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THE SPECIFICITIES OF SMALL MOLECULE INHIBITORS OF THE TGFß AND BMP PATHWAYS

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Abstract

Small molecule inhibitors of type 1 receptor serine threonine kinases (ALKs1-7), the mediators of TGFβ and BMP signals, have been employed extensively to assess their physiological roles in cells and organisms. While all of these inhibitors have been reported as “selective” inhibitors of specific ALKs, extensive specificity tests against a wide array of protein kinases have not been performed. In this study, we examine the specificities and potencies of the most frequently used small molecule inhibitors of the TGFβ pathway (SB-431542, SB-505124, LY-364947 and A-83-01) and the BMP pathway (Dorsomorphin and LDN-193189) against a panel of up to 123 protein kinases covering a broad spectrum of the human kinome. We demonstrate that the inhibitors of the TGFβ pathway are relatively more selective than the inhibitors of the BMP pathway. Based on our specificity and potency profile and published data, we recommend SB-505124 as the most suitable molecule for use as an inhibitor of ALKs 4, 5 & 7 and the TGFβ pathway. We do not recommend Dorsomorphin, also called Compound C, for use as an inhibitor of the BMP pathway. Although LDN-193189, a Dorsomorphin derivative, is a very potent inhibitor of ALK2/3 and the BMP-pathway, we found that it potently inhibited a number of other protein kinases at concentrations sufficient to inhibit ALK2/3 and its use as a selective BMP-pathway inhibitor has to be considered cautiously. Our observations have highlighted the need for caution when using these small molecule inhibitors to assess the physiological roles of BMP and TGFβ pathways.

Keywords: TGF-beta, BMP, Kinase, Inhibitors, LDN-193189, SB-505124

Abbreviations: ActR-IA, activin A receptor, type IIA; ActR-IIB, activin A receptor, type IIB; ALK, Activin receptor-like kinase; AMH, anti-müllerian hormone; AMHR-II, anti-müllerian hormone receptor type II; BMP, bone morphogenetic protein; BMPR-II, bone morphogenetic protein receptor type II; GDF, growth differentiation factor; TGFβ, transforming growth factor-β; TGFβR-II, transforming growth factor-β receptor type II.
1. Introduction

The signalling pathways downstream of the transforming growth factor beta (TGFβ) family of cytokines, that comprise some 42 members, control plethora of cellular processes including proliferation, differentiation, extra-cellular matrix production, motility, survival and fate [1]. Aberrant TGFβ signalling pathways are associated with many human diseases, including bone diseases, immune-suppression, fibrosis, cancer progression and metastasis [2-7]. Hence targeted disruption of specific TGFβ signalling components by small molecules or other means provides potential therapeutic opportunities. Over the past few years, BMP/TGFβ type I and type II receptor serine threonine protein kinases, the transducers of BMP and TGFβ signals, have been targeted for development of small molecule inhibitors. Specific small molecule inhibitors of these protein kinases not only provide a flexible, rapid and cost-effective means of inhibiting their targets in cells and tissues but also potentially could have many therapeutic applications.

The TGFβ family of ligands is broadly divided into two groups based on their ability to trigger the activation of specific Smad transcription factors, the intracellular mediators of TGFβ signals. The TGFβ subfamily (which includes TGFβ, Activin and Nodal) activates Smads 2 and 3, while the BMP subfamily (which includes BMPs, GDFs and AMH) activates Smads 1, 5 and 8 [1]. The ligands exist as homo- or hetero-dimers and bind to specific sets of type II and type I receptors, which are serine-threonine protein kinases, and thus result in a large ligand-receptor complex involving a ligand dimer, two type II and two type I receptor molecules [1, 8]. The formation of ligand-receptor complex facilitates the constitutively active type II receptor kinases to phosphorylate and activate the type I receptor kinases [1, 9]. In all, there are five type II receptors (ActR-IA, ActR-IIB, BMPR-II, AMHR-II and TGFBR-II) and seven type I receptors (also known as Activin-receptor-Like-Kinases: ALKs 1-7). The TGFβ subfamily of ligands form unique receptor complexes by pairing specific type II receptors (TGFβR-II or ActR-IIB) with specific type I (ALK4, ALK5 or ALK7) receptors. Similarly the BMP family of ligands construct receptor complexes by pairing specific type II receptors (BMPR-II or ActR-IA/B or AMHR-II) with specific ALKs (ALK1, ALK2, ALK3 or ALK6) [1, 8]. Once activated ALKs 4, 5 and 7 primarily phosphorylate Smad2 and 3 while ALKs 1, 2, 3 and 6 phosphorylate Smads 1, 5, and 8 at the highly conserved C-terminal Ser-Xxx-Ser motif. This phosphorylation of dual residues, often referred to as tail-phosphorylation, triggers the binding of Smads to co-Smad4 and their translocation to the nucleus. In the nucleus Smad4 and tail-phosphorylated Smads form functional complexes with other cofactors and regulatory proteins and regulate the transcription of over 500 genes, which control context-specific cellular outcomes [1, 10].

Given the indispensable roles of ALKs in driving the TGFβ and BMP pathways, they have become attractive targets for the development of small molecule inhibitors to attenuate the cellular effects of TGFβ and BMP ligands. Among TGFβ ligands, TGFβ 1-3, Activin and Nodal lead to the activation of ALK5, ALK4 and ALK7 respectively [1]. The kinase domains of ALKs 4, 5 and 7 are highly related to each other structurally. Similarly the kinase domains of BMP-activated ALKs (ALKs 1, 2, 3, and 6) display a high degree of similarity with one another, although among these ALK1 is more closely related to ALK2, and ALK3 is more closely related to ALK6 [11]. The expression of ALK1 is limited to certain cell types, primarily in endothelial cells, and has also been implicated in mediating TGFβ-induced phosphorylation of Smad1/5/8 in conjunction with ALK5 [12, 13]. Over the past few years several small molecule inhibitors of various ALKs have been developed. SB-431542 [11, 14, 15], SB-505124 [16], SB-525334 [17], LY-364947 [18, 19], A-83-01 [20], LY-2157299 [21], GW-6604 [22] and SD-208 [23] have all been reported as selective inhibitors of the TGFβ-activated ALKs (ALK4, 5 and 7). Similarly, more recently Compound C (but renamed Dorsomorphin to describe its effect in zebrafish embryos) [24] and its derivative LDN-
193189 [6, 25] have been reported as selective inhibitors of the BMP-activated ALKs (ALKs 2, 3 and 6). Although these compounds are described as “selective” inhibitors of specific ALKs, extensive specificity tests against other protein kinases have not been reported, except for Compound C [26]. Here we report the specificities and potencies of the most commonly used chemical inhibitors of the TGFβ pathway (SB-431542, SB-505124, LY-364947 and A-83-01) and BMP pathway (Dorsomorphin and LDN-193189) (Figure 1) by profiling these against a panel of up to 123 protein kinases covering a broad spectrum of the human kinome. Our data indicate that among the TGFβ pathway inhibitors, SB-505124 is a potent and selective inhibitor of TGFβ-activated ALKs. We also demonstrate that both of the BMP pathway inhibitors, Dorsomorphin and LDN-193189, can potently inhibit multiple protein kinases in addition to the BMP-activated ALKs. Our specificity data will be useful for researchers considering the use of these molecules as BMP and TGFβ pathway inhibitors.

2. Materials and Methods

2.1. Materials

SB-431542, SB-505124, LY-364947 and Dorsomorphin (Compound C) were purchased from Sigma. A-83-01 was purchased from Tocris Bioscience. LDN-193189 was purchased from Stemgent. 32P γ-ATP was from Perkin-Elmer. BMP-2 and TGFβ1 were from R&D Biosystems. Meso-diaminopimelic acid (meso-DAP) was synthesised by Natalia Shpiro. DMSO and Tween-20 were from Sigma. Active GST-ALK2 and GST-ALK4 were purchased from Carna Biosciences. Antibodies recognising phospho-Smad1/5/8, phospho-Smad2, GAPDH, phospho-ERK1/2 and total ERK1/2 were from Cell Signalling.

2.2. General Methods

Tissue culture, immunoblotting, restriction enzyme digests, DNA ligations and other recombinant DNA procedures were performed using standard protocols. All DNA constructs used were verified by DNA sequencing, performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

2.3. Specificity Kinase panel

All protein kinases in the specificity panel were expressed, purified and assayed at The National Centre for Protein Kinase Profiling (http://www.kinase.screen.mrc.ac.uk/) as previously described [26]. Briefly, all assays (except ALK assays, which are described below) were carried out robotically at room temperature (21 °C) and were linear with respect to time and enzyme concentration under the conditions used. Assays were performed for 30 min using Multidrop Micro reagent dispensers (Thermo Electron Corporation, Waltham, MA, U.S.A.) in a 96-well format. The abbreviations for each of the kinases are defined in the legend to Figure 2. The concentration of magnesium acetate in the assays was 10 mM and [γ-32P]ATP (~800 cpm/pmol) was used at 5 μM for ABL, Aurora A, CK2α, CLK2, DAPK1, DYRK3, EF2K, EIF2AK3, ERK1, ERK8, GSK3β, HER4, HIPK2, IGF-1R, IKKβ, IRAK1, IRR, JAK2, MARK3, MKK1, MKK2, p38α MAPK, p38γ MAPK, PKA, PKCβ, PKCγ, PKD1, PLK1, PIM3,PKBα, PKCζ, PRAK, RIPK2, TAK1, TLK1 and ZAP70, 20 μM for Aurora B, BRK, BRSK1, CAMKKβ, CDK2-Cyclin A, CHK1, CHK2, CK1δ, Csk, EPK-B1, EPK-B2, EPK-B3, ERK2, GCK, HIPK1, HIPK3, IR, IRAK4, JNK1, JNK2, JNK3, LKB1, MAPKAP-K2, MAPKAP-K3, MARK1, MARK2, MEKK1, MLK3, MNK1, MSK1, MST4, NEK2α, OSR1, p38γ MAPK, PKA, PKCα, PKCγ, PKD1, PLK1, PKR2, ROCK2, RSK1, S6K1, SmMLCK, SYK, TAO1, TIE2, TrkA, TTK, VEG-FR and YES1 and 50 μM for AMPK, ASK1, BRK, BTK, CAMK1, DYRK1A, DYRK2, ERK2, ERK4, EPK-A, EPK-B4, IKKε, Lck, MARK4, MELK, MINK1, MKK6, MLK1, MNK2, MPSK1, MST2, NEK6, NUAK1, p38α MAPK, PHK, PKBβ, PKCα, RSK2, S6K1, Src, SRPK1,
STK33 and TBK1 in order to be at or below the $K_m$ for ATP for each enzyme. For kinase assays with CK1 isoforms, 300 µM CK1-peptide KRRRALS*VASLPGGL (where S* is phospho Serine) was used as the substrate.

2.4. Cell Culture, Manipulation and Lysis:

Human keratinocyte (HaCaT) cells were cultured in 10-cm diameter dishes in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Foetal bovine serum, 1% penicillin/streptomycin mix and 2 mM L-Glutamine (D10F). RAW 264.7 cells (Mouse leukaemic macrophage cell line) were cultured as above except that foetal bovine serum was heat inactivated at 65 °C for 1h prior to use. Both cell lines were grown under a humidified atmosphere with 5 % CO$_2$ at a constant temperature of 37 °C. HaCaT cells were deprived of serum for 16h prior to treatment with ligands or inhibitors. Unless stated otherwise, cells were treated with the appropriate small molecule inhibitors or solvent control 2h prior to treating cells with BMP-2 (25 ng/ml final), TGFβ (50 pM final) or Meso-DAP (15 µM) for 1h. Cells were then washed once with ice-cold PBS and lysed in 0.5 ml ice-cold complete lysis buffer (50 mMTris-HCl pH7.5, 1 mM EGTA, 1 mM EDTA, 1 % Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5mM sodium pyrophosphate, 0.27 M sucrose, 5 mM β-glycerophosphate, 0.1 % (v/v) 2-mercaptoethanol, 1 tablet per 25 ml of complete protease inhibitor cocktail). The extracts were spun down at 16,000 g at 4 °C for 10 minutes prior to snap-freezing in liquid nitrogen and storing at -80 °C if not processed immediately.

2.5. SDS-PAGE and Western Blotting:

Cell extracts (20 µg) were heated at 95 °C for 5 min in 1XSDS sample buffer (62.5 mM Tris-HCl pH 6.8, 10% (v/v) Glycerol, 2% (w/v) SDS, 0.02% (w/v) bromophenol blue, and 1% (v/v) β-mercaptoethanol), resolved on a 10% polyacrylamide gel by electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in TBS-T buffer [50 mM Tris-HCl pH 7.5, 0.15 M NaCl and 0.1% (w/v) Tween-20] containing 10% (w/v) non-fat milk. The membranes were then incubated with the indicated antibodies, diluted in TBS-T containing 10% (w/v) milk for 16 h at 4 °C. The membranes were washed 2x10 min in TBS-T buffer, probed with the secondary antibody (either HRP-conjugated or IRDye-800 or -680 conjugated and diluted 1:5000 in TBS-T/5% milk) for 1h at room temperature, and washed 3x10 min in TBS-T buffer. Detection was performed by using enhanced chemiluminescence reagent for HRP-conjugated secondary antibodies and by using the Odyssey Imaging System (LI-COR Biosciences) for IRDye-800 or -680 conjugated antibodies. For IC$_{50}$ determinations, the intensities of the bands corresponding to appropriate phosphorylated Smads and corresponding total Smads were quantified using the Odyssey Imaging System software.

2.6. ALK2, ALK3, ALK4 and ALK5 Kinase assays: N-terminal GST-tagged constitutively active mutant of ALK3 (Q233D, 187-532), wild type GST-ALK5 (200-503) and wild type GST-BMPRII (174-1038) were cloned into pFastBac baculovirus vectors (Invitrogen) and expressed in Sf9 insect cells. For kinase assays, 20 µl reactions were setup consisting of 150 ng of kinase (GST-ALK2, 3, 4 or 5) and 2 µg substrate protein (GST-Smad1 or 2) in a buffer containing 50 mM TrisHCl pH 7.5, 0.1% 2-mercaptoethanol, 0.1 mM EGTA, 10 mM MgCl$_2$, 0.5 µM Microcystein-LR, 0.1 mM γ$^3$P-ATP (500 cpm/pmole) and 5% DMSO or DMSO containing the appropriate concentrations of the small molecule inhibitors. For ALK2 and ALK3 assays, GST-Smad1 was used as a substrate. For ALK4 and ALK5 assays, GST-Smad2 was used as a substrate. ALK3 assays also contained 150 ng of GST-BMPRII. The assays were performed at 30 °C for 30 minutes and stopped by adding 1X SDS sample buffer and heating at 95 °C for 5 minutes. The samples were resolved by SDS-PAGE, the gels stained with Coomassie Blue and dried. The radioactivity was analysed by autoradiography. For IC$_{50}$ and percentage kinase activity remaining determinations, the stained bands representing protein substrates were excised and the radioactivity measured.
3. Results

3.1. Specificities of Inhibitors of the TGFβ pathway

Although multiple small molecule inhibitors have been reported as specific inhibitors of TGFβ pathway, SB-431542, SB-505124, LY-364947 and A-83-01 have been the most widely exploited in studies investigating the physiological roles of TGFβ ligands, together accounting for over 300 publications. In this study we have profiled the specificities of these molecules against a panel of up to 123 protein kinases \textit{in vitro} (Figure 2A-D; Figure 4). Structurally SB-431542 is similar to SB-505124 and LY-364947 is similar to A-83-01 (Figure 1).

3.2. Specificities and Potencies of SB-431542 and SB-505124

SB-431542 and SB-505124 were both developed as ALK5 inhibitors from triarylindazole templates [11, 14-16]. Both compounds are ATP-competitive, reversible inhibitors of ALK5 and can also inhibit ALKs 4 and 7 [11, 14-16]. SB-431542 was the first small molecule inhibitor of ALKs 4, 5 and 7 to be reported and has been the most widely used inhibitor of the TGFβ pathway resulting in over 200 research reports [11, 14, 15]. We tested the ability of both SB-431542 and SB-505124 to inhibit the activity of a panel of over 105 protein kinases at two different concentrations (Figure 2A&B). At 1 µM, besides ALK5, SB-431542 inhibited RIPK2 and CK1δ activities by 77% and 70% respectively, while p38α MAPK was inhibited by 30% (Figure 2A). At 0.1 µM, SB-431542 inhibited RIPK2 and CK1δ by 33% and 29% respectively (Figure 2A). Similarly 1 µM SB-505124 inhibited RIPK2 by about 72% and p38α MAPK by 49% but did not inhibit CK1δ (Figure 2B). At 0.1 µM, SB-505124 inhibited RIPK2 by 18% but CK1δ and p38α MAPK were not inhibited (Figure 2B). At both concentrations, SB-431542 and SB-505124 inhibited ALK5 activity \textit{in vitro} but did not inhibit ALK3 (Figure 2A&B, Figure 4A&B). At both concentrations the activities of all other kinases in the panel were not significantly inhibited by either of these compounds (Figure 2A&B).

SB-431542 inhibits the phosphorylation of Smad3 by ALK5 and ALK4 \textit{in vitro} with an IC\textsubscript{50} of 0.094 µM and 0.14 µM respectively [11] (Table 1A). In contrast, SB-505124 inhibits the phosphorylation of Smad3 by ALK5 and ALK4 with an IC\textsubscript{50} of 0.047 µM and 0.129 µM respectively [16] (Table 1A). We determined that SB-431542 and SB-505124 inhibit RIPK2 with IC\textsubscript{50} of 0.41 µM (4-fold lower potency than that seen for ALK5) and 0.35 µM (7-fold) respectively (Table 2). Because SB-431542 also potently inhibited CK1δ at 1 µM (Figure 2A), we tested the ability of both SB-431542 and SB-505124 to inhibit CK1 isoforms \textit{in vitro}. SB-431542 potently inhibited CK1α, CK1δ and CK1ε isoforms with IC\textsubscript{50} of 1.34 µM, 0.92 µM and 0.38 µM respectively but did not inhibit CK1γ (Table 2). SB-505124 inhibited CK1α, CK1δ and CK1ε isoforms with IC\textsubscript{50} of 19.44 µM, 3.38 µM and 1.60 µM respectively but did not inhibit CK1γ (Table 2). Both SB-431542 and SB-505124 also inhibit p38α MAPK at high concentrations with IC\textsubscript{50} values reported to be >10 µM [11, 16].

SB-505124 is reported to be a more potent inhibitor of the TGFβ pathway in cells than SB-431542 [16]. In multiple cell lines the TGFβ induced phosphorylation of Smad2 was inhibited by SB-505124 and SB-431542 with IC\textsubscript{50} values of ~0.25 µM and 0.5-1 µM respectively [11, 16] (Table 1A). Similarly SB-505124 inhibited the ability of constitutively active ALK5 to induce the expression of CAGA-luciferase reporter activity more potently than SB-431542 [16] (Table 1A). Both inhibitors were also shown to inhibit the phosphorylation of Smad2 and expression of CAGA-Luciferase reporter activities driven by constitutively active ALK4 and ALK7 [11, 16].

3.3. Specificities and Potencies of LY-364947 and A-83-01

LY-364947, a pyrazole-based small molecule, was developed as an inhibitor of ALK5 and is an ATP-competitive, cell permeable inhibitor [18, 19]. \textit{In vitro}, it inhibits ALK5 with an IC\textsubscript{50}...
of 0.058 µM, potency comparable to that of SB-505124 [19](Table 1A). Furthermore it inhibits TGFβ-induced phosphorylation of Smad2 in cells with a similar potency as SB-505124 (Table 1A). When profiled against 123 protein kinases (Figure 2C), LY-364947 at 1 µM inhibited ALK5, RIPK2, VEGF-R, CK1δ and MINK1 activity by more than 50% and at 10 µM, in addition to these kinases, it inhibited p38α MAPK, PKD1, GCK, BRK, Lck, TAK1, YES1, FGF-R1 and p38β MAPK by more than 50%. LY-364947 inhibited RIPK2 and CK1δ with IC₅₀ of 0.11µM and 0.22µM respectively (Table 2). Similarly LY-364947 would be predicted to inhibit VEGF-R and MINK1 with similar IC₅₀ values (Figure 2C). LY-364947 inhibited CK1α, CK1ε and CK1γ isoforms with IC₅₀ of 2.27 µM, 1.34 µM and 44 µM respectively (Table 2).

A-83-01, structurally related to LY-364947 (Figure 1), was developed as an inhibitor of the TGFβ pathway using a cell-based CAGA-Luciferase reporter assay driven by constitutively active ALKs4, 5 and 7 [20]. A-83-01 inhibited TGFβ-induced CAGA-Luciferase reporter activity in Mv1Lu lung epithelial cells with an IC₅₀ of 0.03 µM [20](Table 1A), more potently than SB-431542 (IC₅₀, 0.25µM) and SB-505124 (IC₅₀, 0.1 µM). However detailed kinetic analysis of the ability of A-83-01 to inhibit different ALKs in vitro has not been reported [20]. Nonetheless we tested the ability of A-83-01 to inhibit a panel of 107 kinases at 1 µM and 0.1 µM (Figure 2D). We demonstrate that at 1 µM, A-83-01 inhibited ALK5, VEG-FR, RIPK2, MINK1, p38α MAPK, PKD1 and FGF-R1 by more than 50% (Figure 2D). At 0.1µM, ALK5, VEG-FR, RIPK2 were inhibited by more than 50% while MINK1, p38α MAPK and FGF-R1 were inhibited by more than 30% (Figure 2D). A-83-01 potently inhibited RIPK2 with an IC₅₀ of 0.1 µM (Table 1B) and would be predicted to inhibit VEGF-R with similar potency (Figure 2D). A-83-01 inhibited CK1α, CK1δ and CK1γ isoforms with IC₅₀ of 15.66 µM, 3.42 µM, 4.59 µM and 29 µM respectively (Table 2).

3.4. Specificities of Inhibitors of the BMP pathway

Recently, Dorsomorphin (Compound C) and LDN-193189, a Dorsomorphin derivative, were reported as selective and potent inhibitors of the BMP pathway [6, 24, 25]. Subsequently these compounds have been widely used in cell-based assays and whole organisms to study the physiological roles of the BMP pathway. In this study we have profiled the specificities of these molecules against a panel of up to 121 protein kinases in vitro (Figure 3A&B).

3.5 Specificity of Dorsomorphin (Compound C) as a BMP pathway inhibitor

In vertebrates BMP signalling plays a crucial role in defining dorso-ventral (DV) axis, where inhibition of BMP pathway results in dorsalised axis patterning [27]. A high throughput small molecule screen in zebrafish embryos identified Compound C (this was renamed Dorsomorphin) as an inhibitor of the BMP pathway as it resulted in dorsalised axis patterning of zebrafish embryos [24]. Subsequently Dorsomorphin was reported as a selective small molecule inhibitor of BMP pathway and was shown to inhibit BMP-activated ALKs 2, 3 and 6 [24]. Previously Compound C has been described, and extensively used, as a selective inhibitor of AMPK [28]. However, a study looking at the specificity of Compound C profiled against a panel of 70 kinases found that it inhibited a number of kinases, including ERK8, MNK1, PHK, MELK, DYRK isoforms, HIPK2, Src and Lck, with similar or greater potency than AMPK [26]. This information on the specificity profile of Compound C [26] has been overlooked by all the reports describing or employing Dorsomorphin as a specific inhibitor of the BMP pathway [24, 29-31].

In this study we extended the specificity and the potency tests on Dorsomorphin at three different concentrations against a panel of 119 protein kinases (Figure 3A). At 10 µM, Dorsomorphin inhibited the activities of 64 out of the 119 kinases by >50%. At 1 µM, Dorsomorphin inhibited the activities of 34 out of 119 kinases more potently than it inhibited AMPK and by >50% (Figure 3A). Even at 0.1µM, VEGF-R, ERK8, GCK, CLK2, DYRK1A, PHK, ABL, NUA1, PRK2 and YES1 were inhibited by >50% implying that Dorsomorphin inhibits these kinases with IC₅₀ values lower than 0.1µM (Figure 3A). Dorsomorphin
inhibited ALK3 in vitro with an IC\textsubscript{50} of ~1 µM while it did not inhibit ALK5 in vitro (Figure 4C). Furthermore, Dorsomorphin was reported to inhibit the BMP Responsive Element (BRE)-Luciferase reporter activity driven by constitutively active ALK2, ALK3 and ALK6 with IC\textsubscript{50} values of 0.2 µM, 0.5 µM and 5-10 µM respectively [24] (Table 1B). Clearly Dorsomorphin is not a selective inhibitor of the BMP pathway and is therefore not a good candidate for selective inhibition of BMP-activated ALKs. In fact it was recently reported that in zebrafish, Dorsomorphin, when used at concentrations sufficient to inhibit the BMP pathway, strongly inhibited intersegmental vessel formation by inhibiting VEGF-R2 [32], demonstrating the potential off-target effects of using a non-selective inhibitor.

3.6. Specificity of LDN-193189 as a BMP pathway inhibitor

Using Dorsomorphin as a template, LDN-193189 was developed as a compound with improved potency as a BMP pathway inhibitor [25]. In rat pulmonary artery smooth muscle cells (rPASMC), it was shown that LDN-193189 inhibited BMP4-induced phosphorylation of Smad1/5/8 with an IC\textsubscript{50} of 0.005 µM, an improvement of 94-fold over Dorsomorphin, which it inhibits with an IC\textsubscript{50} of 0.47 µM [25]. In human keratinocyte HaCaT cells, we observed that LDN-193189 inhibits BMP2-induced phosphorylation of Smad1/5/8 with an IC\textsubscript{50} of ~0.005 µM (Figure 5A). In contrast the TGFβ-induced phosphorylation of Smad2 was only slightly affected at >3 µM but TGFβ-induced phosphorylation of Smad1/5/8 was inhibited robustly at 0.3 µM LDN-193189 (Figure 5B). We next assessed the ability of LDN-193189 to inhibit various ALKs in vitro using 100 µM ATP in the assays (Figure 6). LDN-193189 inhibited the ability of ALK2 to phosphorylate GST-Smad1 in vitro with an IC\textsubscript{50} of 45 nM, while its autophosphorylation was inhibited with an IC\textsubscript{50} of 30 nM (Figure 6A). LDN-193189 inhibited the ability of ALK3 to phosphorylate Smad1 in vitro with an IC\textsubscript{50} of 100 nM, although even at 3 µM, ALK3 was not completely inhibited (Figure 6B). It is also noteworthy that autophosphorylation of BMPRII, which was also present in the assay and is required in order to activate ALK3 (data not shown), was not inhibited by LDN-193189, implying that BMPRII is not inhibited by LDN-193189 (Figure 6B). LDN-193189 inhibited ALK4 and ALK5 with much higher IC\textsubscript{50} values of 0.3 µM and 0.5 µM respectively (Figure 6C&D). LDN-193189 has been reported to inhibit the BRE-Luciferase reporter activity driven by constitutively active ALK2 and ALK3 with IC\textsubscript{50} of 0.005 µM and 0.03 µM respectively [6] (Table 1B).

Overall it is evident that LDN-193189 is a very potent inhibitor of the BMP pathway and that it can inhibit BMP-activated ALK2 and ALK3 in vitro. However, despite studies describing LDN-193189 as a selective and potent inhibitor of the BMP pathway, its specificity and potency has not been tested against an extensive array of protein kinases.

We profiled the specificity and potency of LDN-193189 at three different concentrations against a panel of 121 protein kinases covering a broad spectrum of the human kinase (Figure 3B). We noted a very similar specificity and potency profile for LDN-193189 and Dorsomorphin (Figure 3A&B). Like Dorsomorphin, we found that at 10 µM, LDN-193189 inhibited 44 out of the 121 kinases by >50%, majority of them very potently (Figure 3B). At 1 µM, LDN-193189 inhibited 24 out of the 121 protein kinases by >50% and of these RIPK2, FGF-R1, NUAK1, CAMKKβ, MINK1, GCK, VEG-FR, BRK, YES1 and CLK2 were inhibited very potently. Even at 0.1 µM, LDN-193189 inhibited RIPK2, FGF-R1, NUAK1, CAMKKβ, MINK1, GCK, VEG-FR and BRK by >50%, implying that these kinases are inhibited by LDN-193189 with IC\textsubscript{50} values lower than 0.1 µM (Figure 3B). Indeed LDN-193189 inhibits RIPK2 and GCK with IC\textsubscript{50} values of 0.025 µM and 0.08 µM respectively, values similar to those seen against ALK2 and ALK3 in vitro respectively (Table 2; Figure 6A&B).

3.7. Inhibition of RIPK2 by LDN-193189 in RAW macrophage cells

We noted from above that RIPK2, a member of the receptor interacting protein (RIP) family of protein kinases, was inhibited potently in vitro by LDN-193189 (Table 2). RIPK2 is implicated in NOD1 and NOD2 signalling and results in the activation of MAP Kinases, NFκB and inflammatory mediators in response to NOD1/2 agonists. In cells, NOD1 and
NOD2 sense peptidoglycan-related molecules from intracellular bacteria that have evaded recognition by Toll-like receptors at the cell-surface [33]. In RAW macrophages cells, the peptidoglycan-related agonist D-glutamyl-meso-diaminopimelic acid (meso-DAP) induced the phosphorylation of ERK1/2 (Figure 7). This induction was inhibited when cells were treated with 0.1 µM or 10 µM LDN-193189 (Figure 7), concentrations that are sufficient to inhibit BMP signalling (Figure 5A).

4. Discussion

In this report we have examined the specificities of the most commonly used small molecule inhibitors of the TGFβ and BMP pathways against a panel of up to 123 protein kinases. The results highlight the potential off-target effects of these small molecule inhibitors when using them to assess the physiological roles of TGFβ and BMP pathways. Furthermore, because the specificity was profiled against only 23% of the human protein kinases, these inhibitors are likely to inhibit other kinases that have not yet been studied. While caution is recommended in interpreting any impact on the TGFβ and BMP pathways resulting from the use of these inhibitors, the specificity profile provided herein should provide useful information for researchers when deciding which inhibitor to use. We also emphasize the fact that the specificity profiles presented in this report were obtained using in vitro kinase assays. We recommend using the minimum effective concentrations against intended targets when using any chemical inhibitors to inhibit the BMP/TGFβ pathways in cell and animal based assays and to test thoroughly whether at these concentrations the molecules also inhibit other kinases that are inhibited potently in vitro.

4.1. Inhibitors of the TGFβ pathway:

Active TGFβ signalling has been implicated in the development of fibrotic sclerosis of multiple organs including heart, kidney, lungs, liver and skin [2-4]. TGFβ signalling is also associated with promotion of cancer progression and metastasis [5, 34]. As a result, TGFβ-activated ALKs, in particular ALK5, have been targeted for the development of small molecule inhibitors by major pharmaceutical industries [14, 18, 35]. Many ALK5 inhibitors, which also potently inhibit ALK4 and ALK7, have entered pre-clinical trials to treat fibrosis and advanced metastatic cancers and have met with mixed results [35]. The specificity of chemical inhibitors is particularly important when using them in whole organisms, as consequences of off-target effects could lead to undesirable side effects.

Based on specificity and potency of the four inhibitors of the TGFβ pathway, we recommend the use of SB-505124, at or below 1µM, as an inhibitor of ALKs 4, 5 and 7 in cell based assays. While both SB-431542 and SB-505124 are relatively selective inhibitors of ALKs 4, 5 and 7, SB-505124 is a more potent inhibitor of ALK4, 5 and 7 and inhibits CK1 isoforms less potently than SB-431542 (Table 1A & 2). Furthermore, in cell-based assays, SB-505124 was reported to be less cytotoxic than SB-431542 [16]. Both inhibit RIPK2 with similar IC50 values (Table 2) and we recommend that RIPK2 inhibition be assessed at concentrations of SB-505124 used to inhibit TGFβ signalling. SB-525334, which is structurally very closely related to SB-505124 (Figure 1), has been reported to be around 3-fold more potent inhibitor of ALK5 and ALK4 compared to SB-505124 [36], however it has not been used as extensively as other ALK5 inhibitors. At concentrations sufficient to inhibit ALK5, both LY-364947 and A-83-01 inhibited RIPK2, MINK1 and VEGF-R potently. LY-364947 also inhibited CK1 isoforms potently while A-83-01 inhibited p38α MAPK, PKD1 and FGF-R1 potently (Figure 2C&D). When using LY-364947 and A-83-01 as TGFβ pathway inhibitors, these potential off-target effects have to be considered. One of the impediments to using small molecule inhibitors of TGFβ pathway is that they inhibit ALKs 4, 5 and 7 and show no significant selectivity between these ALKs. Knockout models of ALK4, ALK5 or ALK7 display unique phenotypes [37, 38] suggesting unique cellular or contextual roles for these ALKs. Development of ALK-specific inhibitors will be essential to probe the roles of
individual ALKs in cells as well as target selective ALKs that may be responsible for driving a particular disease states.

4.2. Inhibitors of the BMP pathway:

While strong pharmaceutical efforts to develop small molecule inhibitors against the TGFβ pathway have led to multiple ALK4, ALK5 and ALK7 inhibitors, the development of small molecule inhibitors of the BMP pathway has lagged behind. BMP signalling plays critical roles during embryogenesis, in controlling the fate of various progenitor cell populations, including embryonic stem cells and hematopoietic stem cells, and in most differentiated and specialized cells and in skeletogenesis [39-42]. Selective small molecule inhibitors of the BMP pathway are desirable in dissecting the physiological roles of BMP signalling in different cellular contexts. ALKs 2, 3 and 6 mediate BMP signals in most tissues, while ALK1, expressed mainly in endothelial cells, signals through both BMP and TGFβ ligands [12, 13]. Sustained BMP signalling driven by a constitutively active mutants of ALK6 have been implicated in heterotopic ossification [6]. Similarly overexpression of certain BMP ligands and activation of downstream signalling has been reported in some cancers [43]. Selective small molecule inhibitors of BMP activated ALKs could be therapeutically beneficial against these diseases. Dorsomorphin and LDN-193189, the only two small molecule inhibitors of the BMP pathway are rather non-specific as they inhibit a number of other protein kinases potently. Some of the off-target effects of using Dorsomorphin at concentrations sufficient to inhibit BMP signalling have been demonstrated and others likely exist [26, 32]. For these reasons use of Dorsomorphin to inhibit BMP pathway is not recommended.

LDN-193189 on the other hand is a very potent inhibitor of BMP signalling, inhibiting BMP-induced phosphorylation of Smad1 in cells with an IC₅₀ of 5 nM [6, 25] (Figure 5A). Because ALK2 and ALK3 were inhibited in vitro by LDN-193189 with IC₅₀ of 30-45 nM and 100 nM respectively, it was rather surprising that the BMP pathway in cells was inhibited with a substantially lower IC₅₀. This could mean that LDN-193189 binds very efficiently, possibly allosterically, to the BMP-activated ALKs in cells. However crystal structures of LDN-193189 in complex with the kinase domain of ALK1 shows LDN-193189 binding to the ATP-binding pocket of ALK1 kinase domain (Link: http://www.thesgc.org/structures/structure_description/3MY0/). Alternatively the binding of LDN-193189 to BMP-activated ALKs could affect the formation of BMP-induced complexes between these ALKs and the upstream type II receptors, which would thus inhibit the activation of type I receptors. Furthermore like Dorsomorphin, LDN-193189 inhibited a number of other kinases very potently. While LDN-193189 displayed improved potency against BMP-activated ALKs over Dorsomorphin, the potency with which both of these compounds inhibit many other kinases did not change significantly (Figure 3). Therefore it is possible that the effects on LDN-193189 to inhibit BMP signalling so potently could be only partly dependent on its effects on ALKs and partly on its effects on other protein kinases, which may impact on the activation or activity of ALKs or the access of ALKs to their substrates. When using LDN-193189 as an inhibitor of the BMP pathway in cells or whole organisms, the consequences of its ability to inhibit other kinases, notably RIPK2, FGF-R1, NUAK1, CAMKKβ, MINK1, GCK, VEG-FR and BRK should be considered. Nonetheless due to its potency as a BMP pathway inhibitor, LDN-193189 provides a very good platform to design derivatives that could enhance its selectivity for BMP-activated ALKs.

Conclusions:

- Based on in vitro specificity tests against a substantial panel of human kinases, routinely used TGFβ pathway inhibitors (SB-431542, SB-505124, LY-364947 and A-83-01) are relatively more selective than the BMP pathway inhibitors (Dorsomorphin and LDN-193189).
- Of the TGFβ pathway inhibitors, we recommend SB-505124, to be used at or below 1µM for cell-based assays, as the most suitable molecule for use as an inhibitor of ALKs 4, 5 & 7 and the TGFβ pathway.

- Dorsomorphin, also known as Compound C, is a non-specific inhibitor of BMP-activated ALKs and potently inhibits many more kinases in vitro. Therefore we do not recommend the use of Dorsomorphin as a selective BMP pathway inhibitor.

- LDN-193189, while being a potent inhibitor of the BMP pathway, also potently inhibits multiple kinases in vitro. At concentrations sufficient to inhibit the BMP pathway, LDN-193189 inhibits RIPK2-mediated phosphorylation of ERK1/2 in cells. Therefore its use as a selective inhibitor of the BMP pathway has to be considered with caution.

Acknowledgements:
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References


**FIGURE LEGENDS**

**Figure 1.** Chemical structures of the small molecule inhibitors of the TGFβ (A-E) and BMP pathways (F&G).

**Figure 2.** The specificities of TGFβ pathway inhibitors SB-431542 (A), SB-505124 (B), LY-364947 (C) & A-83-01 (D) against a panel of up to 123 protein kinases. The results are presented as bars indicating percentage activity remaining for each kinase (averages of two duplicate determinations) in the presence of the indicated concentration of the inhibitor compared with a control lacking the inhibitor ± standard deviation. Further details of the assays are given in the Methods section. The results are ranked according to the percentage activity remaining when the assays were performed in the presence of the indicated inhibitors at 1 µM. Protein kinases referred to in the Results section are indicated by red arrows. Abbreviations for the protein kinases not described in the text are as follows: ABL, Abelson murine leukemia viral oncogene homolog; AMPK, AMP-activated protein kinase; ASK, Apoptosis signal regulating kinase; BRK, Breast tumour kinase; BRSK, brain-specific kinase; BTK, Bruton agammaglobulinemia tyrosine kinase; CaMK, calmodulin-dependent kinase; CaMKK, CaMK kinase; CDK, cyclin dependent kinase; CHK, checkpoint kinase; CK, casein kinase; CLK, CDC-like Kinase; CSK, C-terminal Src kinase; DAPK, Death-Associated Protein Kinase; DYRK, dual-specificity tyrosine-phosphorylated and regulated kinase; eIF, eukaryotic translation initiation factor; EF2K, elongation-factor-2-kinase; EPH, ephrin; ERK, extracellular-signal-regulated kinase; FGF-R, fibroblast-growth-factor receptor; GCK, germinal centre kinase; GLK, glycogen synthase kinase; HER4, V-erb a erythroblastic leukemia viral oncogene homolog 1; HIPK, homeodomain-interacting protein kinase; IGF, insulin-like growth factor; IKK, inhibitory κB kinase; IR, insulin receptor; IRAK, Interleukin-1 Receptor-Associated Kinase; IRR, insulin related receptor; JAK, Janus Kinase; JNK, c-Jun N-terminal kinase; Lck, lymphocyte cell-specific protein tyrosine kinase; LKB1, MO25, STRAD, Ser/Thr Kinase 11; MAPKAP-K, MAPK-activated protein kinase; MARK, microtubule-affinity-regulating kinase; MEKK, mitogen-activated protein kinase kinase; MELK, maternal embryonic leucine-zipper kinase; MINK, misshapen-like kinase; MLCK, smooth-muscle myosin light-chain kinase; MLK, mixed lineage kinase; MNK, MAPK-integrating protein kinase; MSK, mitogen- and stress-activated protein kinase; MSPK, Myristoylated and Palmitoylated serine/threonine protein Kinase; MST, mammalian homologue Ste20-like kinase; NEK, NIMA (never in mitosis in Aspergillus nidulans)-related kinase; NUAK, Snf1-like Kinase; OSIR, Oxidative Stress Responsive; PAK, p21-activated protein kinase; PHK, phosphorylase kinase; PDK, 3-phosphoinositide-dependent protein kinase; PIM, provirus integration site for Moloney murine leukaemia virus; PKA, cAMP-dependent protein kinase; PKB, protein kinase B (also called Akt); PKC, protein kinase C; PKD, protein kinase D; PLK, polo-like kinase; PRAK, p38-regulated activated kinase; PRK, protein kinase C-related kinase; RIPK, receptor interacting protein kinase; ROCK, Rho-dependent protein kinase; RSK, p90 ribosomal S6 kinase; S6K, S6 kinase; SGK, serum- and glucocorticoid-induced kinase; Src, sarcoma kinase; SRPK, serine-arginine protein kinase; STK, Serine / Threonine Kinase; SYK, spleen tyrosine kinase; TAK, Transforming growth factor beta activated kinase; TAB, TAK1 binding subunit; TAO, thousand and one amino acid protein kinase; TBK, TANK-binding kinase; TIE, Tunica Internal Endothelial cell kinase; TLK, toudled-like kinase; TrkA, Neurotrophic tyrosine kinase, receptor, type 1 TTK, Phosphotyrosine picked threonine kinase; VEGFR, vascular endothelial growth factor receptor; YES, Yamaguchi sarcoma viral oncogene homologue; ZAP, zeta chain associated protein kinase.

**Figure 3.** The specificities of BMP pathway inhibitors, Dorsomorphin (A) and LDN-193189 (B), against a panel of up to 121 kinases. The results are presented as bars indicating percentage activity remaining for each kinase (averages of two duplicate determinations) in the presence of the indicated concentration of the inhibitor compared with a control lacking
the inhibitor ± standard deviation. Further details of the assays are given in the Methods section. The results are ranked according to the percentage activity remaining when the assays were performed in the presence of the indicated inhibitors at 1 µM. Protein kinases referred to in the Results section are indicated by red arrows. Abbreviations for protein kinases are described in the Legends to Figure 2.

**Figure 4:** Inhibition of ALK3 and ALK5 by inhibitors of the TGFβ and BMP pathways. ALK3 (A) and ALK5 (B) were assayed as described in the Methods section in the presence or absence of the indicated concentrations of TGFβ pathway inhibitors SB-431542, SB-505124, LY-364947 and A-83-01. The assay samples were resolved by SDS-PAGE, and the gels were Coomassie-stained, dried and analysed by 32P autoradiography. For percentage activity remaining determinations, Coomassie stained bands corresponding to substrate proteins were excised, 32P-incorporation measured and the resulting cpm used as a percentage of control. (C) As above, except that ALK3 and ALK5 were assayed in the presence or absence of the indicated concentrations of Dorsomorphin.

**Figure 5.** Inhibition of TGFβ and BMP pathways by LDN-193189. Serum-starved Human keratinocyte (HaCaT) cells were treated with the indicated concentrations of LDN-193189 for 2h and then treated with BMP-2 (25 ng/ml) (A) or TGFβ (50 pM) (B) for 1h. Extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes, which were analysed by Western Blotting using phospho-Smad1/5/8, Smad1, phospho-Smad2, Smad2/3 and GAPDH antibodies.

**Figure 6.** LDN-193189 inhibits ALK2 and ALK3 in vitro. ALK2 (A), ALK3 (B), ALK4 (C) and ALK5 (D) were assayed as described in the Methods section in the absence or presence of the indicated concentrations of LDN-193189. The assay samples were resolved by SDS-PAGE, and the gels were Coomassie-stained, dried and analysed by 32P autoradiography. For IC50 determinations, Coomassie stained bands corresponding to substrate proteins were excised, 32P-incorporation measured and the resulting cpm plotted against concentrations of LDN-193189 used.

**Figure 7.** Inhibition of NOD-RIPK2 pathways by LDN-193189. RAW 264.7 cells (Mouse leukaemic monocyte macrophage cells) were incubated with indicated concentrations of LDN-193189 for 2h prior to treatment of cells with 15 µM Meso-DAP for 1h. Extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes, which were analysed by Western Blotting using phospho-ERK1/2 and total ERK1/2 antibodies.
Table 1: Summary of the reported potencies of inhibitors of TGFβ and BMP pathways.

(A) IC₅₀ values for the inhibitors of the TGFβ pathway. The appropriate reference for each determination is indicated. Abbreviations: TGFβ-induced P-Smad2, TGFβ-induced phosphorylation of Smad2 in cells as detected by Western blotting; CAGA-Luc, CAGA-Luciferase Reporter Activity dependent on TGFβ signalling measured in a cell-based assay; ca, constitutively active; nd, not determined. For detailed methodologies, refer to appropriate references indicated. Abbreviations: TGFβ-induced P-Smad2, TGFβ-induced phosphorylation of Smad2 in cells as detected by Western blotting; CAGA-Luc, CAGA-Luciferase Reporter Activity dependent on TGFβ signalling measured in a cell-based assay; ca, constitutively active. BMP-induced P-Smad1, BMP-induced phosphorylation of Smad1 in cells as detected by Western blotting; ca, constitutively active. BRE-Luc, BMP-Responsive Luciferase reporter activity measured in a cell-based assay. For detailed protocols, see indicated references.

(A) Assay | SB-431542 | SB-505124 | LY-364947 | A-83-01
---|---|---|---|---
TGFβ-induced P-Smad2 | 0.5-1 μM [11] | 0.5 μM [16] | 0.135 μM [19] | nd
ALK4 in vitro kinase assay | 0.14 μM [15] | 0.129 μM [16] | nd | nd
ALK5 in vitro kinase assay | 0.094 μM [11] | 0.047 μM [16] | 0.058 μM [19] | nd
CAGA-Luc (Cells) | 0.25 μM [16] | 0.1 μM [16] | nd | nd
caALK4 (CAGA-Luc) | 0.75 μM [11] | nd | nd | 0.100 μM [20]
caALK5 (CAGA-Luc) | 0.5 μM [11] | nd | nd | 0.012 μM [20]
caALK7 (CAGA-Luc) | 1-2 μM [11] | nd | nd | 0.030 μM [20]

(B) Assay | Dorsomorphin | LDN-193189
---|---|---
BMP-induced P-Smad1/5/8 | 0.47 μM [24] | 0.005 μM [25]
caALK2 (BRE-Luc) | 0.20 μM [24] | 0.005 μM [6]
caALK3 (BRE-Luc) | 0.50 μM [24] | 0.03 μM [6]
caALK6 (BRE-Luc) | 5-10 μM [24] | 0.15 μM [6]
Table 2: Potencies of compounds developed as TGFβ and BMP pathway inhibitors against some other kinases. IC₅₀ values were determined from multiple assays carried out at ten different inhibitor concentrations. Abbreviations for protein kinases are described in the Legends to Figure 2.

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Figure 1

SB-431542  SB-505124  SB-525334

LY-364947  A-83-01

Dorsomorphin (Compound C)  LDN-193189
Figure 2

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Figure 3

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Figure 4

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Coomassie

$^{32}$P-Autorad

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Coomassie

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Coomassie

$^{32}$P-Autorad
Figure 5

A

B

LDN-193189 (µM)
BMP-2
0.000 + + + + + + + +
0.001 + + + + + + + +
0.010 + + + + + + + +
0.030 + + + + + + + +
0.100 + + + + + + + +
0.300 + + + + + + + +
1.000 + + + + + + + +
3.000 + + + + + + + +
10.00 + + + + + + + +

IB: P-Smad1/5/8
IB: Smad1
IB: P-Smad2
IB: Smad2/3
IB: Gapdh

LDN-193189 (µM)
TGFβ
0.000 + + + + + + + +
0.001 + + + + + + + +
0.030 + + + + + + + +
0.100 + + + + + + + +
0.300 + + + + + + + +
1.000 + + + + + + + +
3.000 + + + + + + + +
10.00 + + + + + + + +

IB: P-Smad1/5/8
IB: Smad1
IB: P-Smad2
IB: Smad2/3
IB: Gapdh

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Figure 6

A. ALK2

LDN-193189 (µM)

|     | 0.000 | 0.001 | 0.003 | 0.010 | 0.030 | 0.100 | 0.300 | 1.000 | 3.000 | 10.00 |

Coomassie

32P-Autorad

Smad1-phosphorylation

ALK2-autophosphorylation

B. ALK3

LDN-193189 (µM)

|     | 0.000 | 0.001 | 0.003 | 0.010 | 0.030 | 0.100 | 0.300 | 1.000 | 3.000 | 10.00 |

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BMPR2-autophosphorylation

C. ALK4

LDN-193189 (µM)

|     | 0.000 | 0.001 | 0.003 | 0.010 | 0.030 | 0.100 | 0.300 | 1.000 | 3.000 | 10.00 |

Coomassie

32P-Autorad

Smad2-phosphorylation

D. ALK5

LDN-193189 (µM)

|     | 0.000 | 0.001 | 0.003 | 0.010 | 0.030 | 0.100 | 0.300 | 1.000 | 3.000 | 10.00 |

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32P-Autorad

Smad2-phosphorylation

Figure 6

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Figure 7

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IB: P-ERK1
IB: P-ERK2
IB: ERK 1/2

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