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Original Research

Novel Indolyl-Benzimidazole Compounds Promote *in vitro* Wound Healing and Osteogenic Differentiation of Pluripotent Cells

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Abstract

Background: Increasing or restoring Bone Morphogenetic Protein- (BMP-) signaling through administration of recombinant BMPs (rBMPs) has demonstrated therapeutic efficacy for treating bone fractures or to enhance repair following spinal surgeries. However, direct use of rBMPs has come up against significant obstacles like high cost and incidence of adverse effects. Recently, we reported our findings on the novel indolyl-benzimidazoles, SY-LB-35 and SY-LB-57, that fully activated BMP receptor signaling demonstrating activity profiles that mirrored rBMPs. Here, we explored the potential of these compounds to substitute for rBMPs in processes like wound healing and osteogenesis. **Methods**: Cell-based assays including cell viability, short- and long-term phosphorylation, protein expression, wound healing and bone differentiation assays were carried out in the pluripotent myoblast C2C12 cell line with select assays performed in multiple cell lines. Several assays included conditions in the presence of a selective inhibitor of type I BMP receptor, Activin-like kinase 2 (ALK2), or inhibitors of BMP-stimulated downstream signaling. All assays were repeated at least 3 times with replicates per condition where indicated. Statistical tests were carried out using Student's two-tailed, *t*-test. **Results**: Sustained activation of non-canonical BMP signaling pathways was observed after 24-hour exposure to SY-LB-35 and SY-LB-57. Moreover, this treatment increased the expression of targets of BMP-mediated transcription such as the Id1 transcription factor. SY-LB-35 and SY-LB-57 promoted substantial increases in cell viability in three distinct cell types and increased the rate of wound closure in scrape-wounded C2C12 cell cultures. Cell viability and wound closure induced by SY-LB compounds required ALK2-, PI3K- and p38-dependent pathways. In contrast, responses to SY-LB compounds were not affected by ERK inhibition. Expression of bone differentiation markers beginning at 4 hours and evidence of calcium deposition detected after 21 days in C2C12 cell cultures exposed to SY-LB-35 and SY-LB-57 demonstrated the osteogenic potential of these compounds. **Conclusions**: The functional similarities between these novel compounds and rBMPs indicates that SY-LB-35 or SY-LB-57, acting as potent activators of BMP receptor signaling and inducers of osteogenic processes, could potentially replace rBMPs for treating BMP-related pathologies such as bone fracture repair or other wound healing processes.

Keywords: BMP; BMP receptors; indolyl-benzimidazoles; heterocycles; wound healing; bone growth

1. Introduction

Bone Morphogenetic Proteins (BMPs) are a family of extracellular factors that were initially discovered for the ability to differentiate mesenchymal cells into osteoblasts and later induce ectopic bone formation [1]. Now, BMPs are known to play critical roles during embryogenesis and development, and also for maintenance of adult tissue homeostasis in many organ systems. Due to the widespread expression and importance as regulators throughout the body, deficiency in BMP production or functionality usually leads to marked defects or severe pathologies affecting cardiovascular and pulmonary, gastrointestinal, urinary, neurological, ophthalmic and musculoskeletal systems [2,3]. Mouse knockout models targeting different components of BMP signaling leads to embryonic lethality or significant defects, emphasizing the essential developmental functions of BMPs [3,4].

BMPs stimulate intracellular signaling in a variety of cell types such as mesenchymal cells, bone marrow stromal cells, monocytes and sensory spinal interneurons [5–7]. These proteins control important cellular processes like proliferation, differentiation, chemotaxis, axon guidance and apoptosis [8–10]. BMP dimers elicit cellular responses by simultaneously binding pairs of type I and type II serine-threonine kinase transmembrane receptors [11–13]. Ligand binding triggers assembly of receptor pairs into an active tetrameric complex [13]. In the absence of ligand stimulation, small fractions of type I and type II BMP receptors are present as pre-existing homodimers and heterodimers on the cell surface. Binding of ligand increases oligomerization of the receptors, which may induce conformational changes of the receptor molecules [14,15]. Moreover, the composition of the tetrameric complex may impart specificity in signaling outcomes [10,15–17].

Following activation of the tetrameric BMP receptor complex, type I BMP receptors phosphorylate downstream, pathway restricted Smads (R-Smads – Smad1, Smad5, and Smad8), which are important mediators of BMP transcriptional responses [18–20]. Phosphorylated R-Smads (p-Smads) dissociate from the receptors and form a complex with Smad4. In the nucleus, the R-Smad/Smad4 complex binds to BMP-responsive DNA elements and regulates gene expression [21–23]. BMPs also activate non-Smad intracellular signaling including p38 and Extracellular-regulated kinase (ERK) pathways, which are also responsible for regulation of gene transcription and known to play an important role in BMP-induced osteogenesis [24–26].

Because of its diverse functions and osteogenic potential, the Food and Drug Administration (FDA) approved use of human recombinant BMP2 (rBMP2) during spinal fusion and maxillary sinus reconstructive surgeries and for tibial shaft repair [27,28]. Although stimulation of BMP receptor-dependent pathways has been shown to be beneficial, the clinical use of rBMPs poses significant obstacles [28,29]. For example, the high concentrations of rBMP2 required to regenerate or repair bone leads to translational barriers due to high formulation costs. Moreover, clinical use of rBMPs requires potentially harmful doses to achieve efficacy and penetration of proteinaceous rBMP-based therapeutics is poor [27–29]. Thus, alternative strategies are needed to exploit the beneficial effects of BMP signaling. The cost-effective production of small molecule activators of BMP pathways, which can be formulated in large quantities with minimal difficulty and efficiently targeted to diseased or injured areas, would constitute a substantial advance in the BMP field. Such small molecules could replace rBMPs for the treatment of bone injuries or other BMP-related pathological conditions.

Our lab has recently demonstrated that select members of a novel series of small synthetic benzimidazole compounds robustly stimulate BMP signaling and act as BMP receptor agonists [30]. The current study extends our initial investigation of the indolyl-benzimidazole small molecules, SY-LB-35 and SY-LB-57. Assessment of cell viability in multiple cell types demonstrated a common cellular response to the SY-LB compounds. In C2C12 cells, cell viability responses to SY-LB-35 and SY-LB-57 were blocked by a selective inhibitor of Activin-like kinase (ALK2), a type I BMP receptor subunit, as well as inhibitors to Phosphatidyl inositol 3-kinase (PI3K)- and p-38-dependent pathways, but not by an inhibitor to ERK signaling. Amplification of cell viability and activation of non-canonical BMP signaling were detected at picomolar concentrations of SY-LB-35 and SY-LB-57. Interestingly, Smad phosphorylation is undetectable at these low concentrations. In response to SY-LB compounds, Western blots showed sustained activation of p38 and ERK pathways, as well as increases in the expression of BMP-regulated proteins, Id1 and BMP receptor type 2 (BMPR2). Wound healing assays revealed that wound closure promoted by SY-LB compounds was dependent upon ALK2, PI3K and p38 but not ERK activity. With respect to osteogenesis, bone-related gene products, such as β-catenin and Runx2, were detected after a 4-hour exposure to the small molecules, SY-LB-35 and SY-LB-57. Moreover, considerable upregulation of osteogenic markers, alkaline phosphatase (ALP) and osteocalcin (OCN), were observed after 3 and 7 days, respectively. Furthermore, long-term exposure to SY-LB-35 produced cultures that exhibited evidence of calcium deposition. These results establish the indolyl-benzimidazole compounds as small molecule, BMP receptor agonists and demonstrate the osteogenic potential of these novel compounds. Collectively, our findings reveal that SY-LB-35 and SY-LB-57 are potent replacements for rBMPs *in vitro* and, as small molecules that are efficiently synthesized at minimal cost, warrant further development as prospective therapeutics for bone growth and repair.

2. Materials and Methods

2.1 Synthesis and Purification of Indolyl-Benzimidazole Compounds

SY-LB-35 and SY-LB-57 are indolyl-benzimidazoles that were synthesized and purified by HPLC by Dr. Leonard Barasa in the laboratory of Dr. Sabesan Yoganathan at St. John's University (Queens, NY, USA). Details of SY-LB synthesis, purification and characterization were previously reported [30,31].

2.2 Cell Biology-Related Materials

C2C12 mouse myoblast cells (CRL-1772), WEHI 274.1 (WEHI) cells (CRL-1702), and primary Pulmonary Artery Endothelial Cells (PAECs) (PCS-100-022) were obtained from American Type Culture Collection (ATCC®) (Manassas, VA, USA). All three cell lines were certified to be free of mycoplasma contamination by using the MycoStrip testing kit (#rep-mys-10) from InvivoGen (San Diego, CA, USA) for C2C12 cells or by DAPI staining of fixed WEHI cells and PAECs. The authenticity of the C2C12 cell line was established using ATCC Mouse Cell Authentication Services in accordance with the Consortium for Mouse Cell Line Authentication. Moreover, the C2C12 and WEHI 274.1 cell lines do not appear as cross-contaminated or misidentified in either the Cell Line Authentication Committee or the ExPASy database. Dulbecco's Modified Eagle's Medium (DMEM), Penicillin/Streptomycin/Glutamine solution (PSG), Penicillin/Streptomycin solution (PS), 0.25% Trypsin-EDTA, Ca^{2+}-, Mg^{2+}-free Dulbecco's-Phosphate Buffered Saline (D-PBS) were purchased from Gibco by Life Technologies (Gaithersburg, MD, USA). Fetal Bovine Serum (FBS) was obtained from Atlanta Biologicals (Flowery Branch, GA, USA). Vascular Cell Basal medium, Endothelial Cell Growth Kit – BBE, Trypsin/EDTA solution for Primary cells, and Trypsin Neutralizing Solution for Primary cells were obtained from ATCC® (Manassas, VA, USA).

Cell Lysis Buffer (10×) was obtained from Cell Signaling Technologies (Danvers, MA, USA). TGX Fast Cast Acrylamide gel kit (12%), 4× Laemmli Sample Buffer, 10× Tris/Glycine/SDS Running Buffer and Precision Plus Protein Standards were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Amersham™ Protran™ 0.2 µm Nitrocellulose membranes were obtained from GE Healthcare Life Sciences (Pittsburgh, PA, USA). Bovine Serum Albumin (30% solution) was from Alfa Aesar (Ward Hill, MA, USA). SuperSignal™ West Femto HRP Substrate solutions for chemiluminescent imaging was from Thermo Scientific (Rockford, IL, USA).

Recombinant BMP2 was purchased from R&D Systems (Minneapolis, MN, USA). The RealTime-Glo™ MT Cell Viability Assay Kit was purchased from Promega Corporation (Madison, WI, USA). PD98059 (PD), LDN193189 dihydrochloride (LDN), and SB202190 (SB) were purchased from Tocris Biosciences (Bristol, UK). LY294002 (LY) was obtained from Cell Signaling Technology (Danvers, MA, USA) and Mitomycin C (MC) was from Cayman Chemical Company (Ann Arbor, MI, USA). Recombinant mouse ALP and mouse OCN ELISA kits were obtained from Novus Biologicals (Centennial, CO, USA). p-N-Phenyl-Phosphate Substrate (p-NPP) was purchased from Life Technologies (Fredrick, MD, USA). Alizarin Red S was procured from Sigma Aldrich (St. Louis, MO, USA).

2.3 Antibodies

Phospho-Smad1/5(S463/465)/9(S465/467) rabbit monoclonal antibody (mAb) (#13820S), Smad1 (D5907) XP® rabbit mAb (#6944), phospho-Akt (Ser473) (D9E) XP® rabbit mAb (#4060), Akt (pan) (C67E7) rabbit mAb (#4691), β-actin mAb (#4970) were obtained from Cell Signaling Technology (Danvers, MA, USA). The BMPR2 mouse mAb (#612292) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). β-catenin rabbit polyclonal antibody (pAb) (#A11932), phospho-p38 rabbit pAb (#AP0526), p38 rabbit pAb (#A11340), phospho-ERK1 rabbit pAb (#AP0472), ERK1/2 rabbit mAb (#A4782), phospho-Cyclin-dependent kinase 1 (p-CDK1) rabbit pAb (#AP0015), CDK1 rabbit pAb (#A2861), Smad4 rabbit pAb (#A5657), Smad6 rabbit pAb (#A0579), Smad7 rabbit pAb (#A12343), Runx2 rabbit pAb (#A2851) and Id1 rabbit pAb (#A8432) were obtained from Abclonal (Woburn, MA, USA).

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (#sc-2004) was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). HRP-conjugated goat anti-mouse IgG (#115035068) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

2.4 Cell Culture

C2C12 and WEHI cells were cultured in complete growth medium (DMEM/10% FBS/1X PSG) incubated at 37 °C in 5% CO_2. Adherent C2C12 cells were washed once with D-PBS and passaged using 0.25% Trypsin-EDTA to detach cells. For WEHI cells, fractions of suspension cultures were transferred to flasks containing fresh growth medium. PAECs were maintained in Vascular Cell Basal medium supplemented with the Endothelial Cell Growth Kit-BBE and the subculture of PAECs was carried out using Trypsin/EDTA solution for Primary cells and Trypsin Neutralizing Solution for Primary cells according to specifications from ATCC®. Determination of cell concentrations for all subsequent assays for these cell lines were performed using the Luna-FL™ Dual Fluorescence Cell Counter from Logos Biosystems (Annandale, VA, USA) following instructions from the manufacturer.

2.5 Cell Viability Assays

C2C12 cells (100 µL at 5×10^5 cells/mL) or PAECs (100 µL at 1×10^4 cells/mL) were seeded into 96-well plates and incubated overnight to achieve ~80% confluency. For suspension cultures of WEHI cells, the cells were washed in unsupplemented medium (DMEM/1 × PS) and seeded into a 96-well plate (100 µL) at 5×10^5 cells/mL. C2C12 and WEHI cultures were serum-starved in DMEM/1 × PS for 16–18 hours and PAECs were starved in unsupplemented Vascular Cell Basal Medium for 2 hours. The next day, serum-starved C2C12, WEHI or PAECs were treated with unsupplemented medium, 125 µM Triton X-100 and SY-LB-35 or SY-LB-57 (0.01–1000 µM) for 24 hours. For assays including ALK2 or signaling pathway inhibitors, serum-starved cultures were pre-treated with the inhibitor for 1 hour before BMP2, SY-LB-35 or SY-LB-57 treatment.

Cell viability of the treated cultures was obtained by using the RealTime-Glo™ MT Cell Viability Assay Kit to detect cellular luminescence with a FilterMax F5 Multimode Microplate Reader (Molecular Devices, San Jose, CA, USA) as previously described [30].

2.6 Stimulation Assays and Preparation of Whole Cell Lysates

Cell treatments, whole cell lysate preparation and quantification of total cellular protein were carried out as previously described [30]. Briefly, C2C12 cells (3 mL at 7.5×10^4 cells/mL) were seeded into 35 mm dishes in fully supplemented growth medium and incubated until the cells occupied ~80% of the culture dish. Next, cells were serum-starved in unsupplemented medium for 16–18 hours followed by treatment with unsupplemented medium, BMP2 (50 ng/mL or 2 nM) or the indicated SY-LB-35 and SY-LB-57 concentrations for 15 minutes, 30 minutes, 4 hours or 24 hours. For ALK2 inhibitor (LDN193189) and Noggin

experiments, serum-starved cultures were pre-treated with LDN193189 or Noggin for 1 hour prior to stimulation with BMP2, SY-LB-35 or SY-LB-57.

2.7 Western Blotting

A detailed method for Western blotting was previously reported [30]. Briefly, samples of whole cell lysates (20 µg) were separated on 12% TGX Fast Cast Acrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA/0.1% Tween 20/ 1 × TBS for 30 minutes at room temperature (RT) and incubated overnight at 4 °C with the desired primary antibody diluted in 1% BSA/0.1% Tween 20/ 1 × TBS. Next, membranes were washed with 1 × TBST (0.1% Tween 20/1 × TBS) three times followed by incubation with the appropriate HRP-conjugated secondary antibody for 1 hour at RT. The membranes were washed again with 1 × TBST three times. Finally, SuperSignal Femto Western HRP substrate solutions were used to develop the Western blot signals, which were captured with the Omega Lum™ G Imaging System (Gel Company, San Francisco, CA, USA). ImageJ (Image Processing and Analysis in Java 1.8.0_112) developed at NIH was used to quantify Western blot signals.

2.8 Wound Healing Assay

In 35 mm culture dishes, C2C12 cells (3 mL) were seeded at 7 × 10^4 cells/mL and grown to form a monolayer, then near confluent cultures were serum-starved for 2 hours in unsupplemented medium. Next, a fresh and sterile 200 µL pipet tip was used to generate a wound in the cell monolayer. Two wounds were made on opposite sides of the dish and the cultures were washed with unsupplemented medium to remove loose cells. For assays including the selective ALK2 inhibitor (5 µM LDN193189), various pathway-specific inhibitors (15 µM LY294002, 10 µM SB202190 and 5 µM PD98059) or an inhibitor of proliferation (30 nM Mitomycin C), C2C12 cell cultures were pre-treated with inhibitors for 1 hour and then stimulated with BMP2 (50 ng/mL or 2 nM) as positive control or 1 µM SY-LB compounds for 18 hours. The following day, cultures were fixed in ice-cold 4% paraformaldehyde/ PBS for 10 minutes at RT, washed twice with ice-cold D-PBS and stored in D-PBS at 4 °C. Phase contrast images (20×) of wound closure were captured at 18 hours for each condition. Wound closure was quantified by counting the number of cells in the scratched area at 18 hours. To analyze the cultures, the borders of the wound were determined from an average of 10 measurements taken from images at time = 0 hours. The number of migrating cells invading the wound was quantified (~25 images per condition per experiment; n = 3) following 18 hours of treatment for each condition.

2.9 ALP Assay

C2C12 cells were seeded in 24-well tissue culture plates at 7 × 10^4 cells/mL and incubated at 37 °C in 5% CO_2 until nearly 100% confluent. The cultures were then treated with a single treatment of BMP2 (200 ng/mL or 7.7 nM), SY-LB-35 (10 µM) or SY-LB-57 (10 µM). The samples were freshly prepared in low serum media (2% FBS/DMEM/1 × PS). Cells grown in low serum medium served as a control.

At 24-, 48- and 72-hours following treatment, 200 µL culture medium from each well of the assay was transferred to a fresh 48-well plate. Low serum media (200 µL) was added back to each culture to replace the collected media. Next, an equal volume of the substrate, p-N-Phenyl-Phosphate (200 µL p-NPP, 10 mM) prepared in Assay Buffer (2.7 g 2-Amino-2-methyl-1,3-propanediol/50 µM $MgCl_2$/50 mL dH_2O, pH = 10) was added to the collected media in the 48-well plate and incubated for 1 hour at 37 °C. Reactions were stopped by addition of 0.3 N NaOH (400 µL) and optical density was measured at 405 nm on a FilterMax F5 Multi-mode Microplate Reader.

At the 72-hour time point, the cultures were washed with D-PBS and trypsinized as described above. Pelleted cells were resuspended in 500 µL D-PBS and cell concentration was determined. A sample of 5 × 10^5 cells was prepared from each treatment. The samples were pelleted and resuspended in 200 µL Assay Buffer. Next, cells were subjected to three freeze/thaw cycles in liquid nitrogen to lyse the cells followed by centrifugation for 10 minutes at 4 °C. The supernatant was transferred to a fresh microcentrifuge tube and 200 µL p-NPP in Assay Buffer was added. Reactions were incubated for 1 hour at 37 °C and stopped by adding 0.3 N NaOH. The optical density of the samples was measured at 405 nm.

2.10 OCN ELISA

C2C12 cells were seeded in 35-mm dishes at 7 × 10^4 cells/mL (3 mL) and grown to near confluency. Cultures were then treated every other day with BMP2 (200 ng/mL or 7.7 nM) as positive control, SY-LB-35 (10 µM) or SY-LB-57 (5 µM) diluted in complete growth medium. After 7 days, the cells were washed gently with cold D-PBS and trypsinized to dissociate the cells. Pelleted cells were washed 3 times with cold D-PBS and the cell concentration was measured. A total of 1 × 10^6 cells were transferred to a fresh microcentrifuge tube and cold D-PBS was added to a total volume of 250 µL. The cells were lysed by three freeze/thaw cycles in liquid nitrogen. Samples were centrifuged for 10 minutes at 1500 ×g at 4 °C and supernatants were transferred to a fresh microcentrifuge tube for use in the OCN ELISA.

Duplicate solutions of OCN protein (0, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 ng/mL) were used to generate a standard curve. Samples of supernatants (100 µL/ well) were transferred to the designated wells of the assay plate and the ELISA was carried out according to instructions from the manufacturer. The optical density was measured at 450 nm.

2.11 Differentiation Assay

C2C12 cells were seeded in 35 mm dishes at 7×10^4 cells/mL and incubated at 37 °C in 5% CO_2 to reach nearly 100% confluency. Next, the cells were treated with 100 ng/mL (3.8 nM) or 200 ng/mL (7.7 nM) BMP2, 10 µM SY-LB-35 or 5 µM SY-LB-57 every other day for 3–10 days (depending on the experiment). The samples were prepared in low serum media (2% FBS/DMEM/1 × PS). The cultures were monitored daily for changes in morphology. Phase contrast images (20×) of treated cultures for each condition were collected at the indicated time points.

2.12 Alizarin Staining Assay

C2C12 cells were seeded at 7×10^4 cells/mL in a 24-well plate and incubated until nearly confluent. Next, the cells were treated with 200 ng/mL (7.7 nM) BMP2, as a positive control, or 10 µM SY-LB-35 every other day for 21 days with treatments freshly prepared in complete growth medium. After 3 weeks, the cells were fixed in 70% ethanol for 15 minutes at RT, washed twice with 1 × PBS, and subsequently, stained with 2% Alizarin Red S for 15 minutes at RT with gentle agitation. Next, the cells were washed 3 times with 1 × PBS to remove excess stain and stored in PBS for imaging. Bright field images (20×) of the cells for each condition were collected.

2.13 Imaging

Phase contrast and bright field images (20×) were collected on a Zeiss Axiovert 25CFL inverted microscope equipped with a Luminera Infinity 3-1 CCD camera and Infinity capture software (version 6.5.4).

2.14 Statistical Analysis

Significance is defined as $p \leq 0.05$. Levels of significance are indicated as follows $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and $****p \leq 0.0001$. Either standard deviation (SD) or standard error (SEM) is reported. All statistical analyses of cell viability assays, Western blots, ALP assay, OCN ELISA, and scratch assay results were performed by Microsoft Excel using Student's two-tailed *t*-tests. The IC_{50} values obtained from cell viability assays were determined by using non-linear regression analyses by GraphPad Prism v.5.0™.

3. Results

3.1 SY-LB Compounds Considerably Amplify Cell Viability in Multiple Cell Types

Three distinct cell types were evaluated for cell viability responses in the presence of the two benzimidazole small molecules. The C2C12 mouse myoblast cell line has been commonly used to evaluate BMP-induced responses and demonstrate robust canonical and non-canonical pathway activation evoked by rBMPs [32,33]. Indeed, our group recently demonstrated large increases in cell viability in C2C12 cells in response to various rBMPs, including BMP2 [30]. WEHI suspension cells were originally shown to respond to a gradient of BMP2 or BMP7 in transwell chemotaxis assays [34,35]. Later, WEHI cells were demonstrated to require select subunits of type II BMP receptors for BMP7-induced chemotaxis and growth cone collapse [36,37]. The primary cells derived from murine pulmonary arteries (PAECs) have been targeted in a search for a cure for pulmonary arterial hypertension (PAH), since defects in BMP receptor-dependent signaling are a major cause of PAH [38]. The increases in cell viability induced by 0.01 µM to 1000 µM SY-LB-35 and SY-LB-57 in C2C12 cells were previously reported [30] and are reproduced in **Supplementary Table 1**. To determine if these effects were a common cellular response to these novel indolyl-benzimidazoles, WEHI cells and primary PAECs were serum-starved and exposed to 0.01 µM to 1000 µM SY-LB-35 or SY-LB-57 for 24 hours. Triton X-100 (125 µM) served as a toxic control.

In WEHI cells, cell viability response to 0.01 µM to 100 µM SY-LB-35 was significantly increased compared with control, untreated WEHI cell cultures (Fig. 1A,C and **Supplementary Table 2**). In contrast, treatment with 1000 µM SY-LB-35 caused a substantial decrease in WEHI cell viability (Fig. 1A,C and **Supplementary Table 2**). Cell viability in WEHI cells in response to 0.01 µM to 10 µM SY-LB-57 was significantly increased compared with control, untreated cultures, however, the response to 100 µM SY-LB-57 was not different from control (Fig. 1B,C and **Supplementary Table 2**). A significant decrease in WEHI cell viability was detected in cultures exposed to 1000 µM compared with control, untreated cultures (Fig. 1B,C and **Supplementary Table 2**). The IC_{50} values for SY-LB-35 and SY-LB-57 in WEHI cells are 1074.2 µM and 1089.5 µM, respectively (Fig. 1C). Responses to SY-LB-57 were significantly more robust than SY-LB-35-evoked responses between 0.01 and 10 µM (**Supplementary Table 2**).

SY-LB-35 and SY-LB-57 induced significant changes in cell viability in primary PAECs after 24 hours at every concentration tested between 0.01–1000 µM. Cell viability after exposing PAECs to SY-LB-35 was significantly increased in response to 0.01 µM to 100 µM SY-LB-35, (Fig. 1D,F and **Supplementary Table 3**). Treatment with 1000 µM SY-LB-35 substantially decreased PAEC cell viability (Fig. 1D,F and **Supplementary Table 3**). Similarly, significant increases in PAEC cell viability were detected in response to 0.01 µM to 100 µM SY-LB-57 (Fig. 1E,F and **Supplementary Table 3**) and a significant decrease in PAEC viability was measured in cultures exposed to 1000 µM SY-LB-57 (Fig. 1E,F and **Supplementary Table 3**). The IC_{50} values for SY-LB-35 and SY-LB-57 in PAECs are 797.9 µM and 1148.9 µM, respectively (Fig. 1F). Responses to SY-LB-57 were significantly stronger than SY-LB-35-induced responses in PAECs at all concentrations except at 10 µM (**Supplementary Table 3**).

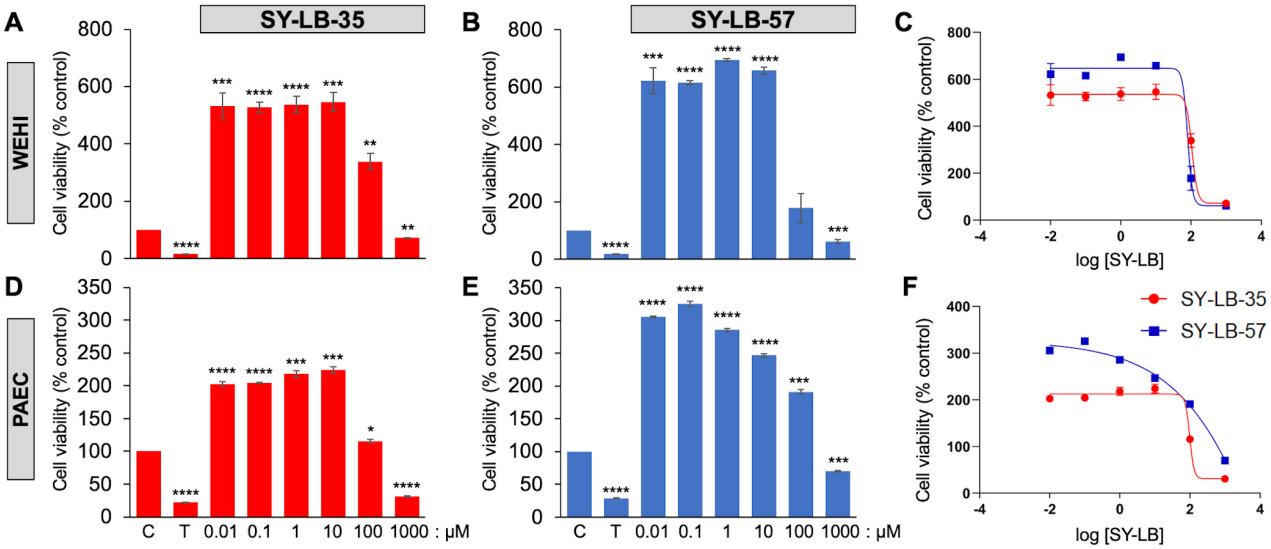

Fig. 1. SY-LBs stimulate robust increases in WEHI cell viability and in Pulmonary Artery Endothelial Cells (PAECs). The viability of WEHI cells (A,B,C) and PAECs (D,E,F) following treatment of cultures for 24 hours with the indicated concentrations of (A,D) SY-LB-35 and (B,E) SY-LB-57. Triton X-100 (T, 125 µM) served as a negative control. SY-LB compounds at 1000 µM strongly reduced cell viability when compared to control (C), untreated cells (****$p < 0.0001$). At lower concentrations between 0.01 µM and 100 µM, large increases in cell viability were observed in WEHI cells and PAECs compared with control (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). Cell viability is expressed as the mean ± SEM (n = 3) with each experiment performed in triplicate. Statistical comparisons *vs.* control were carried out using Student's two-tailed *t*-test. WEHI cells - SY-LB-35 *vs.* control. Full data sets are reported in **Supplementary Tables 1–3**. (E) Non-linear regression analysis reveals an IC_{50} value of 1074.2 µM for SY-LB-35 and 1089.5 µM for SY-LB-57 in WEHI cells. (F) Non-linear regression analysis reveals an IC_{50} value of 797.9 µM for SY-LB-35 and 1148.9 µM for SY-LB-57 in PAECs.

3.2 Robust Expression of Major BMP-Regulated Gene Products Induced by SY-LB-35 and SY-LB-57

If stimulation with SY-LB-35 and SY-LB-57 induce such significant changes to cell viability in 24 hours, then these BMP-like compounds would be expected to promote the expression of major BMP-regulated gene products like the Id1 transcription factor, the type II BMP receptor, BMP receptor type 2 (BMPR2), and the inhibitory Smads, Smad6 and Smad7 [39–41]. To determine if exposure to the SY-LB compounds results in the expected upregulation of Id1, BMPR2, Smad6 and Smad7, C2C12 cell cultures were serum-starved, then treated with BMP2 (50 ng/mL) and 0.01 µM to 10 µM SY-LB-35 or SY-LB-57 for 24 hours. Western blot analysis of whole cell lysates from stimulated cultures demonstrated strong increases in Id1 and BMPR2 expression in response to BMP2 and the SY-LB compounds relative to β-actin expression (Fig. 2A). Robust increases in Smad6 relative to β-actin were observed at all SY-LB-35 and SY-LB-57 concentrations tested (Fig. 2B). A concentration-dependent increase in Smad7 relative to β-actin was detected in response to rising concentrations of SY-LB-35, whereas in C2C12 cultures treated with SY-LB-57, Smad7 expression relative to β-actin was strongly upregulated in all cases except at 10 µM SY-LB-57 (Fig. 2B).

3.3 Sustained Activation of Non-Canonical BMP Signaling Pathways by SY-LB Compounds

To determine if non-canonical BMP signaling pathways remain activated after 24-hour exposure to the SY-LB compounds. C2C12 cell cultures were serum-starved, then stimulated with the positive control (BMP2) or 0.01 µM to 10 µM SY-LB-35 or SY-LB-57. Whole cell lysates were analyzed by Western blot using phospho-specific and pan antibodies against p38, ERK and Cyclin-Dependent Kinase1 (CDK1). The SY-LB compounds strongly increased phospho-ERK (p-ERK) and p-p38 levels at all concentrations analyzed relative to total ERK and p38 expression levels, respectively (Fig. 3). While the enhancing effect of SY-LB-35 on p-CDK1 levels tended to decrease over time, p-CDK1 levels remained significantly higher than control after 24 hours (Fig. 3). In contrast, SY-LB-57 maintained a robust, concentration-dependent stimulatory effect on p-CDK1 levels at 24 hours at all concentrations tested (Fig. 3).

Western blots from three independent experiments for each set of antibodies were quantified and demonstrated significant increases in p-p38, p-ERK and p-CDK1 in response to 0.01 µM to 10 µM SY-LB-35 and SY-LB-57 compared with control, untreated cultures (**Supplementary Fig. 1**). Normalized results are expressed as a percentage of control, untreated cultures (mean ± SD; n = 3; Student's

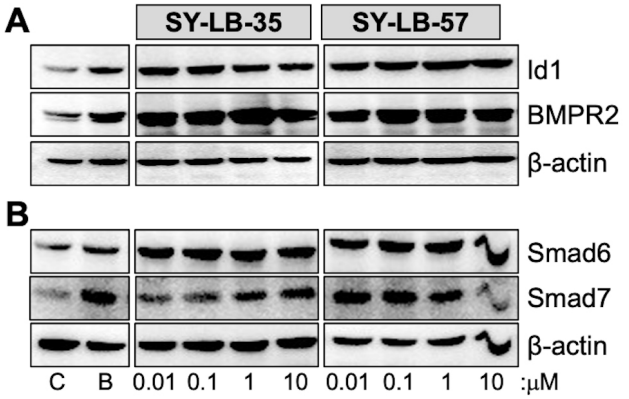

Fig. 2. Major targets of BMP-mediated transcription are up-regulated by SY-LB-35 and SY-LB-57. Serum-starved C2C12 cell cultures were stimulated with 50 ng/mL BMP2 (B), unsupplemented medium (C), SY-LB-35 (0.01–10 μM) or SY-LB-57 (0.01–10 μM) for 24 hours. (A,B) Whole cell lysates (20 μg) were analyzed by Western blot by first probing the membranes with antibodies that recognize (A) Id1 or (B) Smad6. The membranes were gently stripped and re-probed with antibodies against (A) BMPR2 or (B) Smad7, respectively. The membranes were stripped a second time and probed with anti-β-actin antibodies. Representative Western blots are shown.

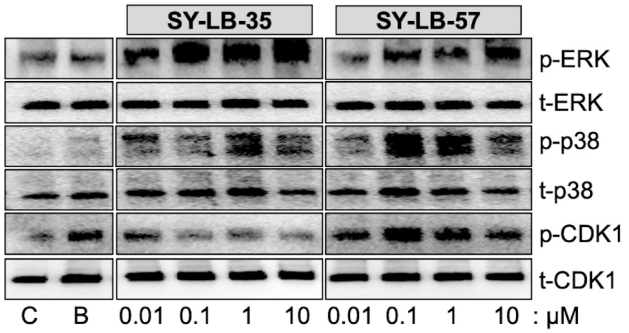

Fig. 3. SY-LB-35 and SY-LB-57 promote long-term increases in non-canonical BMP signaling pathways. Serum-starved C2C12 cell cultures were exposed to 50 ng/mL BMP2 (B) as a positive control, unsupplemented medium as a negative control (C), SY-LB-35 (0.01–10 μM) or SY-LB-57 (0.01–10 μM) for 24 hours. Whole cell lysates (20 μg) were analyzed by Western blot by first probing the membranes with phospho-specific antibodies to ERK (p-ERK), p38 (p-p38) and CDK1 (p-CDK1). The membranes were stripped and re-probed with antibodies to total cellular ERK, p38 and CDK1. Representative Western blots are shown. Full quantitative analysis of the experiment (n = 3) is reported in **Supplementary Fig. 1** and **Supplementary Tables 4,5,6**.

two-tailed *t*-test). The mean, SD and statistical tests are reported in **Supplementary Tables 4,5,6**. Phospho-p38 levels (**Supplementary Fig. 1A,B** and **Supplementary Table 4**) and p-ERK levels (**Supplementary Fig. 1C,D** and **Supplementary Table 5**) at 24 hours from this study are plotted against quantitation of previously reported Western blots of C2C12 whole cell lysates exposed to the SY-LB compounds for 15 minutes [30]. Plots of 15-minute and 24-hour p-CDK1 levels relative to total CDK1 levels are reported in **Supplementary Fig. 1E,F**, respectively, and **Supplementary Table 6**. The p-CDK1 and total CDK1 Western blots of SY-LB-stimulated C2C12 cell cultures at 15 minutes were not previously reported and representative blots are shown in **Supplementary Fig. 1G**.

Comparison of phosphorylation in response to the SY-LB compounds at 15 minutes and 24 hours revealed that levels of p-p38 are significantly greater at 24 hours than at 15 minutes (**Supplementary Fig. 1A,B** and **Supplementary Table 4**), while the profiles for p-ERK responding to SY-LB-35 and SY-LB-57 did not display such a difference (**Supplementary Fig. 1C,D** and **Supplementary Table 5**). Instead, the p-ERK profiles stimulated by both SY-LB compounds exhibited a concentration-dependent increase in ERK phosphorylation. Moreover, the percentage increases measured at 15 minutes and 24 hours in SY-LB-stimulated p38 phosphorylation at 24 hours were significantly elevated compared with 24-hour p-ERK responses (**Supplementary Fig. 1A–D** and **Supplementary Tables 4,5**).

3.4 Cell Viability Promoted by SY-LB-35 and SY-LB-57 Requires PI3K and p38 but not ERK Signaling

To examine the contribution of the PI3K/Akt, p38 and ERK signaling pathways on SY-LB-induced activity, cell viability assays were conducted in primary PAECs in the absence and presence of an inhibitor of PI3K (LY294002, 15 μM), p38 (SB202190, 10 μM) or ERK (PD98059, 5 μM) activity. In serum-starved PAECs stimulated for 24 hours with 0.01 μM, 0.1 μM and 1 μM SY-LB-35 or SY-LB-57, inhibition of PI3K (Fig. 4A,B) or p38 (Fig. 4C,D) signaling completely blocked SY-LB-induced increases in cell viability. In contrast, inhibition of ERK activity had no effect on the responses to SY-LB-35 or SY-LB-57 in C2C12 cell cultures (Fig. 4E,F).

PI3K and p38 inhibitors caused significant inhibition of 0.01 μM to 1 μM SY-LB-35- and SY-LB-57-stimulated responses in C2C12 cells compared to cell viability increases in the absence of these inhibitors (Fig. 4A–D). For ERK inhibition, no significant differences were detected in SY-LB-induced cell viability responses in absence or presence of PD98059 at any concentration tested (Fig. 4E,F). There was no difference in cell viability between control cultures and cultures treated with 15 μM LY294002, 10 μM SB203190 or 5 μM PD98059 alone (Fig. 4). Decreases in BMP-induced phosphorylation of Akt, p-38 and ERK in the presence of LY294002, SB202190 and PD98059, respectively, were observed ensuring that the inhibitors were active (**Supplementary Fig. 2**).

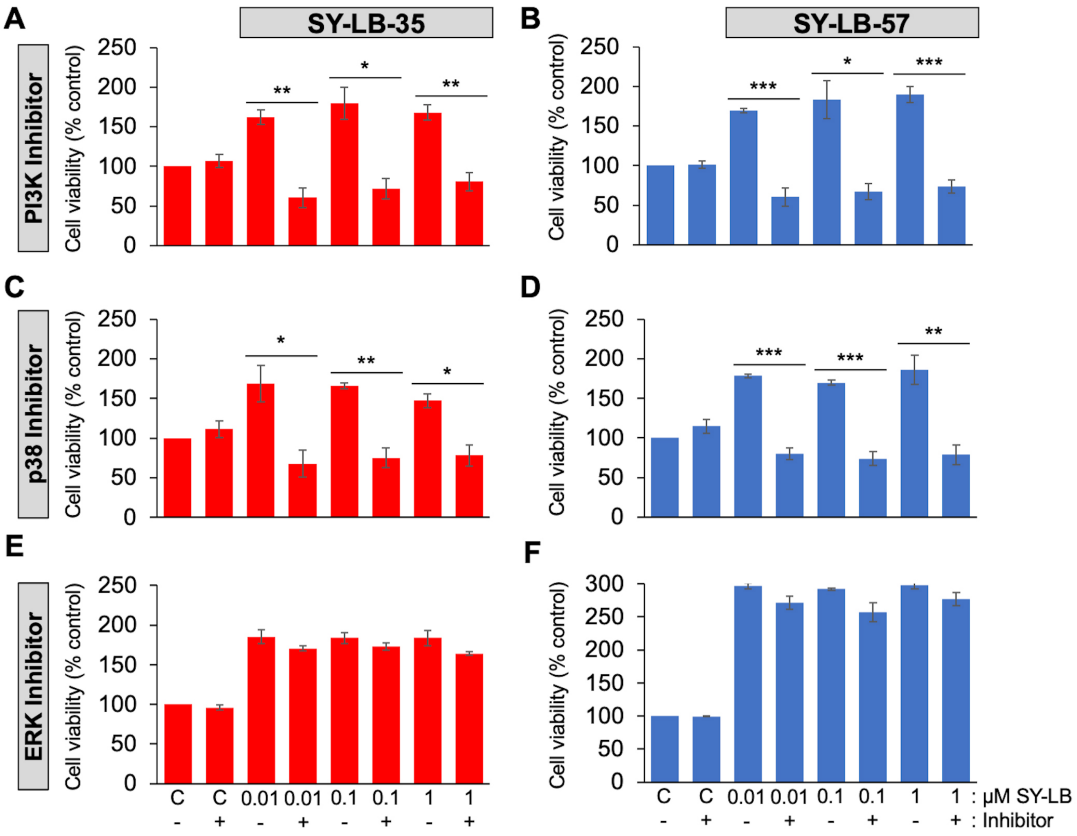

Fig. 4. Cell viability increases evoked by SY-LB-35 and SY-LB-57 are blocked by inhibitors to PI3K- and p38-dependent signaling but not to ERK-dependent pathways. (A–D) Cell viability in PAECs or (E,F) C2C12 cells following treatment of cultures for 24 hours with 0.01 µM to 1 µM (A,C,E) SY-LB-35 or (B,D,F) SY-LB-57 in the absence or presence of inhibitors to the (A, B) PI3K pathway (15 µM LY294002), (C,D) the p38 MAPK pathway (10 µM SB202190) or (E,F) the ERK MAPK pathway (5 µM PD98059). Cultures treated with unsupplemented medium (C-) or inhibitor alone (C+) served as controls. Cell viability increases stimulated by SY-LBs were completely blocked by PI3K inhibition and a p38 pathway inhibitor at every concentration tested compared with the respective control sample. The ERK inhibitor had no effect on SY-LB-stimulated increases in cell viability. (LY(-) *vs.* LY(+), SY-LB-35: 0.01 µM, 162% *vs.* 60%, $^{**}p = 0.0025$; 0.1 µM, 179% *vs.* 72%, $^{*}p = 0.0110$; 1 µM, 168% *vs.* 81%, $^{**}p = 0.0044$; SY-LB-57: 0.01 µM, 169% *vs.* 60%, $^{***}p = 0.00076$; 0.1 µM, 183% *vs.* 67%, $^{*}p = 0.0115$; 1 µM, 190% *vs.* 74%, $^{***}p = 0.00083$. Control *vs.* LY, SY-LB-35: 100% *vs.* 107%, $p = 0.4567$; SY-LB-57: 100% *vs.* 102%, $p = 0.7444$) (SB(-) *vs.* SB(+), SY-LB-35: 0.01 µM, 169% *vs.* 68%, $^{*}p = 0.0237$; 0.1 µM, 166% *vs.* 75%, $^{**}p = 0.0022$; 1 µM, 147% *vs.* 78%, $^{*}p = 0.0124$; SY-LB-57,: 0.01 µM, 178% *vs.* 80%, $^{***}p = 0.00021$; 0.1 µM, 170% *vs.* 74%, $^{***}p = 0.00046$; 1 µM, 186% *vs.* 79%, $^{**}p = 0.0086$. Control *vs.* SB, SY-LB-35: 100% *vs.* 111%, $p = 0.3469$; SY-LB-57: 100% *vs.* 115%, $p = 0.1634$). (PD(-) *vs.* PD(+), SY-LB-35: 0.01 µM, 185% *vs.* 170%, $p = 0.1865$; 0.1 µM, 184% *vs.* 173%, $p = 0.2482$; 1 µM, 184% *vs.* 164%, $p = 0.1148$; SY-LB-57: 0.01 µM, 297% *vs.* 271%, $p = 0.0730$; 0.1 µM, 292% *vs.* 257%, $p = 0.0695$: 1 µM, 298% *vs.* 277%, $p = 0.1349$. Control *vs.* PD, SY-LB-35: 100% *vs.* 96%, $p = 0.2268$; SY-LB-57: 100% *vs.* 99%, $p = 0.5046$). Cell viability is expressed as the mean ± SEM (n = 3) with each experiment performed in triplicate. Statistical comparisons were carried out using Student's two-tailed *t*-test.

3.5 A Selective ALK2 Inhibitor Blocks SY-LB-Induced Increases in C2C12 Cell Viability

SY-LB-35- and SY-LB-57-induced cell viability increases, and Smad phosphorylation were previously shown to depend on the activity of type I BMP receptors using Dorsomorphin [30]. However, Dorsomorphin is a pan type I BMP receptor inhibitor and more selective inhibitors have since been developed including LDN193189, which selectively inhibits the type I BMP receptor subunit, ALK2 [35]. To investigate whether the SY-LB compounds stimulate increases in WEHI cell viability through a mechanism dependent upon ALK2 activity, serum-starved WEHI cell cultures were exposed to 0.01 µM, 0.1 µM or 1 µM SY-LB-35 or SY-LB-57 for 24 hours in the absence or presence of 5 µM LDN193189 (Fig. 5A,B, respectively). After 24 hours, WEHI cell responses evoked by the SY-LB compounds were completely inhibited by the presence of LDN193189 at all concentrations tested (Fig. 5A,B).

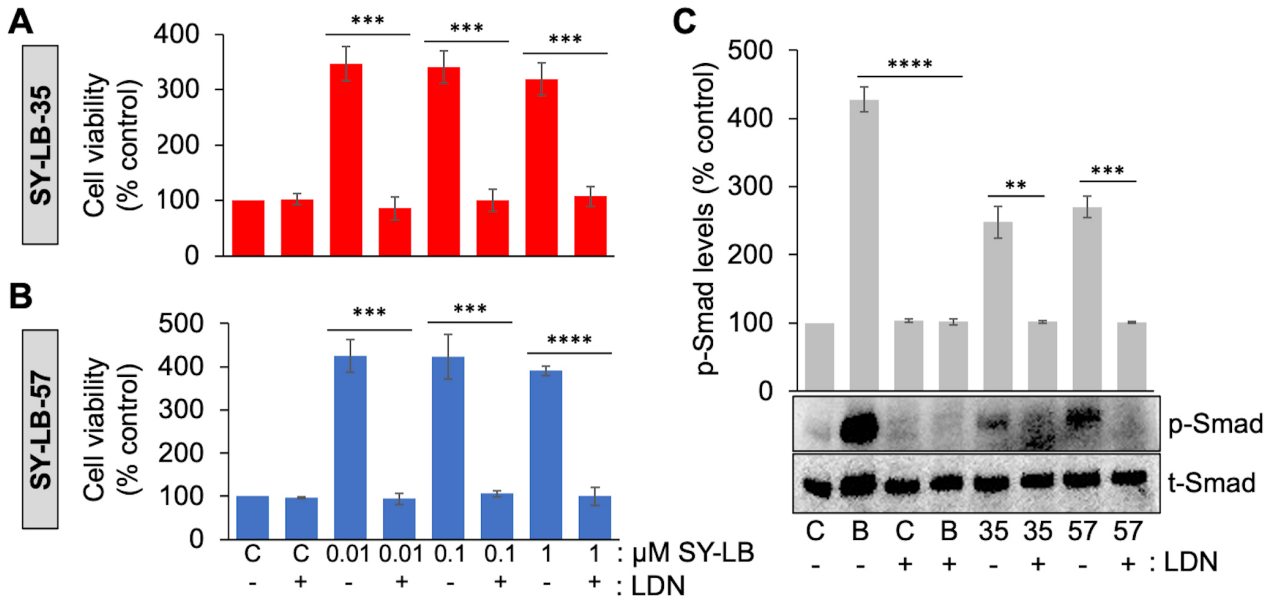

Fig. 5. Increases in cell viability and Smad phosphorylation levels depend on ALK2 activity. (A, B) Cell viability in WEHI cells following treatment of cultures for 24 hours with 0.01 μM to 1 μM (A) SY-LB-35 or (B) SY-LB-57 in the absence or presence of an inhibitor of ALK2 activity (5 μM LDN193189 (LDN)). Cultures treated with unsupplemented medium (C-) or inhibitor (C+) alone served as controls. Cell viability increases stimulated by SY-LB-35 or SY-LB-57 were completely blocked by the ALK2 inhibitor at every concentration tested compared with the respective control sample. LDN(-) *vs.* LDN(+), SY-LB-35: 0.01 μM, 347% *vs.* 85%, ***p = 0.00028; 0.1 μM, 340% *vs.* 85%, ***p = 0.00031; 1 μM: 319% *vs.* 85%, ***p = 0.0004; SY-LB-57: 0.01 μM, 425% *vs.* 94%, ***p = 0.00015; 0.1 μM, 423% *vs.* 106%, ***p = 0.00046; 1 μM, 359% *vs.* 97%, ****$p = 2.69 \times 10^{-5}$. Cell viability is expressed as the mean ± SEM (n = 3) with each experiment performed in triplicate. (C) Following serum starvation, C2C12 cell cultures were exposed to 1 μM SY-LB-35 or SY-LB-57 for 30 minutes in the absence (-) and presence (+) of 5 μM LDN193189. BMP2 (B, 50 ng/mL) stimulation served as a positive control. Whole cell lysates of the cultures were analyzed by Western blot using a phospho-specific anti-Smad1/5/8 antibody (p-Smad) to probe the membrane. The membrane was gently stripped and re-probed with an anti-pan Smad antibody (t-Smad). Levels of p-Smad were normalized to t-Smad levels and expressed as a percentage of control, untreated (C-) cultures (mean ± SEM (n = 3)). BMP2- and SY-LB-evoked increases in Smad phosphorylation were eliminated by ALK2 inhibition. (Control *vs.* BMP2, ****$p = 5.54 \times 10^{-5}$; Control *vs.* SY-LB-35, **p = 0.0031; Control *vs.* SY-LB-57, ***p = 0.00047. LDN(-) *vs.* LDN(+), Control: 100% *vs.* 104%, p = 0.2113; BMP2: 428% *vs.* 102%, ****$p = 6.30 \times 10^{-5}$; SY-LB-35: 248% *vs.* 101%, **p = 0.0033; SY-LB-57: 269% *vs.* 101%, ***p = 0.00048). Statistical comparisons were carried out using Student's two-tailed *t*-test.

To demonstrate the activity of the ALK2 inhibitor and to confirm the dependence of SY-LB-35 and SY-LB-57 on ALK2 activity specifically, C2C12 cells were starved, pre-treated with 5 μM LDN193189 and exposed to BMP2 (50 ng/mL) or the SY-LB compounds at 1 μM for 30 minutes. C2C12 whole cell lysates were analyzed by Western blot using antibodies against p-Smad and total Smad (t-Smad) (Fig. 5C). Levels of p-Smad normalized to t-Smad expression in the absence and presence of LDN193189 is reported as the mean ± SEM (n = 3). BMP2, SY-LB-35 and SY-LB-57 evoked significant increases in p-Smad levels compared with control, untreated C2C12 cell cultures (Fig. 5C). Inhibition of BMP2- and SY-LB-induced Smad phosphorylation by ALK2 inhibition was significantly different from p-Smad levels in the absence of LDN193189 (Fig. 5C).

3.6 SY-LB Compounds May Extracellularly Activate the BMP Receptor Complex

The endogenous, extracellular BMP antagonist, Noggin, binds BMPs with high affinities and has a marked preference for BMP2 and BMP4 over BMP7 [42,43]. The Noggin/BMP interaction prevents BMPs from binding to cell surface receptors, thus serves to regulate active BMP levels and, consequentially, BMP receptor signaling [43]. To determine whether SY-LB-35 or SY-LB-57 are antagonized by Noggin, C2C12 cells were starved and pre-treated for 1 hour with recombinant Noggin (400 ng/mL), followed by treatment with the positive control, BMP2 at 50 ng/mL and 1 μM SY-LB-35 or SY-LB-57 for 30 minutes. Western blot analysis of p-Smad and t-Smad levels in C2C12 whole cell lysates reveals that in the absence of Noggin, Smad phosphorylation levels stimulated by SY-LB-35 and SY-LB-57 at 1 μM were increased significantly compared with

control, untreated cells (**Supplementary Fig. 3A**). The p-Smad responses to BMP2, SY-LB-35 and SY-LB-57 were eliminated by pre-treatment with Noggin (**Supplementary Fig. 3A**).

To determine whether concentrations greater than 1 µM SY-LB-35 or SY-LB-57 can overcome the blockade of Smad signaling by Noggin, C2C12 cells were starved and exposed to SY-LB compounds at 10 µM, 100 µM and 1000 µM in absence or presence of 400 ng/mL Noggin for 30 minutes. Treating C2C12 cells with SY-LB-35 or SY-LB-57 at concentrations greater than 10 µM can overcome the antagonism of Smad phosphorylation by Noggin (**Supplementary Fig. 3B,C**, respectively).

3.7 Smad4 Knockdown Affects Non-Canonical BMP Signaling Stimulated by SY-LB Compounds

To investigate potential crosstalk between the canonical and non-canonical BMP signaling pathways, RNA interference experiments were carried out to knockdown the level of Smad4 as a vital player of the canonical pathway. C2C12 cells were transfected with empty vector as a negative control or a lenti-viral shRNA plasmid targeting Smad4 and incubated for 48 hours [36]. Down-regulation of Smad4 expression relative to β-actin expression was confirmed by Western blot using an anti-Smad4 antibody (**Supplementary Fig. 4A**). Next, BMP2 (50 ng/mL) and 1 µM SY-LB-35 or SY-LB-57 were added to transfected C2C12 cells for 30 minutes. The C2C12 cell lysates were probed by Western blot for non-canonical BMP signaling components p-p38, p-ERK and p-CDK1. The results demonstrated that the absence of Smad4 did not reduce the baseline or unstimulated levels of these signaling components. In contrast, stimulation of Smad4 shRNA-transfected C2C12 cells with BMP2, SY-LB-35 or SY-LB-57 resulted in further reduction of the phosphorylated forms of p38, ERK and CDK1 (**Supplementary Fig. 4B**).

Smad4 shRNA-transfected C2C12 cell lysates were stimulated with 50 ng/mL BMP2 or 1 µM SY-LB-35 and probed for Smad6 and Smad7 expression (**Supplementary Fig. 4C**). In unstimulated cultures, a significant increase in Smad6 and Smad7 expression levels was detected in response to Smad4 shRNA transfection alone (**Supplementary Fig. 4C**), which was significantly amplified by 30-minute exposure to BMP2 or SY-LB-35, compared with control, untransfected, untreated C2C12 cell cultures (**Supplementary Fig. 4C**).

3.8 SY-LB-35 and SY-LB-57 Are Active at Picomolar Concentrations

To determine the lower limit for increasing cell viability, serum-starved C2C12 cell cultures were exposed to SY-LB-35 and SY-LB-57 over a very low concentration range (0.001–1000 nM) for 24 hours. SY-LB-35 and SY-LB-57 continued to stimulate significant increases in cell viability at concentrations as low as 1 pM (Fig. 6A,B). Moreover, concentration-dependent reduction of cell viability was observed as the concentrations decreased. The cell viability induced by SY-LB compounds demonstrated significantly higher percentages of cell viability than the control, untreated cells at all tested concentrations (Fig. 6A,B).

The canonical and non-canonical BMP signaling pathways were analyzed by Western blot of C2C12 cell lysates following 15- and 30-minute treatment with picomolar concentrations of SY-LB-35 or SY-LB-57. BMP2 at 50 ng/mL was used as a positive control. Low concentrations (0.001–10 nM) of SY-LB-35 (Fig. 6C) and SY-LB-57 (Fig. 6D) were able to stimulate phosphorylation of Akt (as a readout for PI3K activity), p38, ERK, and CDK1 at concentrations as low as 1 pM. In contrast, Smad phosphorylation was not detected in response to SY-LB-35 or SY-LB-57 at either 100 pM or 1 nM following a 30-minute stimulation (Fig. 6E).

3.9 SY-LB-35 and SY-LB-57 Promote Wound Healing in Vitro

To explore how SY-LB-35 and SY-LB-57 impact cell migration, *in vitro* wound healing/scratch assays were conducted. C2C12 cell monolayers were scrape-wounded followed by exposure to BMP2 (50 ng/mL) or 1 µM SY-LB compounds for 18 hours. The wounds were documented at 0-hour and 18-hour time points to assess wound closure over time. Migration of cells into the wounded area was quantified by counting the number of cells in the scratch area at 18 hours (Fig. 7A,B). Significantly more C2C12 cells invaded the scratched area in the presence of BMP2 or the SY-LB compounds compared with control, untreated cultures (Fig. 7A,B).

To explore the contribution of ALK2 activity to SY-LB-induced wound healing, *in vitro* scratch assays were carried out as described above. The monolayers were scrape-wounded followed by addition of BMP2 (50 ng/mL) or the SY-LB compounds at 1 µM in absence or presence of the selective ALK2 inhibitor, LDN193189, at 5 µM. Representative images at 18 hours are shown in **Supplementary Fig. 5**. LDN193189 treatment alone had a small, but significant effect on the Invasion rate of C2C12 cells (Fig. 7C and **Supplementary Fig. 5**). Mimicking the effect of LDN193189 on BMP2-evoked C2C12 cell migration, the presence of LDN193189 completely blocked C2C12 cell invasion into the wounded area promoted by the SY-LB compounds (Fig. 7C).

To assess the involvement of PI3K/Akt, p38 and ERK signaling pathways in SY-LB-induced cell migration, C2C12 cells were scrape-wounded and incubated with BMP2 (50 ng/mL) and 1 µM SY-LB compounds for 18 hours in the absence or presence of inhibitors to PI3K (15 µM LY294002), p38 (10 µM SB203190) and ERK (5 µM PD98059). Representative images at 18 hours are shown in **Supplementary Fig. 5**. Inhibition of PI3K and p38 had a profound effect on C2C12 cell migration and significantly eliminated SY-LB-induced increases in Invasion

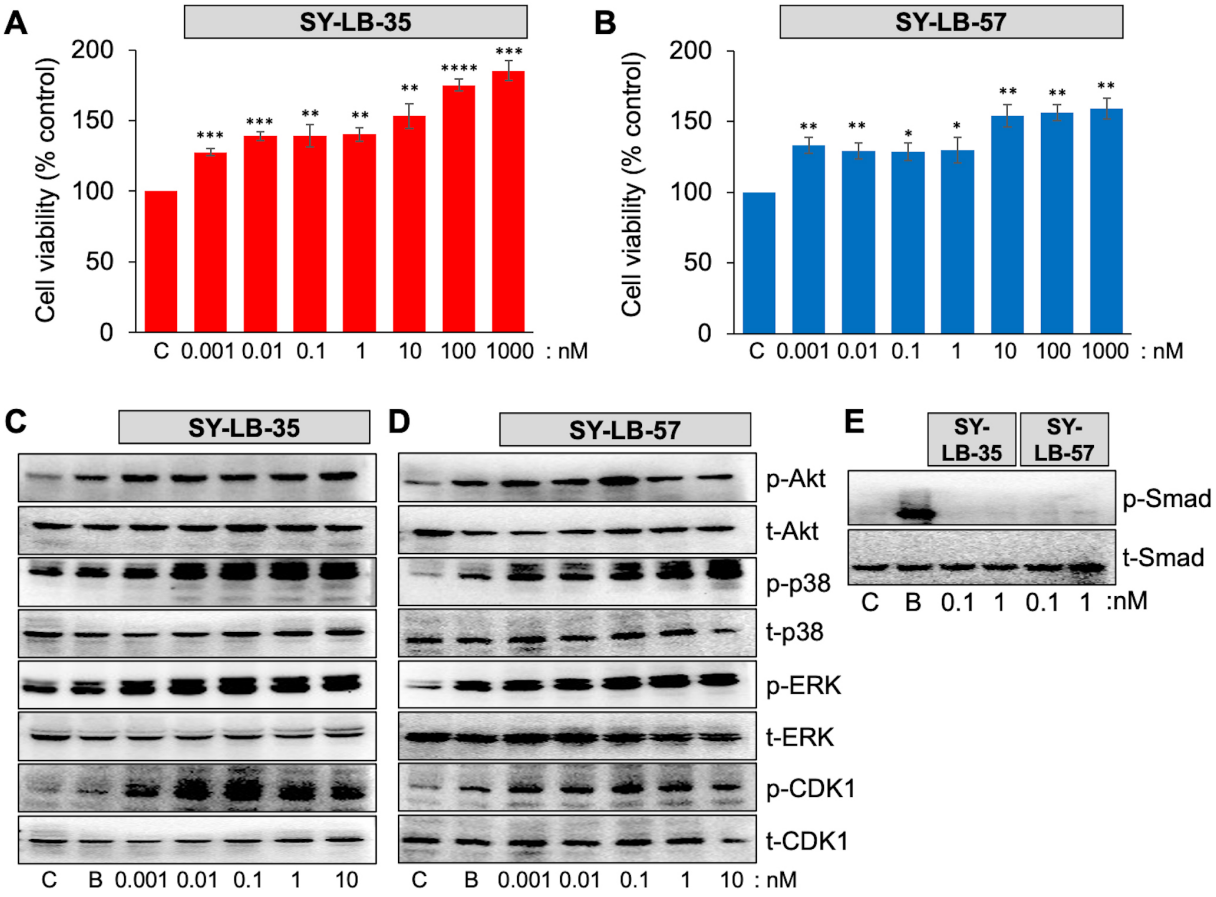

Fig. 6. SY-LBs stimulate increases in cell viability and non-canonical signaling, but not canonical, Smad signaling at picomolar concentrations. (A,B) Cell viability in C2C12 cells following treatment of cultures for 24 hours with 0.001 nM to 1000 nM of (A) SY-LB-35 and (B) SY-LB-57. SY-LB compounds significantly increased cell viability across the entire concentration range compared with control (C), untreated cells. Cell viability is expressed as the mean ± SEM (n = 3) with each experiment performed in triplicate. Control *vs.* SY-LB-35: 0.001 nM, 128% ± 2.4%, ***p = 0.00030; 0.01 nM, 139% ± 3.1%, ***p = 0.00023; 0.1 nM, 139% ± 8.0%, **p = 0.0081; 1 nM, 140% ± 4.8%, **p = 0.0011; 10 nM, 153% ± 8.9%, **p = 0.0039; 100 nM, 175% ± 4.3%, ****p = 6.52 × 10^{-5}; 1000 nM, 185% ± 7.0%, ***p = 0.00026; Control *vs.* SY-LB-57: 0.001 nM, 133% ± 5.6%, **p = 0.0049; 0.01 nM, 129% ± 5.4%, **p = 0.0097; 0.1 nM, 129% ± 6.1%, *p = 0.0139; 1 nM, 130% ± 9.0%, *p = 0.0420; 10 nM, 154% ± 7.9%, **p = 0.0045; 100 nM, 156% ± 5.8%, **p = 0.0012; 1000 nM, 159% ± 7.6%, **p = 0.0029. Statistical comparisons were carried out using Student's two-tailed, paired *t*-test. (C,D) Representative Western blots of cell lysates from starved C2C12 cells following a 15-minute exposure to BMP2 (B, 50 ng/mL) and the indicated concentrations of (C) SY-LB-35 or (D) SY-LB-57. The membranes were probed with phospho-specific antibodies to Akt (p-Akt), p38 (p-p38), ERK, (p-ERK) and CDK1 (p-CDK1) followed by gentle stripping and re-probing with the respective pan antibodies (t-Akt, t-p38, t-ERK and t-CDK1). (E) Following serum starvation, 0.1 nM and 1 nM SY-LB-35 or SY-LB-57 were added to C2C12 cells for 30 minutes. BMP2 (B, 50 ng/mL) served as a positive control. Whole cell lysates of the cultures were analyzed using an anti-p-Smad antibody to probe the Western blot membrane. The membrane was gently stripped and re-probed with an anti-t-Smad antibody. SY-LB compounds are unable to stimulate increases in p-Smad levels at nanomolar concentrations. Representative Western blots are shown.

rates (Fig. 7D,E, respectively). LY294002 had no effect on the Invasion rate of control, untreated C2C12 cell cultures (Fig. 7D). Inhibition of p38 with SB203190 produced a minor but significant decrease in the Invasion rate of control, untreated cultures (Fig. 7E).

In contrast to the effects of PI3K and p38 inhibition on C2C12 cell invasion rates, inhibition of the ERK pathway alone significantly increased the Invasion rate in control, untreated cultures and in cultures treated with BMP2 (Fig. 7F). PD98059 had no significant effect on C2C12 cell migration evoked by SY-LB-35 or SY-LB-57 with all conditions strongly stimulating migration in the presence of PD98059 (Fig. 7F).

To exclude cell proliferation as a cofounding factor in wound healing assays, C2C12 cells were subjected to scratch assays in the absence or presence of Mitomycin C

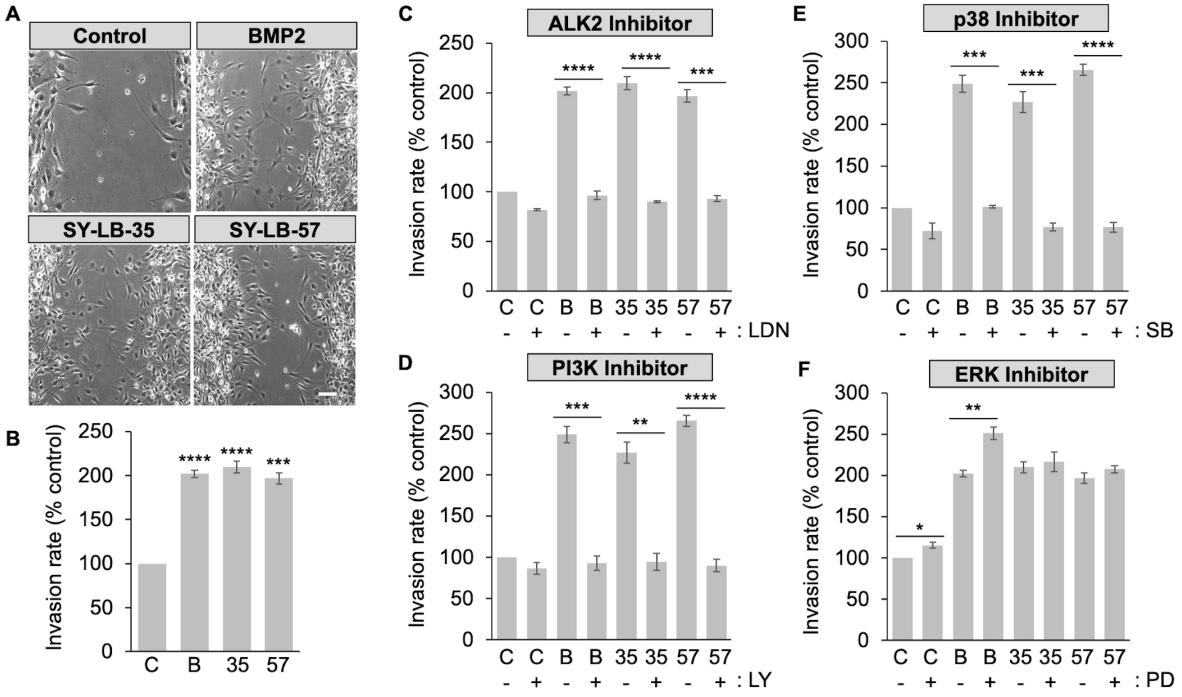

Fig. 7. SY-LB-promoted wound healing is dependent upon ALK2, PI3K and p38 but does not rely on ERK signaling. (A) Representative phase contrast images of C2C12 scratch assays incubated for 18 hours in the presence of 50 ng/mL BMP2 and 1 μM SY-LB-35 or SY-LB-57. Control cultures were maintained in unsupplemented medium. The wounds were examined at 0- and 18-hour time points to assess wound closure over time. BMP2 and SY-LB compounds promote invasion of C2C12 cells into the wounded area, where few cells have entered the wounded area in control cultures after 18 hours. (B) A quantitative assessment of wound healing shown in (A) was carried out by counting the number of cells invading the wounded area after 18 hours. The Invasion rate is expressed as the number of cells invading the wound as a percentage of invading cells in control (C-), untreated cultures. BMP2 (B, 50 ng/mL, SY-LB-35 (35, 1 μM) and SY-LB-57 (57, 1 μM) significantly increased the migration of C2C12 cells into the scrape-wounded area compared with control (mean ± SEM (n = 3). Control *vs.* BMP2: 202% ± 4.0%, ****$p = 1.43 \times 10^{-5}$; Control *vs.* SY-LB-35: 210% ± 6.8%, ****$p = 8.73 \times 10^{-5}$; Control *vs.* SY-LB-57: 197% ± 6.3%, ***$p = 0.00011$). Statistical comparisons were carried out using Student's two-tailed *t*-test. (C–F) Wound healing assays were carried out in C2C12 cell cultures treated with 50 ng/mL BMP2, 1 μM SY-LB-35 or 1 μM SY-LB-57 in the absence (-) or presence (+) of various inhibitors including (C) an ALK2 inhibitor (5 μM LDN193189 (LDN)), (D) a PI3K inhibitor (15 μM LY294002 (LY)), (E) a p38 inhibitor (10 μM SB202190 (SB)) or (F) an ERK inhibitor (5 μM PD98059 (PD)). ALK2 inhibitor, LDN(-) *vs.* LDN(+), Control: 100% *vs.* 82%, ****$p = 3.38 \times 10^{-5}$; BMP2: 202% *vs.* 96%, ****$p = 5.25 \times 10^{-5}$; SY-LB-35: 210% *vs.* 90%, ****$p = 6.39 \times 10^{-5}$; SY-LB-57: 197% + 6.3% *vs.* 93%, ***$p = 0.00011$), PI3K inhibitor, LY(-) *vs.* LY(+), Control: 100% *vs.* 86%, $p = 0.1182$; BMP2: 249% *vs.* 93%, ***$p = 0.00029$; SY-LB-35: 227% *vs.* 94%, **$p = 0.0012$; SY-LB-57: 266% *vs.* 92%, ****$p = 6.20 \times 10^{-5}$; Fig. 7D), p38 inhibitor, SB(-) *vs.* SB(+), Control: 100% *vs.* 72%, *$p = 0.0425$; BMP2: 249% *vs.* 101%, ***$p = 0.00013$; SY-LB-35: 227% *vs.* 77%, ***$p = 0.00037$; SY-LB-57: 266% *vs.* 7%, ****$p = 3.11 \times 10^{-5}$) ERK inhibitor, PD(-) *vs.* PD(+), Control: 100% *vs.* 115%, *$p = 0.0119$; BMP2, 202% *vs.* 251%, **$p = 0.0045$; SY-LB-35: 210% *vs.* 217%, $p = 0.6465$; SY-LB-57: 197% *vs.* 207%, $p = 0.2349$). After 18 hours, the cultures were fixed and the number of cells invading the wounded area was determined. The Invasion rate is expressed as the number of cells invading the wound as a percentage of invading cells in control (C-), untreated cultures. Inhibition of ALK2, PI3K and p38 activity dramatically inhibited the Invasion rate compared with the respective control sample, whereas inhibiting ERK activity had no effect on Invasion rate (mean ± SEM (n = 3)). Statistical comparisons were carried out using Student's two-tailed, paired *t*-test. Scale bar = 100 μm.

(30 nM), a DNA synthesis inhibitor, and exposed to BMP2 (50 ng/mL) or 1 μM SY-LB compounds for 18 hours. Treatment with Mitomycin C (MC) alone had no effect on C2C12 cell migration compared with control, untreated cultures (**Supplementary Fig. 6A**). Importantly, the increases in C2C12 Invasion rates in response to BMP2, SY-LB-35 or SY-LB-57 were not significantly different whether in the absence or presence of Mitomycin C (**Supplementary Fig. 6A**). Representative phase contrast images are shown in **Supplementary Fig. 6B**.

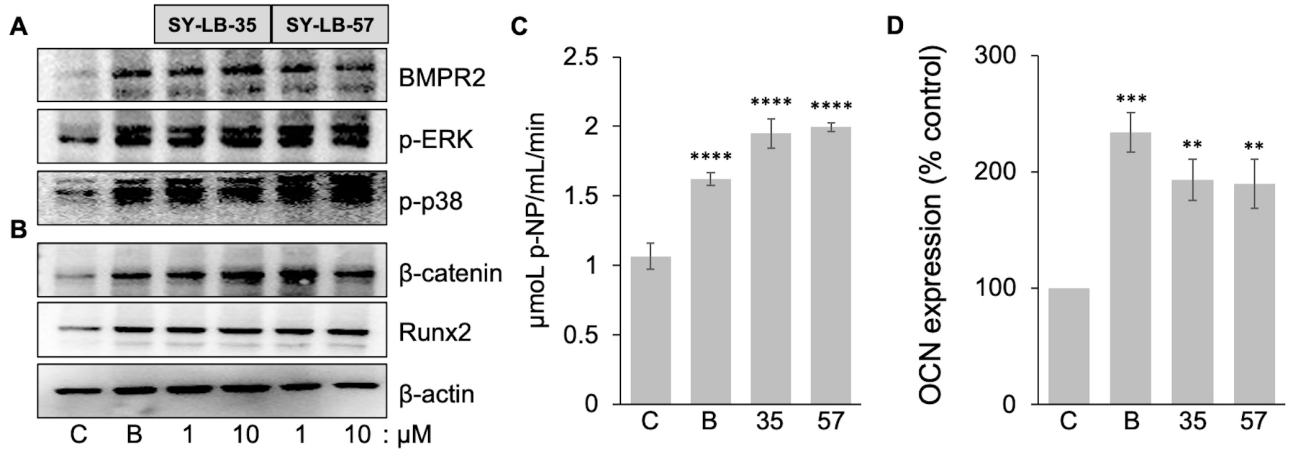

Fig. 8. Early-, mid- and late-stage markers of bone differentiation are upregulated by SY-LB-35 and SY-LB-57. (A,B) C2C12 cultures were serum-starved and incubated for 4 hours with BMP2 (50 ng/mL) and SY-LB-35 or SY-LB-57 at 1 μM and 10 μM. Whole cell lysates of the cultures were analyzed by Western blot using antibodies that recognize (A) BMPR2 or (B) β-catenin to probe the membrane. The membranes were gently stripped and re-probed with (A) anti-p-ERK or (B) anti-Runx2 antibodies. Finally, the membranes were stripped again and incubated with (A) anti-p-p38 or (B) anti-β-actin antibodies. Representative Western blots are shown. (C) BMP2 (200 ng/mL) or SY-LB compounds (10 μM) were added to confluent cultures of C2C12 cells for 72 h (single treatment at day 1). Whole cell lysates were collected and subjected to Alkaline phosphatase (ALP) assays using soluble p-nitrophenol phosphate (p-NPP) as a substrate. ALP enzymatic activity is represented as the amount of p-nitrophenol (p-NP) product per mL of medium per minute. The amount of p-NP in each treatment group was calculated using the standard curve of p-NP versus absorbance at 405 nm. SY-LB-35 and SY-LB-57 significantly increase the activity of ALP after 72 hours. Data is expressed as the fold change in ALP activity (mean ± SEM, n = 3). (Control: 1.07 ± 0.10; BMP2: 1.62 ± 0.04, ***p = 0.00079; SY-LB-35: 1.95 ± 0.11, ***p = 0.00042; SY-LB-57: 1.99 ± 0.03, ****p = 8.60×10^{-5}). (D) Confluent C2C12 cell cultures were incubated with SY-LB-35 (10 μM) or SY-LB-57 (5 μM) every other day for 7 days. BMP2 (B, 200 ng/mL) served as a positive control. Whole cell lysates of treated C2C12 cultures were used in an osteocalcin (OCN) ELISA. Expression of OCN is reported as a percentage of OCN expression in control, untreated C2C12 cultures (mean ± SEM (n = 3)). Similar to BMP2, SY-LB compounds enhanced expression of OCN significantly compared to control (C), untreated cells (BMP2: 234% ± 17%, ***p = 0.00018; SY-LB-35: 193% ± 18%, ***p = 0.00082; SY-LB-57: 190% ± 21%, **p = 0.0018). Statistical comparisons were carried out using Student's two-tailed *t*-test.

3.10 SY-LB Compounds Induce the Expression of Osteogenic Signaling Markers

Exposure to BMP2 causes C2C12 cells to differentiate into an osteoblast lineage accompanied by the expected expression markers for bone formation [32,44]. To examine the osteogenic potential of SY-LB-35 and SY-LB-57, the expression of markers related to bone differentiation was examined in C2C12 cells that were serum-starved and incubated with 50 ng/mL BMP2 or SY-LB compounds (1 μM and 10 μM) for 4 hours. Western blot analysis revealed an increase in the expression of early osteogenic markers such as Runx2, β-catenin and BMPR2 relative to β-actin expression after 4 hours of treatment with BMP2 or the SY-LB compounds (Fig. 8A). In the same C2C12 cell whole lysates, SY-LB-35 (Fig. 8A) and SY-LB-57 (Fig. 8B) stimulated robust increases in p-ERK and p-p38 levels relative to β-actin expression.

Alkaline phosphatase (ALP) is widely regarded as an early pre-osteoblast marker, while proteins such as osteocalcin (OCN) are considered to be markers of differentiated osteoblasts [45]. To explore the activation of ALP enzymatic activity by SY-LB compounds, confluent C2C12 cultures were stimulated with a single treatment of BMP2 (200 ng/mL) or 10 μM SY-LB compounds. The culture media was assessed at 24, 48 and 72 hours for secreted, active ALP in the presence of the soluble p-nitrophenol phosphate (p-NPP) substrate, which is converted to p-nitrophenol (p-NP) by the ALP enzyme. ALP activity in the whole cell lysates of C2C12 cultures was measured after 72 hours of the treatment. The whole cell lysates were assessed for ALP activity by incubation with p-NPP substrate and demonstrated robust increases in ALP activity following exposure to BMP2, SY-LB-35 and SY-LB-57 compared with control lysates (Fig. 8C). A time course of ALP activity in the culture media is presented in **Supplementary Fig. 7A** and a standard curve for the ALP assay is shown in **Supplementary Fig. 7B**.

To determine if the SY-LB compounds increase the expression of OCN, fully confluent C2C12 cells were exposed to BMP2 (200 ng/mL), SY-LB-35 (10 μM) or SY-LB-57 (5 μM). After day 7, the cells were collected, and the resulting cell lysate was used in the OCN ELISA. The protein

level of osteocalcin in the samples was obtained from concentration of standard solutions of OCN plotted versus absorbance at 450 nm (**Supplementary Fig. 7C**). Quantitative analysis of OCN expression revealed that BMP2, SY-LB-35 and SY-LB-57 caused a significant increase in the level of OCN expression compared with control, untreated C2C12 cell cultures (Fig. 8D).

3.11 Long-Term Treatment with SY-LB-35 Induces Morphological Changes and Calcium Deposition in C2C12 Cells

To examine the effects of SY-LB-35 on differentiation of C2C12 cells into osteoblasts following long-term treatment, C2C12 cell cultures were grown to a fully confluent state and exposed to BMP2 (200 ng/mL) or SY-LB-35 (10 µM) every 24 hours for 72 hours. Treatments were prepared in Low Serum media (2% FBS/DMEM/1 × PSG). After 72 hours, C2C12 cells exhibited morphological changes in BMP2- and SY-LB-35-treated cells compared with control and Low Serum cultures (**Supplementary Fig. 8**). C2C12 cells grown in Low Serum conditions in the absence of BMP2 or SY-LB-35 show signs of myofiber formation (**Supplementary Fig. 8**, yellow arrowheads), typical of myogenic differentiation of these cells following serum depravation [32]. In contrast, C2C12 cells grown under low serum conditions and exposed to BMP2 or SY-LB-35 appear to be undergoing a similar program of differentiation with the appearance of round, enlarged cells and areas of cobblestone-like arrangements (**Supplementary Fig. 8**, yellow arrows and asterisks, respectively).

In longer-term cultures, fully confluent C2C12 cultures were serum-starved for 16 hours followed by treatment with BMP2 (100 ng/mL) or SY-LB-35 (10 µM) every other day for 10 days in complete growth medium containing 10% FBS. Unstimulated cells develop a radial branching morphology consisting of long fibers extending in many directions (Fig. 9A). While long-term stimulation of C2C12 cells with SY-LB-35 (Fig. 9C) exhibited morphological changes, differentiating into large, round cells and losing the branching morphology, distinct from the control group but similar to BMP2-treated cultures (Fig. 9B).

To determine whether evidence of calcium deposition could be detected in cultures of differentiating C2C12 cells, Alizarin Red S staining was carried out, which indicates mineralization in the cultures by binding to calcium deposits in the extracellular matrix. To this end, fully confluent C2C12 cell cultures were incubated with BMP2 (200 ng/mL) or SY-LB-35 (10 µM) for 21 days in complete growth medium followed by staining with 2% Alizarin Red S solution. Alizarin Red S staining in control, untreated C2C12 cells showed diffuse, light red staining (Fig. 9D). In contrast, cultures treated with BMP2, or SY-LB-35 showed strong calcium depositions with large, intensely staining clusters of cells (Fig. 9E,F, respectively).

4. Discussion

The initial characterization of SY-LB-35 and SY-LB-57 was carried out in the pluripotent C2C12 cell line and demonstrated that these novel, small molecule heterocyclic compounds strongly stimulated canonical Smad signaling through a type I BMP receptor-dependent mechanism [30]. Prior to our study, several small molecules were reported to stimulate BMP receptor signaling but exhibited weak responses or acted through a BMP receptor-independent mechanism or both [46–50]. In our previous study, the substantial SY-LB-induced amplification of Smad phosphorylation and cell viability was blocked by non-selective inhibition of type I BMP receptor activity [30]. The dependence on type I BMP receptor activity set SY-LB-35 and SY-LB-57 apart from previously reported small molecule activators of BMP signaling and suggested that the SY-LB compounds may act at the BMP receptor complex to regulate activity [30,46–50]. The SY-LB compounds also exhibited rapid activation of signaling via non-canonical BMP receptor pathways through PI3K/Akt-, p38- and ERK-mediated signaling [30]. These findings suggested that SY-LB-35 and SY-LB-57 function as true BMP receptor agonists with the ability to strongly activate Smad-dependent and Smad-independent signaling.

There is an unmet clinical need for the development of BMP receptor agonists or for agents that can enhance BMP-regulated intracellular signaling. Not only are formulations of rBMPs being used clinically to promote bone growth following reconstructive surgery or after injury [27–29], but preclinical experiments suggest that enhancing or restoring BMP receptor-dependent intracellular signaling would be beneficial for the treatment of diseases like chronic kidney disease or pulmonary arterial hypertension [2,38,46,51,52]. While rBMPs are able to stimulate *in vivo* repair processes [27,43], the use of recombinant proteins as therapeutics has significant drawbacks [29,53–55]. Recombinant BMPs, which are dimeric ligands, require complicated biosynthesis processes to produce active proteins, which ultimately translates to high patient costs [28,29]. Moreover, numerous reports of adverse effects resulting from *in vivo* rBMP administration call into question whether the benefits outweigh the harm rBMP therapeutics offer [53,54,56,57]. Small molecules can be synthesized at a fraction of the cost and can overcome obstacles regarding bioavailability or tissue penetration that often plague protein-based therapies such as rBMP2 or monoclonal antibody therapies. SY-LB-35 and SY-LB-57 are efficiently synthesized at large scale from commercially available compounds by a highly efficient one-pot synthesis method [30,31]. Thus, a strong case can be made for the development of these novel indolylbenzimidazole compounds as therapeutics for the treatment of injuries or disorders for which the production of new bone is needed. These compounds might also be beneficial in disorders like hereditary pulmonary arterial hypertension, where patients are typically lacking functional

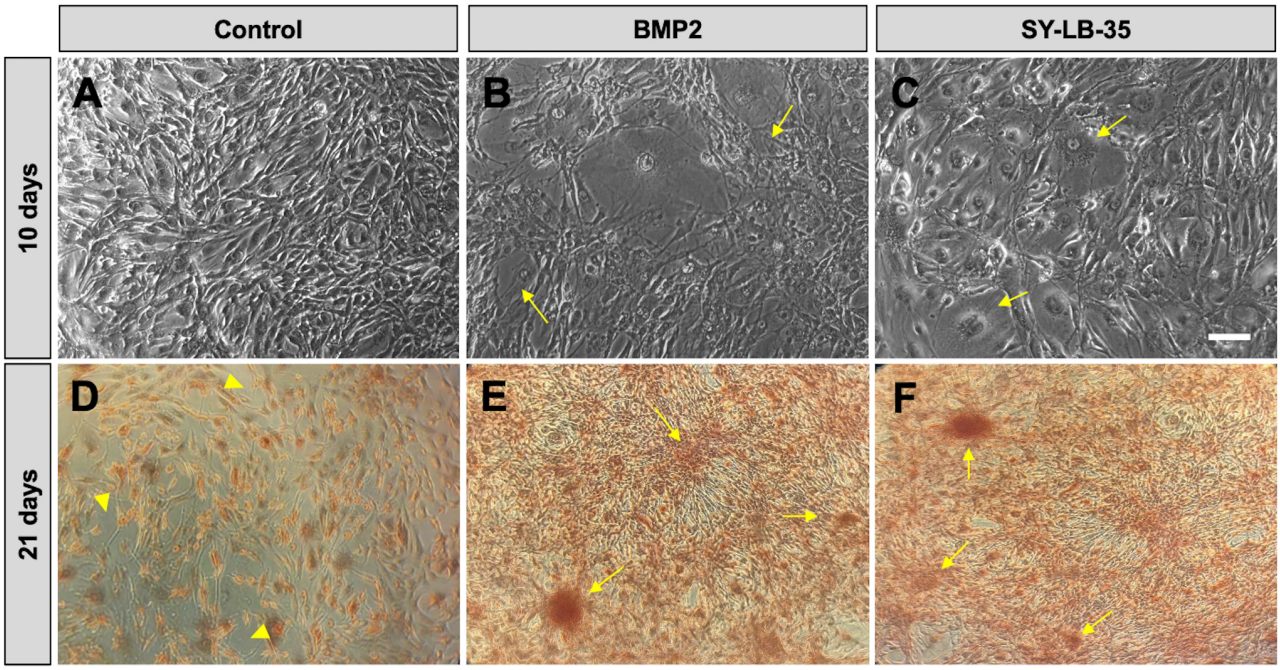

Fig. 9. Morphological changes and evidence of calcium deposition in long-term C2C12 cell cultures treated with SY-LB-35. (A–C) Representative 20× phase contrast images of serum-starved, confluent C2C12 cell cultures incubated with BMP2 (100 ng/mL) or SY-LB-35 (10 µM) treated every other day for 10 days in complete growth medium. Cultures maintained in (A) complete growth medium alone demonstrate typical spindle C2C12 cell morphology. Exposure of C2C12 cultures to (B) BMP2 or (C) SY-LB-35 resulted in cultures that were morphologically similar with an increased incidence of large, rounded cells (yellow arrows). (D–F) BMP2 (200 ng/mL) or SY-LB-35 (10 µM) were added to confluent C2C12 cultures every other day for three weeks. The cultures were then fixed and stained with Alizarin Red S, an indicator of extracellular calcium deposits (bright orange/red staining). (D) No strong Alizarin Red S staining is observed in cultures grown in complete growth medium for 21 days. Fewer cells are also present after 21 days in control cultures evidenced by more space between cells (yellow arrowheads). In contrast, 21-day treatment of C2C12 cell cultures with (E) BMP2 or (F) SY-LB-35 induced wide-spread calcium deposition in the cultures with strong staining in multiple areas in both cultures (yellow arrows). Scale bar = 100 µm.

BMPR2 receptor subunits or other key signaling components downstream of receptor activation [52,58–60].

The present study expands the results of the original study to include similarly robust activation by SY-LB-35 and SY-LB-57 of intracellular signaling regulated by BMPs in multiple cell types, the C2C12 mouse myoblast cell line, the WEHI 274.1 mouse monocytic cell line and primary endothelial cells isolated from mouse pulmonary arteries. SY-LB compounds stimulated highly significant increases in cell viability and wound healing that were specifically dependent upon signaling through PI3K and p38 activity, but, interestingly, not ERK activity. Moreover, these compounds exhibited specificity for type I BMP receptor, ALK2, where the substantial changes in Smad phosphorylation, cell viability and wound healing produced by SY-LB-35 and SY-LB-57 were completely eliminated by the selective ALK2 inhibitor, LDN193189. The prior study examined SY-LB-induced intracellular responses over a short time course using a field standard of 30 minutes for peak Smad phosphorylation and 15 minutes for phosphorylation events occurring through non-canonical BMP signaling pathways. Herein, the responses to SY-LB-35 and SY-LB-57 were surveyed at 24 hours in C2C12 cells and showed upregulation of Id1, BMPR2 and the inhibitory Smads, Smad6 and Smad7, and sustained phosphorylation of p38, ERK and CDK1. Moreover, increased cell viability and non-canonical BMP-regulated signaling was elicited by SY-LB-35 and SY-LB-57 at concentrations as low as 1 pM. In contrast, canonical Smad signaling is undetectable at these low concentrations.

Given the remarkable parallels between the responses in C2C12 cells to these novel benzimidazoles and rBMP2, SY-LB-35 and SY-LB-57 were assessed for the ability to induce the differentiation of the pluripotent C2C12 cell line into osteoblasts. C2C12 cells are widely used to assess the osteogenic activity of rBMPs and candidate BMP receptor activators [32,44,48,61]. In C2C12 cells, expression of Runx2, β-catenin, the type II BMP receptor, BMPR2, as well as increases in ALP activity are early markers of pre-osteoblast differentiation [24,26,62–64]. Upregulation of OCN expression in rBMP-treated C2C12 cells is a marker of committed osteoblasts [25,32,65] and matrix mineraliza-

tion of the extracellular matrix is indicative of mature osteoblasts [25,45,66]. Exposing C2C12 cells to SY-LB-35 or SY-LB-57 for 4 hours resulted in robust increases in expression of these early markers of osteoblast differentiation, as well as strong upregulation of p38 and ERK phosphorylation. Moreover, significant increases in ALP activity were observed after 72 hours and OCN expression after 7 days of SY-LB-35 or SY-LB-57 treatment. Furthermore, C2C12 cultures incubated with the SY-LB-35 for 10 days resulted in similar morphological changes in BMP2- and SY-LB-treated C2C12 cultures that were distinct from control cultures. Finally, after 21 days, these cultures exhibited evidence of calcium deposition and matrix mineralization. Taken together, the results of SY-LB-induced differentiation of C2C12 cells into osteoblasts demonstrates the osteogenic potential of the novel indolyl-benzimidazoles, SY-LB-35 and SY-LB-57, and establishes these compounds as potent BMP receptor agonists that merit further investigation as an attractive alternative to rBMP-based bone repair treatments.

4.1 SY-LB-35 and SY-LB-57 Are Potent BMP Receptor Agonists

The current study makes clear that the SY-LB compounds are potent BMP receptor agonists that stimulate robust BMP-regulated signaling through a common mechanism in multiple cell types. Moreover, rBMP-like increases in intracellular signaling through Smad, PI3K/Akt and p38 signaling pathways stimulated by the SY-LB compounds are associated with functional outcomes since inhibiting type I BMP receptor-, PI3K/Akt- and p38-dependent activity completely blocked increases in cell proliferation and wound healing in response to SY-LB-35 and SY-LB-57. The ability of SY-LB-35 and SY-LB-57 to stimulate increases in p-Akt, p-p38, p-ERK and p-CDK1 at picomolar concentrations and, importantly, significantly increased cell viability at the same very low concentrations demonstrates the potency of these novel compounds. It remains to be seen what concentrations would be required to achieve efficacy *in vivo*.

Therapies involving rBMPs in humans require potentially dangerous concentrations of rBMPs [27–29]. One reason for the need to use such high concentrations of rBMPs is due to endogenous antagonism of BMPs. BMP antagonists are families of extracellular factors that bind directly to BMPs and prevent BMP ligands from interacting with the BMP receptor complex [42]. Noggin is a BMP antagonist with a particularly high affinity for BMP2, which is precisely the BMP that is approved by the FDA for human use [27,67]. Although SY-LB-35- and SY-LB-57-evoked p-Smad responses were blocked by pre-incubation of C2C12 cells with Noggin, the SY-LB compounds were able to overcome inhibition by Noggin in competition assays suggesting that these potent agents may be able to do so *in vivo* as well. Whether these compounds are sensitive to other classes of BMP antagonists is still unclear. The ability of Noggin to block SY-LB-stimulated Smad phosphorylation also indicates that these compounds might interact with the extracellular domains of the BMP receptor complex.

4.2 Specificity in SY-LB-Stimulated Signaling

Previously, SY-LB-35 and SY-LB-57 were shown to stimulate Smad phosphorylation through a type I BMP receptor-dependent mechanism using the non-selective inhibitor, Dorsomorphin [30]. Here, the SY-LB compounds were shown to increase p-Smad levels, cell viability and the rate of wound healing through a specific type I BMP receptor subunit, ALK2. The selective ALK2 inhibitor, LDN193189, eliminated the responses to the SY-LB compounds in C2C12 and WEHI cells indicating a conserved mechanism involving ALK2 as part of the BMP receptor complex activated by SY-LB-35 and SY-LB-57. How and where these small molecule activators of BMP receptor signaling interact with an ALK2-containing BMP receptor complex is an area of ongoing investigation.

The activation of particular intracellular signaling pathways by the SY-LB compounds to increase cell viability and the rate of wound healing raises an enduring problem in the BMP/BMP receptor field of how the choice of downstream pathway is regulated [9,15,19,68–70]. Inhibition of PI3K/Akt and p38 signaling prevented the two indolyl-benzimidazoles from increasing cell viability or Invasion rates. In contrast, inhibition of ERK signaling had no effect on these processes indicating p-ERK stimulation may not have a role in cell proliferation or motility in C2C12 cells. Thus, it appears that these small molecules selectively utilize the PI3K/Akt and p38 signaling pathways to increase cell viability and rates of wound closure. Functional consequences of robust increases in ERK phosphorylation stimulated by SY-LB compounds remains to be determined.

A less appreciated feature of BMP/BMP receptor-dependent mechanisms is the distinct concentration range at which canonical and non-canonical BMP-regulated signaling is activated. For example, the PI3K/Akt pathway can be stimulated at picomolar concentrations of BMP7 [34,36,71]. Moreover, the peak of BMP7-stimulated WEHI monocyte chemotaxis was at 1 pg/mL BMP7 and found to be dependent upon the PI3K/Akt pathway [36]. Here, Smad phosphorylation was not detected in response to SY-LB-35 or SY-LB-57 at 0.1 nM and 1 nM, which is 100–1000 times the concentrations needed to activate non-canonical signaling pathways, given that p-Akt, p-p38, p-ERK, p-CDK1 and increases in cell viability were all stimulated by concentrations as low as 0.001 nM or 1 pM. Collectively, these results indicate that regulation of BMP receptor subunit activity by indolyl-benzimidazoles at low concentration is selective for non-canonical BMP-regulated signaling.

4.3 Osteogenic Differentiation by SY-LB Compounds Through Known Osteogenic Pathways

BMP2 prevents the myogenic differentiation of C2C12 cells and directs the differentiation of these cells into an osteoblast lineage [32]. Id1 is a negative regulatory protein that is upregulated during C2C12 osteoblast differentiation and is the most significantly upregulated gene in response to BMP2, BMP6 and BMP9 in various human and murine cell types [39,44]. BMP2 induces osteogenic differentiation in C2C12 cells by upregulating the expression of Id1 and the activity of ALP, an early marker of osteogenesis [45,65]. Osteoblast-specific markers include transcription factors such as Runx2, DLX5 and Osterix, while markers that are related to matrix formation include OCN and fibromodulin [25,26,72]. Moreover, *in vitro* and *in vivo* studies suggest a relevant role for p38 and ERK throughout the osteoblastic commitment process, from a mesenchymal progenitor into a fully functional anabolic bone cell [24,26,63,64]. Furthermore, it is clear that Wnt-dependent activation of β-catenin is required for bone formation and that BMP2 and β-catenin synergize to promote osteoblast differentiation [66,73].

Exposure of C2C12 cell cultures to SY-LB-35 and SY-LB-57 caused upregulation in the expression of Id1 and BMPR2, as would expected of any BMP-like ligand. The SY-LB compounds also stimulated the expression of protein markers specific to osteogenesis such as Runx2, β-catenin, ALP and OCN. Moreover, morphological changes and matrix mineralization consistent with differentiation of C2C12 cells into osteoblasts was observed in long-term cultures treated with SY-LB-35. Taken together, the evidence strongly supports the conclusion that SY-LB-35 and SY-LB-57 are osteogenic and raises the question of whether these novel indolyl-benzimidazole compounds or potential derivatives could be beneficial for bone repair *in vivo*.

SY-LB-35 and SY-LB-57 mimicked rBMP2-induced responses in numerous *in vitro* assays but did not behave identically. SY-LB-57 consistently induced responses that were significantly greater in magnitude than responses to SY-LB-35 demonstrating greater efficacy of SY-LB-57 for stimulating BMP-regulated processes. Given that the two compounds differ by a single substitution, a hydroxyl group on SY-LB-35 or a methoxy group in the equivalent position on SY-LB-57, it will be of interest to vary the substitutions and/or the position of the substitutions to evaluate how such changes might change the activity of the benzimidazole compounds.

5. Conclusions

SY-LB-35 and SY-LB-57 are heterocyclic, indolyl-benzimidazole small molecules that faithfully and robustly reproduce BMP-stimulated *in vitro* responses in multiple cell types. Interestingly, the SY-LB compounds activate non-canonical BMP-regulated signaling pathways and stimulate increases in cell viability at concentrations at which canonical Smad phosphorylation is not detected demonstrating differential regulation of downstream signaling by SY-LB-35 and SY-LB-57. The functional consequences of exposure to the SY-LB compounds include substantially increasing cell viability and the rate of wound closure. In addition to promoting cellular proliferation or migration, the indolyl-benzimidazole compounds exhibit clear evidence of promoting osteogensis. Markers of early-, mid- and late-stage osteoblast differentiation are all upregulated in C2C12 cells exposed to these small molecules. Taken together, the evidence presented confirms that these SY-LB compounds are potent BMP receptor agonists with osteogenic activity. Future efforts to explore the osteogenic potential of the compounds include rodent models of subcutaneous ectopic bone growth to first screen for responses to *in vivo* exposure to SY-LB-35 or SY-LB-57 [74]. Next, bone defect and ovariectomy-induced postmenopausal osteoporosis are attractive models that could be used to test the *in vivo* efficacy of the indolyl-benzimidazoles [55,75,76]. Moreover, inducible models of PAH or chronic kidney disease are also appealing avenues for pre-clinical investigation of the *in vivo* efficacy of these small molecules [2,77,78]. With the advantages associated with small molecules, the SY-LB compounds could potentially be cost-effective replacements for rBMP-based therapies targeting a number of organ systems.

Abbreviations

ALK2, activin-like kinase 2; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; BMPR2, BMP receptor type 2; OCN, osteocalcin.

Availability of Data and Materials

All data and materials obtained from this study are reported in the submitted article.

Author Contributions

SN and JCP designed the research study. SY designed the synthesis method for the indolyl-benzimidazoles. LB carried out the chemical synthesis, purification, and characterization of the indolyl-benzimidazoles. SN, JMJF and JHA performed the research. SN, JHA and JCP analyzed the data. SN and LB wrote the original manuscript. SN, JCP, LB, SY, JHA and JMJF contributed to revisions of the manuscript. All authors have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2810268.

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