LCB 03-0110 inhibits both DDR1 and DDR2 tyrosine kinase activity with almost the same potency. DDR1 and DDR2 were reported to share 89% homology in their tyrosine kinase domain (Vogel

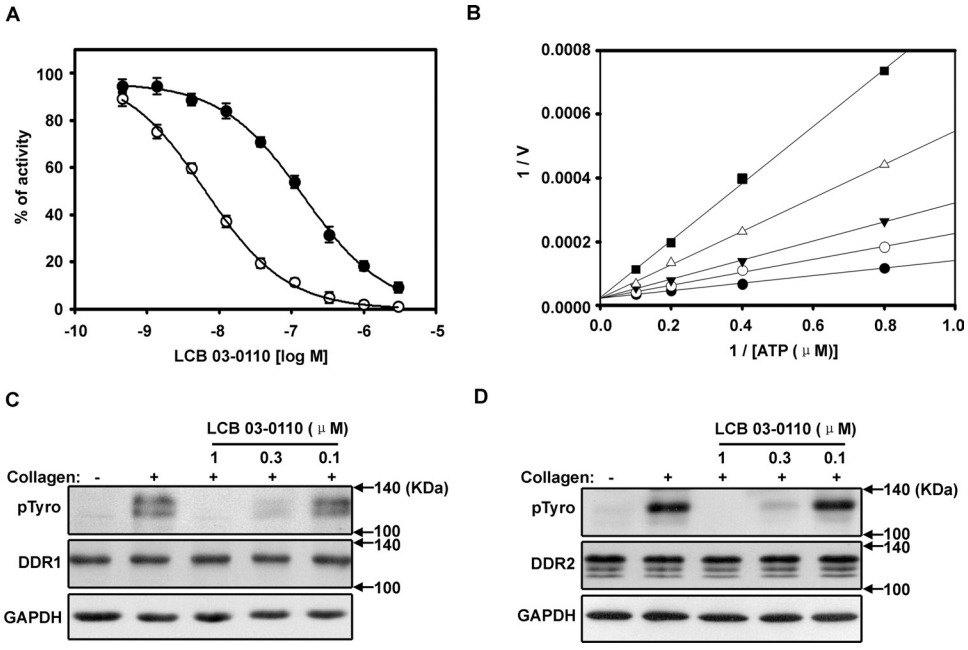

Fig. 2. LCB03-0110 inhibits the tyrosine kinase activity of DDR1 and DDR2. A, dose-dependent inhibition against the in vitro activity of activated (○) and nonactivated DDR2 tyrosine kinase (●) by LCB 03-0110. Data were fitted on a sigmoid curve by using SigmaPlot software (Systat Software Inc.). B, reciprocal plotting of DDR2 kinase activity in the presence of various concentrations of LCB 03-0110, using the least-square method. LCB 03-0110 concentrations: no inhibitor (●), 1.1 nM (○), 3.3 nM (▲), 10.0 nM (△), and 30.0 nM (■). C and D, type 1 collagen-induced autotyrosine phosphorylation of DDR1 (C) and DDR2 (D) expressed in HEK293 cells and their inhibition by LCB 03-0110. Tyrosine phosphorylation in DDR protein was detected by Western blotting with phosphotyrosine antibody and quantitated by densitometry for estimating IC_{50} values. GAPDH was used as the equal loading control.

et al., 2006). In a kinase panel inhibition assay against 60 kinases with 10 μM concentration of LCB 03-0110, the compound produced more than 90% inhibition against 20 tyrosine kinases: all eight Src family kinases, Btk, Syk, Tie2, FLT1, FLT3, FLT4, EphA3, EphB4, VEGF receptor 2, misshapen/niks-related kinase 1, c-Abl, and RET (rearranged during transfection) (see Supplemental Table 1). This suggests that LCB 03-0110 is a pan tyrosine kinase inhibitor. Therefore, we further estimated IC_{50} values of 21 more tyrosine kinases, and the values are shown with that of activated form of DDR2 in Table 1. We chose these 21 kinases with the ones inhibited strongly by LCB 03-0110 as described in Supplemental Table 1 and ones to help us guess its selectivity. It is noteworthy that it was confirmed that LCB 03-0110 is able to potently inhibit tyrosine kinases that play important roles in the signaling of various immune cells, for instance, c-Src family proteins, Btk, and Syk, in addition to its strong inhibition against the DDR tyrosine kinase family.

Inhibition of Macrophage and Fibroblast Activation In Vitro. Because LCB 03-0110 showed potent inhibition against the DDR tyrosine kinase family as well as c-Src family tyrosine kinases, and these two family kinases are involved in cellular signaling pathways for the activation of macrophage and fibroblast cells, we were interested in testing whether this compound could have an antifibroinflammatory activity by inhibiting the activation of both macrophage and fibroblast cells.

To test whether this chemical could suppress the activation of macrophages, we stimulated J774A.1 macrophage cells with LPS in the presence of LCB 03-0110 for 1 h and examined alterations in the activation of cellular molecules involved in the activation of macrophage cells. The activation of macrophage cells by LPS induced the activating phosphorylation of p38 and JNK. However, LCB 03-0110 potently suppressed the induction of these proteins at IC_{50} values of approximately 140 and 160 nM for p38 and JNK, respectively, although it did not alter the cellular amount of these two proteins (Fig. 3A). These results suggest that LCB 03-0110 inhibits the pathways for the activation of p38 and JNK. In contrast, LCB 03-0110 had an almost negligible effect on the activation of MAPK even at 10 μM concentration (Fig. 3B). On the other hand, the activation by LPS reduced the amount of cellular IκB-α protein level, and this reduction was associated with the increase of the phosphorylation at serine 32 at IκB-α. However, LCB 03-0110 treatment could not prevent the decrease of the IκB-α level by LPS at all, but instead even further reduced the IκB-α level at 10 μM concentration. This was correlated with an observation that LCB 03-0110 did not significantly prevent the LPS induced phosphorylation of IκB-α at serine 32 (Fig. 3B). These data show that LCB 03-0110 does not modulate the pathways involved in the phosphorylation of MAPK and IκB in the activation of macrophages. Taken together, these results suggest that LCB 03-0110 could suppress macrophage activation by inhibiting the signaling pathways leading to the activation of p38 and JNK. Next, we examined whether LCB 03-0110 could inhibit the enhanced production of inflammatory mediators when macrophage cells were activated. The induction of iNOS and COX-2 by treating J774A.1 macrophage cells with LPS was suppressed considerably by LCB 03-0110 at IC_{50} values of approximately 240 and 190 nM, respectively (Fig. 3C). In addition, LCB 03-0110 inhibited the induction of NO and TNF-α at IC_{50} values of approximately 750 nM and 1.99 μM, respectively, in the activated macrophage cells (Fig. 3, D and E). One of the important characteristics for macrophages to participate in inflammatory reactions is an enhanced migratory capability. J774A.1 macrophage cells activated by LPS showed an elevated migration through type VI collagen matrix when the migration was induced by monocyte chemotactic protein-1. However, treatment with LCB 03-0110 inhibited the migration at an IC_{50} of approximately 1.05 μM (Fig. 3F). Taken together, it is clear that LCB 03-0110 can inhibit the inflammatory activities of activated macrophage cells.

Next, we tested whether LCB 03-0110 could also suppress the activation of fibroblasts. When fibroblast cells become activated into myofibroblast-type cells during wound healing, they show an enhanced expression of α-smooth muscle actin and an increased capacity for migration. To activate primary dermal fibroblasts in an in vitro cell culture, we treated the cells with TGF-β1 in culture plates with or without type I collagen coating. TGF-β1 treatment considerably induced the expression of α-smooth muscle actin, whereas plating the cells on the collagen-coated dish only slightly increased the expression. The highest induction of α-smooth muscle actin was observed to be approximately 16-fold when TGF-β1 was applied to cells in the collagen-coated plate. However, this induction was abolished by LCB 03-0110 at an IC_{50} of approximately 170 nM (Fig. 4A). In addition, we examined the activation of signaling mediators such as FAK and Akt1. TGF-β1 significantly induced the activating phosphorylation of FAK at tyrosines 397, 407, 577, and 861, but relatively slightly at tyrosine 576. On the other hand, plating cells on the collagen-coated surface slightly increased the phosphorylation at all five tyrosine residues. It is noteworthy that treatment with TGF-β1 of dermal fibroblast cells plated on the collagen-coated plate showed a markedly enhanced phosphorylation at two tyrosines (residues 576 and 861) among the five phosphorylated tyrosines, suggesting that TGF-β1 and collagen signaling synergistically induce the phosphorylation of these two residues. However, it is noteworthy that LCB 03-0110 treatment of cells exposed to TGF-β1 and type I collagen showed a marked inhibition preferentially in the phosphorylation of tyrosine 861, to give approximately 50% reduction at 1 μM and an almost complete abolishment at 3 μM, whereas more or less suppression of phosphorylation

TABLE 1
IC_{50} values of LCB 03-0110 for the inhibition of protein tyrosine kinases

Kinase	IC_{50}	Kinase	IC_{50}
	nM		*nM*
DDR2	6.0	Syk	25.2
c-Src	1.3	ZAP70	1732
Lck	21.6	VEGFR2	4.6
Lyn	4.3	EphA3	5.1
Fyn	2.3	FLT3	26.1
Yes	2.1	EGFR	273
Fgr	3.7	IR	10,750
Hck	4.4	PDGFRα	69
Blk	50.7	PDGFRβ	409
Btk	17.7	c-Kit	591.7
Itk	>10,000	FGFR1	827.8

Blk, B-cell lymphocyte kinase; Itk, IL2-inducible T-cell kinase; ZAP70, ζ-chain-associated protein kinase 70; VEGFR2, VEGF receptor 2; EGFR, endothelial growth factor receptor; IR, insulin receptor; PDGFR, PDGFR receptor; FGFR, fibroblast growth factor receptor.

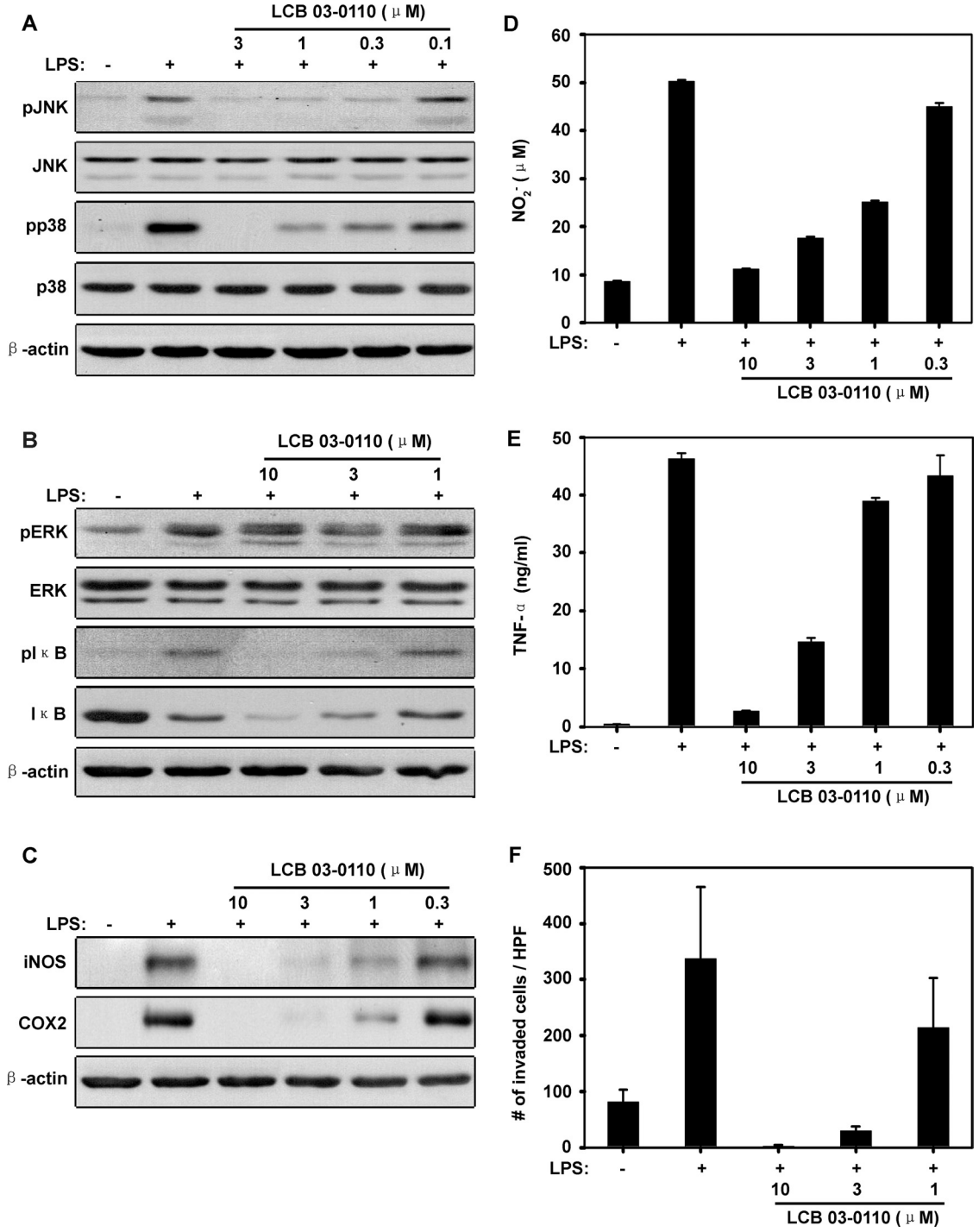

Fig. 3. LCB 03-0110 suppresses the activation of J774A.1 macrophage cells. A to C, after treating J774A.1 cells with LPS and LCB 03-0110 for 1 h (A and B) or 18 h (C), Western blot analysis was performed by using the total cell lysate. The degree of the induced phosphorylation of each protein and its inhibition by LCB 03-0110 was quantitated by densitometry for estimating IC_{50} values. β-Actin was used as the equal loading control. ERK, extracellular signal-regulated kinase. D and E, nitrite accumulation assay (D) and enzyme-linked immunosorbent assay for TNF-α expression (E) were performed by using culture media of cells treated with LPS and LCB 03-0110 for 18 h, and the average values with S.D. from three independent experiments are shown as a bar graph. F, J774A.1 cells treated (+) or not (−) with LPS were allowed to invade through type IV collagen gel in the presence of LCB 03-0110. A total of 10 digital images of high-power fields (HPFs) at 400× magnification for invaded cells were obtained at each condition from three independent experiments. The average values with S.D. of invaded cells/HPF are shown as a bar graph.

was observed in the other four tyrosines (Fig. 4B). This result suggests that LCB 03-0110 can inhibit FAK activation in the activated fibroblasts mainly by inhibiting cellular signaling to stimulate the phosphorylation of its tyrosine 861. We also studied the activation of Akt1 in primary dermal fibroblasts treated with TGF-β1 and type I collagen and its inhibition by LCB 03-0110. In this study, we found that the activating phosphorylation of Akt1 at threonine 371 was not induced by TGF-β1 treatment and was even suppressed slightly by the presence of type I collagen. However, it is noteworthy that the simultaneous stimulation of cells with TGF-β1 and type I collagen considerably enhanced its phosphorylation and

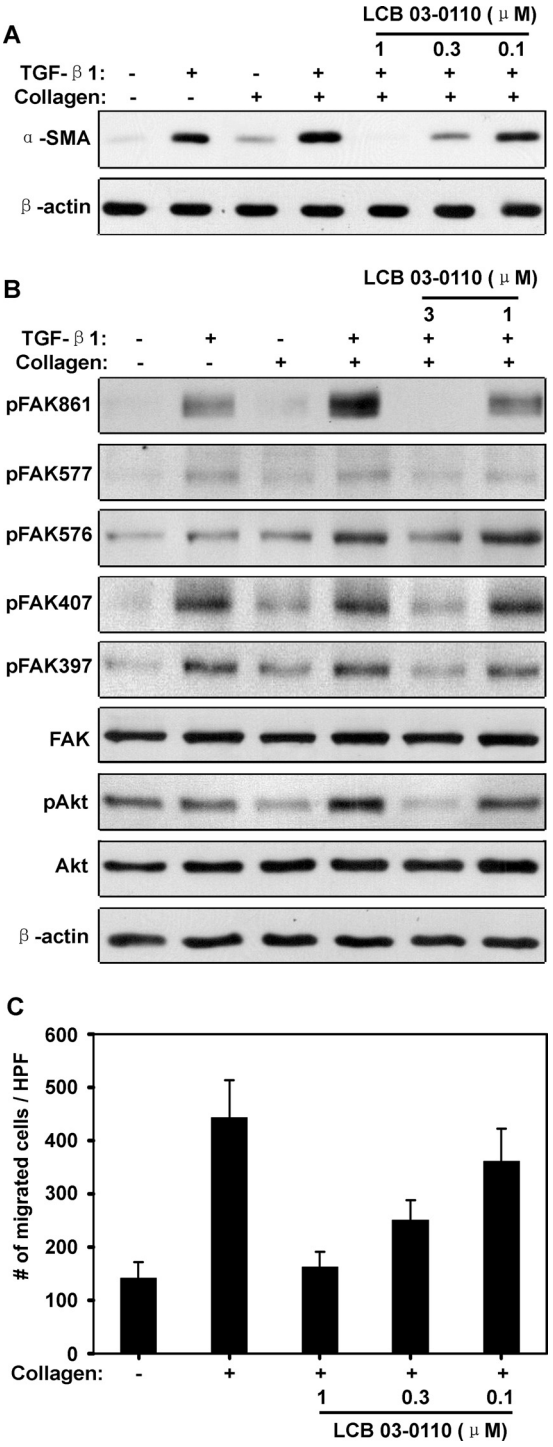

Fig. 4. LCB 03-0110 suppresses the activation of dermal fibroblast cells. A and B, primary dermal fibroblast cells plated on the control 12-well plates (−) or on plates coated with type I collagen (+), using DMEM containing 0.5% FBS, were treated with 5 ng/ml TGF-β and LCB 03-0110 for 48 h. The total cell lysate was subjected to Western blot analysis. β-Actin was used as the equal loading control. C, the migratory ability of the dermal fibroblast cells plated either on type I collagen-coated (+) or noncoated (−) wells was estimated by incubating the cells for 24 h in the presence of LCB 03-0110 after generating a scratch wound in the confluent monolayer culture. A total of six digital images of HPFs at 400× magnification for cells that migrated into the scratched area were obtained at each condition from three independent assays. Each bar represents the mean with S.D. of the counted cells per HPF.

LCB 03-0110 treatment abolished this enhancement almost completely at 3 μM (Fig. 4B). Taken together, these results show that LCB 03-0110 can suppress the activation of α-smooth muscle actin, FAK, and Akt1 in fibroblast cells. Because these three proteins are involved in the activation of fibroblast cells, these results suggest that LCB 03-0110 could suppress fibroblast activation. To support this conclusion, we tested whether LCB 03-0110 could suppress the ability of fibroblast cells to acquire an increased capacity for migration. We observed that dermal fibroblast cells exhibited a significantly increased migration when plated on the type I collagen-coated surface compared with the noncoated surface of the culture dish. However, LCB 03-0110 treatment inhibited the collagen-induced migration of dermal fibroblast cells at IC_{50} of 194 nM (Fig. 4C).

Suppression of Macrophage and Fibroblast Activation in a Rabbit Ear Wound Healing Model. Upon observing that LCB 03-0110 could suppress the activation of both fibroblasts and macrophages in the in vitro cell culture experiment, we wanted to test whether this compound could also inhibit the activation of the two cell types in an animal model of wound healing. We generated full excisional wounds on rabbit ears and applied the compound topically on the wound sites. After 2 days, we performed an immunohistochemical examination of the population of activated myofibroblast cells and the accumulation of macrophage cells in the area of regenerating tissue in the wound site. A 0.1% solution of LCB 03-0110 treatment significantly reduced the number of α-smooth muscle actin-positive myofibroblast cells by 57.7% and the F4/80 positive macrophage cells by 37.7%, indicating that LCB 03-0110 suppressed the accumulation of macrophage and myofibroblast cells in the wound site by inhibiting their activation (Fig. 5). This result suggests that LCB 03-0110 can suppress the activation of fibroblasts and macrophages in an animal model of wound healing, which is consistent with the results from the cell culture experiment.

Suppression of Hypertrophic Scar Formation in the Rabbit Ear Wound Healing Model. Fibroinflammation during skin wound healing results in a hypertrophic scar after wound closing. The elevation of the scar over the original skin tissue reflects the intensity of scar formation. Using a full excisional wound healing model on rabbit ears, we attempted to estimate how much potently LCB 03-0110 could reduce scar formation by measuring the scar elevation index of the scar formed after wound closing. When we applied 0.1% solution of LCB 03-0110 topically into the wound site every 3 days after the generation of wound, this compound significantly prevented hypertrophic scar formation by reducing the scar elevation index by 38.5% compared with that in the control group treated with only the carrier solution (Fig. 6, top). Considering that scar formation resulted from fibroinflammation by activated macrophage and myofibroblast cells, this result is consistent with the conclusion that LCB 03-0110 exhibits antifibroinflammatory activity by suppressing the activation of fibroblasts and macrophages. In this wound healing experiment, we observed that treatment with LCB 03-0110 did not delay wound closing but seems to have stimulated it, as shown by the wound closing rate obtained by monitoring the reduction of the unclosed wound area (Fig. 6, bottom). This suggests that LCB 03-0110 does not interfere with the regeneration of the epidermal layer,

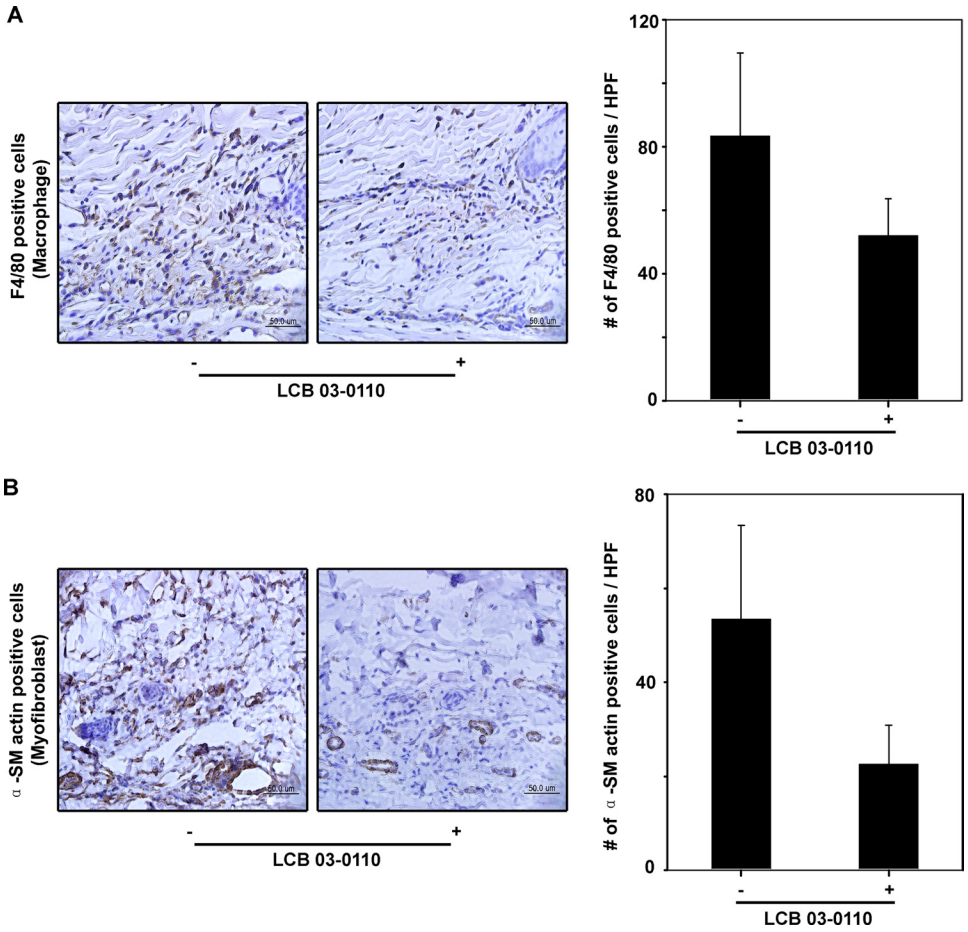

Fig. 5. LCB 03-0110 inhibits the accumulation of macrophage and myofibroblast cells in the wound site. Either 0.1% LCB 03-0110 or the carrier solution as a control was applied topically to full excisional wounds generated on the ears of New Zealand white rabbits with occlusive dressing. After 2 days, a 5-μm slice of the wound tissue section was immunostained. A, left, a representative image of immunostaining of macrophage cells in the regenerating deep dermis with the F4/80 antibody. Right, the bar graph shows the mean (S.D.) of the number of positively stained cells per HPF at 400× magnification from 12 digital images in the $n = 4$ experiment. LCB 03-0110 significantly reduced the number of macrophage cells ($p < 0.01$). B, left, a representative image of immunostained myofibroblast cells in the regenerating deep dermis with antibody against α-smooth muscle actin. Right, the bar graph represents the mean with S.D. of the number of positively stained cells/HPF at 400× magnification from 12 digital images in the $n = 4$ experiment. LCB 03-0110 significantly reduced the number of myofibroblast cells ($p < 0.01$).

which is responsible for wound closing, whereas it acts on fibroblast and macrophage cells with suppressing their activation.

Discussion

Whenever inner and outer bodily organs acquire injury caused by various damaging stimuli, a preprogrammed wound healing mechanism is immediately turned on as a natural defense mechanism of the body. However, an inflammatory response during the process activates an undesirable fibrotic pathology that results in scar formation and organ fibrosis. Although it is suggested that activated macrophage and myofibroblast cells are primarily involved in fibrosis-associated inflammation during the wound healing process (Martin et al., 2003; Gurtner et al., 2008; Wynn, 2008), to date there have been few attempts to simultaneously suppress the activation of macrophage and fibroblast cells to prevent scar formation and fibrosis. In this work, we demonstrated that LCB 03-0110 can reduce fibroinflammation and scar formation in a skin wound healing model, and this function is tightly associated with the ability of the compound to simultaneously suppress the activation of both macrophage and fibroblast cells. This suggests that inhibiting the activation of both macrophages and fibroblasts by a small molecule, as we demonstrated here, could be an effective strategy in developing an antifibroinflammatory agent.

LCB 03-0110 was identified as a pan kinase inhibitor because it inhibits various tyrosine kinases as shown in Table 1. We think that the activity of LCB 03-0110 to inhibit scar formation and activations of macrophage and fibroblast would be based on its nature as a multikinase inhibitor. Among these kinases, we propose that its inhibitory activity against DDR family and c-Src family tyrosine kinases might have a major role in the suppression of the activation of both cell types. This argument can be supported by the fact that DDR family proteins have been reported to be involved in the activation of fibroblasts (Olaso et al., 2002) and macrophages (Franco et al., 2009), and c-Src family tyrosine kinases such as Hck, Fgr, and Lyn were shown to be involved in cell signaling to activate macrophages (Cohen, 2002).

Although LCB 03-0110 is a pan kinase inhibitor, it seems to work with a certain degree of specificity in modulating cellular signaling in activated macrophage and fibroblast cells. LCB 03-0110 inhibited p38 and JNK activation, whereas it has no influence on the pathways that induce MAPK and Iκb phosphorylation although all four pathways are associated with the activation of macrophage by LPS (Guha and Mackman, 2001). This might be a reason we needed higher concentrations of LCB 03-0110 to inhibit the production of NO and TNF-α and cell migration than those required to suppress the activated p38 and JNK in J774A.1 macrophage cells stimulated by LPS. Furthermore, among the pathways that lead to an activating phosphorylation at tyrosine residues within the FAK protein in fibroblast cells, LCB 03-0110 interferes preferentially with the pathway that induces phosphorylation at tyrosine 861. The phosphorylation of tyrosine 861 of FAK was suggested to be involved in stimulating cell migration (Lim et al., 2004), and this pro-

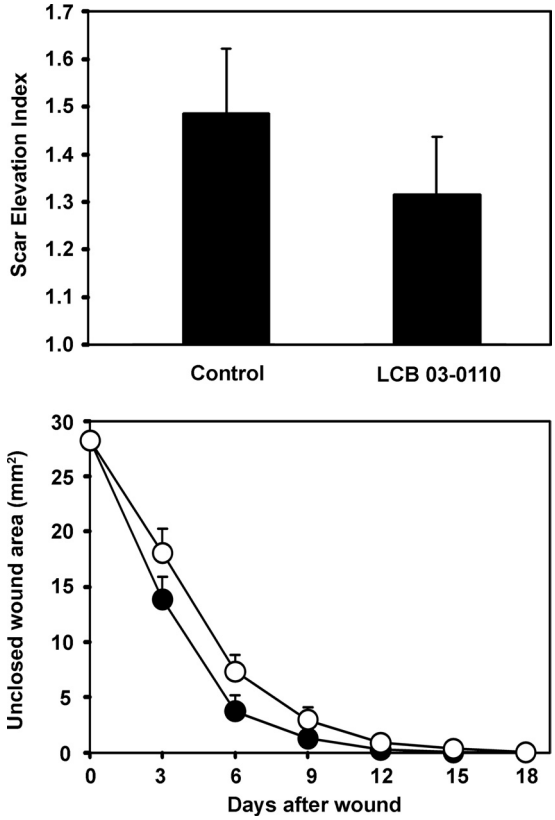

Fig. 6. LCB 03-0110 suppresses hypertrophic scar formation in a wound healing model. Full excisional wounds on the ear of New Zealand white rabbits were treated every 3 days topically with either 0.1% LCB 03-0110 or the carrier solution for 21 days. Top, a total of 12 closed wounds for each treated group were analyzed to measure the scar elevation index, as described under *Materials and Methods*. The mean (S.D.) values are depicted as a bar graph. LCB 03-0110 significantly suppressed hypertrophic scar formation ($p < 0.01$). Bottom, the unclosed wound areas of 12 wounds for each group were calculated by using the following formula: 3.14 × (the longest diameter and the shortest diameter of the unclosed wound area)/2 every 3 days. The mean (S.D.) values are depicted as a line graph. ○, LCB 03-0110-treated group; ●, carrier-only group. The increase of wound closing rate by treating LCB 03-0110 was significant ($p < 0.01$).

vides an explanation for the ability of LCB 03-0110 to suppress the migration of fibroblast cells. It is noteworthy that LCB 03-0110 did not interfere with cellular activities that promote wound repair and closing, whereas it significantly reduced the populations of macrophage and myofibroblast cells during wound healing, and the scar formation after wound closing, in our skin wound healing model experiment. Taken together, these results suggest that LCB 03-0110 works with a certain degree of specificity in modulating cell signaling pathways and cellular activities.

We noted that the in vitro kinase inhibition profile of LCB 03-0110 is similar to that of dasatinib (SPRYCEL, BMS-354825). In addition to its strong inhibitory activity against tyrosine kinases of c-Abl, c-Src, and the DDR family, dasatinib inhibited Btk potently to suppress inflammatory activity by eosinophils (Hantschel et al., 2007). In addition, it inhibited T-cell activation in a mechanism involving its inhibition of the Lck kinase (Schade et al., 2008) and suppressed TNF-α production after stimulation of Toll-like receptor signaling with LPS, in vitro and in vivo (Fraser et al., 2009). Dasatinib was suggested to be an antifibroinflammatory agent on the basis of these results, although no concrete study has been published yet to confirm this conclusion (Day et al., 2008). However, when we applied dasatinib to our rabbit ear wound healing model experiment, it severely interfered with the wound closing process in contrast to LCB 03-0110, which stimulated wound healing (X. Sun, unpublished data). We think that a certain difference in the kinase inhibition profile between LCB 03-0110 and dasatinib might be responsible for this different activity concerning wound healing, despite having a largely similar kinase inhibition profile, including their potent inhibition activity against DDR and c-Src family tyrosine kinases.

Authorship Contributions

Participated in research design: Sun, Phan, Jung, Kim, Cho, Lee, Woo, Park, and Yang.

Conducted experiments: Sun, Phan, Jung, Kim, Cho, Lee, and Woo.

Performed data analysis: Sun, Phan, Jung, Kim, Cho, Lee, Woo, Park, and Yang.

Wrote or contributed to the writing of the manuscript Sun, Phan, Kim, Park, and Yang.

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