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Pharmacologic strategies for assaying BMP signaling function

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Abstract

The bone morphogenetic protein (BMP) signaling pathway, a subset of the transforming growth factor β (TGF- β) signaling family, consists of structurally diverse receptors and ligands whose combinatorial specificity encode autocrine, paracrine and endocrine signals essential for regulating tissue growth, differentiation and survival during embryonic patterning and postnatal tissue remodeling. Aberrant signaling of these receptors and ligands are implicated in a variety of inborn and acquired diseases. The roles of various receptors and their ligands can be explored using small molecule inhibitors of the BMP receptor kinases. Several BMP type I receptor kinase inhibitor tool compounds have been described that exhibit sufficient selectivity to discriminate BMP receptor signaling *in vitro* or *in vivo*, with various trade-offs in selectivity, potency, cell permeability, and pharmacokinetics. Several methods for assaying BMP function via pharmacologic inhibition are presented. Two *in vitro* methods, an In-Cell Western assay of BMP-mediated SMAD1/5/8 phosphorylation, and an alkaline phosphatase osteogenic differentiation assay, represent efficient high-throughput methodologies for assaying pharmacologic inhibitors. Two *in vivo* methods are described for assaying the effects of BMP signaling inhibition in embryonic zebrafish and mouse development. Small molecule inhibitors of BMP receptor kinases represent an important complementary strategy to genetic gain- and loss-of-function and ligand-trap approaches for targeting this signaling system in biology and disease.

Keywords

bone morphogenetic protein (BMP) signaling; kinase inhibitor; Dorsomorphin; LDN-193189; LDN-214117; LDN-212854; SB-431452; DMH1; K02288a

1. Introduction

The bone morphogenetic protein (BMP) signal transduction pathway is a subset of the larger TGF- β signaling family, which includes 7 type I receptors, 5 type II receptors, and over 30 ligands of the BMP, TGF- β , Activin and Growth and Differentiation Factor (GDF) ligand families (1). Much progress has been made in understanding the multifaceted roles of BMPs and their signaling mechanisms as a result of genetic, molecular, biochemical and structural biology tools. In addition to specifying developmental patterning of dorsoventral axis and key aspects of organogenesis, BMPs are now known to play a critical role in the

maintenance of normal physiology beyond development, such as homeostasis of the vasculature, iron metabolism, and bone mineralization. A wide variety of inborn and acquired imbalances in the regulation of this signaling pathway can lead to disease, including hereditary hemorrhagic telangiectasia (HHT) syndromes, heritable pulmonary arterial hypertension (HPAH), juvenile familial polyposis (JP), and fibrodysplasia ossificans progressiva (FOP). Pharmacologic modulators of the BMP pathway are a useful tool in elucidating the role of these receptors and ligands in normal physiology and disease.

The first described small molecule inhibitor of BMP signaling was dorsomorphin (Fig. 1), a.k.a. compound C, a pyrazolo[1,5-a]pyrimidine compound previously known to inhibit AMP activated kinase at micromolar concentrations, identified for its potent dorsalization activity in a chemical biological screen examining embryonic zebrafish development (2). Dorsalization is a phenotype that is known to reflect inhibition of BMP signaling during dorsoventral axis specification in multiple vertebrate species (3–5), leading to the inference that dorsomorphin might act as a BMP inhibitor. Biochemical analyses and co-crystallization with several type I receptor kinase domains subsequently identified the mechanism of this activity as resulting from the inhibition of BMP type I receptors kinases ALK2, ALK3, and ALK6 as an ATP-competitive antagonist (2, 6). Cell-based assays confirmed sub-micromolar potency in inhibiting the BMP type I receptors, as well as a variety of other receptor tyrosine kinases, including VEGFR2 and PDGFR β .

Multiple pharmaceutical modulators have since been designed to inhibit receptors of the BMP pathway with greater potency and/or selectivity, some of which are pyrazolo-pyrimidine derivatives, while others are based on novel scaffolds, each with distinct physiochemical properties and selectivity for individual BMP receptors. LDN-193189 is pyrazolo-pyrimidine derivative with modifications to the solvent-exposed and hydrophobic pocket domains (Fig. 1) that result in greater potency while retaining similar BMP selectivity as compared to dorsomorphin. LDN-193189 is a low nanomolar inhibitor of BMP signaling in cells with improved metabolic stability (>30 min in microsomal degradation assays), cell permeability and *in vivo* bioavailability ($T_{1/2}$ ~3.7 h in mice by IP injection) (7). LDN-193189 has been shown to inhibit BMP type I receptors ALK2 (IC₅₀~10 nM in cells), ALK3 (IC₅₀~11 nM), ALK1 (310 nM), and ALK6 in preference to TGF- β /GDF/Activin type I receptors ALK5 (IC₅₀~1 μ M in cells), ALK4, and ALK7. It exhibits improved selectivity for this family of receptors versus receptor tyrosine kinases and AMPK as compared to dorsomorphin, yet still exhibits potent activity against RIPK2 and ABL family kinases. Despite its limitations in kinome selectivity, LDN-193189 has been useful tool in probing BMP-mediated effects due to its high degree of potency, provided appropriate complementary methods are utilized for confirmation. The 2-aminopyridine compound K02288 (Fig. 1B), identified by a melting point shift screen with ALK2 kinase versus TGF β type I kinase ALK5, shows comparable high selectivity for BMP versus TGF- β /Activin type I receptors, as seen in LDN-193189, but with a substantially less off-target effects in the kinome (8). K02288 exhibits greater potency in biochemical kinase assays than cell-based assays of signaling, suggesting that cell permeability may limit its activity in cells. Several derivative compounds of both scaffolds have been described as having a greater degree of selectivity for particular receptors, or having altered physiochemical or drug-like properties. LDN-212854 is a structural isomer of LDN-193189 with similar physiochemical properties,

but by virtue of its 5-quinoline versus 4-quinoline pendant group has altered complementarity with residues in the deep hydrophobic binding pocket of ALK2 (8), and thus exhibits several fold greater selectivity for ALK2 (IC_{50} ~16 nM in cells) than for ALK3 (IC_{50} ~166 nM in cells) and ALK1 (IC_{50} ~100 nM in cells) as compared to LDN-193189. LDN-212854 has relatively similar kinome selectivity to LDN-193189 and with similar off-target effects. DMH1 is an analog of LDN-193189 with its solvent exposed domain modified to an isopropyl ether, resulting in improved selectivity for ALK2 (IC_{50} ~107 nM), and very low activity against ALK5, VEGFR2, and other targets in biochemical kinase assays (7–9). The potency of DMH1 in cell-based applications appears to be diminished in proportion to its biochemical activity, suggesting diminished permeability as a result of the solvent-exposed domain changes, and requires higher concentrations in the micromolar range to achieve its effects, potentially limiting the gains in selectivity. DMH2, DHM3, and DMH4 are other derivatives of LDN-193189 with altered solvent exposed-domains which result in further tradeoffs in selectivity for potency (9). LDN-214117 is a methyl-pyridine derivative of K02288 (Figure 1) which exhibits increased selectivity for ALK2, improved cell permeability, and in vivo bioavailability as compared to K02288 (10). With careful dose titration experiments, compounds with greater selectivity within the BMP signaling family such as LDN-212854 and LDN-214117 may be used in vitro to distinguish between the effects of ALK2 ligand-mediated versus ALK3 and ALK1 ligand-mediated signaling. For example, LDN-214117 can be used to resolve the activity of classic BMP ligands BMP2 and BMP4 transduced by ALK3 (IC_{50} ~1 μ M in cell-based BRE-luciferase reporter assays) as distinct from BMP6 transduced by ALK2 (IC_{50} ~100 nM in the same assay). Due to their different off-target effects, compounds from the pyrazolo-pyrimidine versus 2-aminopyridine scaffold families may be used together to diminish the likelihood that their effects are not due to inhibition of off-target kinases.

Two of the procedures provided represent potentially high-throughput in vitro approaches for demonstrating pathway-specific effects of these inhibitors, and thus confirm effects of signaling inhibition in parallel with ligand-traps (noggin)(11), and soluble BMP extracellular domain proteins such as ALK1-Fc or ALK3-Fc (12, 13), or with siRNA (14, 15). The other two procedures describe the application of inhibitors to disrupt or attenuate embryonic and early postnatal BMP signaling via delivery to embryonic zebrafish following fertilization, or to pregnant dams at a safe interval following fertilization, and offer the potential to confirm by pharmacologic epistasis the impact of BMP signaling upon a given developmental phenotype or process.

Procedure 1 demonstrates how inhibitors can be applied to various mesenchymal cell lineages which demonstrate the capacity for osteogenic-like differentiation in response to BMP signaling, a response that is measured through by an increase in alkaline phosphatase activity based on a colorimetric assay. In some cases, the capacity of BMPs to induce alkaline phosphatase expression in these cells is a surrogate for the osteogenic effects of these BMPs to induce endochondral bone in vivo, whereas the inhibition of this activity by various BMP type I receptor inhibitors is a pathway-specific anti-osteogenic effect. This assay can be adjusted to different genetically modified cell lines (RNAi vs. global or conditional BMP receptor knockout cell lines) or in the presence of specific BMP ligands to yield data on the osteogenic properties, responsible ligands, and receptors that mediate a

given osteogenic effect in such cells (11, 16). This procedure will work with C2C12, NIH3T3 cells, and other similar mesenchyme derived cell lines or primary cultures.

Procedure 2 describes a versatile In-Cell Western technique, in which levels of phosphorylated SMAD proteins, the immediate downstream effectors of canonical BMP signaling can be detected as a measure of BMP signaling activation. This technique can be applied for the identification and characterization of novel inhibitors as a high-throughput screening methodology (Fig. 2A), and can be used to determine concentration-dependent effects of various BMP inhibitors (Fig. 2B) (7, 8).

Procedure 3 illustrates how various BMP modulators can be tested for their impact on dorso-ventral axis development in zebrafish embryos. This assay is also easily modified for studying knockout zebrafish strains or for determining the contribution of BMP receptors in a given early embryonic or neonatal process, provided that the compounds have been shown to penetrate the embryo based on dorsalization, as has been shown for dorsomorphin, LDN-193189, LDN-212854, K02288a, and DMH1 (2, 7–9).

Procedure 4 describes an application of these compounds to inhibit BMP signaling during fetal and early neonatal murine development. This technique has been successfully applied to rescue skull malformations due to excessive BMP signaling in osteoblast lineages. The ability to modulate phenotypes in other tissues would need to be determined empirically, and ideally would include demonstrating the engagement of target by detecting decreased abundance of phosphorylated SMAD1/5/8, or decreased expression of the BMP transcriptional target *Id1* in target tissues, as was shown previously for LDN-193189 in such an application (17).

These methods are meant to serve as examples of potential applications in which the effects of pharmacologic inhibition of BMP signaling can be assayed. It should be emphasized that given the various off target effects of each of the published BMP inhibitor compounds (18) and concentration dependent effects, confirmation with two distinct chemotypes (pyrazolo-pyrimidine versus pyridine scaffold-based inhibitors), recombinant protein inhibitors and ligand-traps, or genetic manipulation of receptor expression should be used to raise confidence in the conclusion. Due to the limitations of recombinant and genetic approaches to antagonizing the redundant activities of this pathway with over 30 identified structurally diverse ligands, the pharmacologic approach to inhibition is most effective as an initial screen for ascertaining a potential role of BMP signaling in a given biological process.

2. Materials

Pharmacologic Modulators:

1. LDN-214117 (Tocris Bioscience)
2. LDN-212854 (Tocris Bioscience)
3. LDN-193189 (Tocris Bioscience)
4. SB-431452 (Sigma)

5. DMH1 (Sigma)
6. K02288a (Sigma)
7. Dorsomorphin, a.k.a., Compound C (Tocris Bioscience)

Antibodies

1. Rabbit monoclonal anti-phospho-SMAD1/5/8 (Cell Signaling Technology #41D10)
2. Polyclonal goat anti-Rabbit HRP conjugate (Cell Signaling Technology #7074)

2.1 Determining kinetics of SMAD signaling and function in vitro

1. Cells of interest with appropriate media (*see* Note 1)
2. 10% FBS containing media for growth phase (*see* Note 2)
3. 1%–2% FBS containing media for starvation phase (*see* Note 2)
4. 1% Triton X-100/TBS: 1% Triton X-100, 1X Tris buffered saline (TBS)
5. Phosphate Buffered Saline (PBS)
6. PnPP substrate Sigma P7998
7. Plate reader

2.2 In-Cell Western for phosphorylated SMAD 1/5/8

1. 96-well clear bottom plate with white walls, tissue culture treated. For cells that are poorly adherent, use appropriate coatings specific to cell type or consider using plate with improved binding such as lysine-coated or CellBind (Corning) plates.
2. Cells of interest with appropriate media (*see* Note 1)
3. 10% FBS containing media for growth phase (*see* Note 2)
4. 1%–2% FBS containing media for starvation phase (*see* Note 2)
5. Primary antibody anti-*p*-SMAD 1/5/8 Rabbit mAb, Cell Signaling #9516
6. HRP-anti-rabbit IgG, Cell Signaling
7. ECL Reagent (Bio-Fx Ultra Sensitive HRP Chemiluminescent Substrate, Surmodics #CHMI-0060)
8. 1X Phosphate Buffered Saline (PBS)
9. 0.5% glutaraldehyde (diluted from stock solution in PBS)

¹For In-Cell Western, C2C12 cells (ATCC) are commonly for BMP 2,4,6,7 or TGF- β 1/2/3 signaling experiments. Bovine Aortic Endothelial Cells (BAECs; Lonza or Genlantis) are used for BMP9 or BMP10 experiments.

²The recommended media for C2C12 cells is RPMI with 10% FBS. The recommended media for BAECs is DMEM supplemented with high glucose and glutamine with 10% FBS. For other mesenchymal-derived cell lines, more custom types of media can also be used, such as MCDB131.

10. 2% BSA (bovine serum albumin) in PBS
11. BMP and TGF β ligands from R&D Systems diluted in 0.1% BSA PBS to desired concentrations (see Note 3)

2.3 Determining kinetics of SMAD signaling and function in vivo in zebrafish

1. Zebrafish embryos isolated following fertilization
2. E3 buffer: 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄ (2)

2.4 Protocol for murine in utero suppression of SMAD signaling

1. Mice breeding pair with timed matings judged by plug
2. 31G insulin needle syringe combination for i.p. injection
3. CO₂/method for euthanasia

3. Methods

Inhibitor(s) to be used should be chosen based on the potency, selectivity and cell permeability that are most suitable for the specific application, or have been previously established empirically (Table 1, *see* Note 4, Note 5). Preliminary experiments to determine optimal dose ranges of inhibitors are recommended (*see* Note 6).

3.1 Determining kinetics of BMP-induced osteogenic differentiation via alkaline phosphatase expression in vitro

Goal: to determine the temporal requirements of BMP-SMAD signaling in recruiting osteogenic differentiation

1. Seed 2000 cells/well in 10% FBS culture media. Incubate at 37°C and 5% CO₂ overnight
2. Remove media and replace with 2% FBS media in total volume of 50 μ L in a 96 well-plate and incubate overnight
3. Stimulate with inhibitor at different time-points (*see* Note 6)
 - a. T0, T1hr, T2hr, T4hr, T8hr, T16hr, T24hr, T32hr, T48hr, T72hr, T96hr
4. At T120hr, wash cells by replacing media with 200 μ L PBS three times, discarding liquid after each wash, and then permeabilize/solubilize by the

³For the In-Cell Western, concentrations close to the EC₅₀ of the BMP ligands is often used. Common ranges tested are: 10–25 ng/mL for BMP 2,4; 50 ng/mL for BMP6,7; 0.5–1 ng/mL for BMP9/10 (specifically for endothelial cells).

⁴To identify the optimal inhibitor for a given application, consider PK, IC₅₀, and selectivity data that have been previously published for LDN-214117, LDN-212854 (8), LDN-193189 (8, 20, 21), SB-431452 (22), DMH1 (8), K02288a (8, 20), Dorsomorphin (Compound C) (8, 21))

⁵At moderate to high micromolar concentrations, several of these BMP inhibitor compounds lose their selectivity and act as pan-BMP/TGF- β /Activin type I receptor inhibitors (8, 10) with significant off-target effects against other kinases. This quality can be advantageous if blocking several or all of the BMP/TGF β type I receptors is desirable, but can generate non-specific results.

⁶Prior to the experiment, it may be helpful to run a pilot study testing varying concentrations of inhibitor that are relevant for the cell type, the complement of BMP/TGF- β /Activin receptors being targeted, and the assay in question. The examination of signaling kinetics at different time points, may also be helpful for optimizing the sensitivity of the assay and tailoring to biological endpoints.

addition of 50 μ L of 1% Triton X-100/TBS. Seal plate, and agitate or vortex for 30 sec gently enough to avoid cross-contamination or loss of samples.

5. Transfer 20 μ L of each sample to a clear 96-well microplate, and then add 100 μ L of PnPP substrate to each well.
6. Incubate for 1 hour at room temperature.
7. Read absorbance at 405 nm in a microplate spectrophotometer.

3.2 In-Cell Western for phosphorylated SMAD 1/5/8

1. Grow adherent cells to confluence in a tissue-culture treated clear bottom, white walled 96-well plate. Seed the suspension of cells, in 100 μ L 10% FBS media per well, and allow cells to achieve confluence at 37°C and 5% CO₂ with humidification (*see* Note 1).
2. Wash confluent cells with 1X PBS in a sterile manner, and serum-starve cells overnight in 45 μ L 1% FBS media per well.
3. Add 5 μ L of varying dilutions of inhibitor compounds in PBS or vehicle control, and incubate for 10–30 min at 37°C and 5% CO₂ (*see* Note 7,8), being careful not to allow plate to cool.
4. Add BMP/TGF- β ligands in a volume of 5.5 μ L to wells and incubate for 30min at 37°C and 5% CO₂, being careful not to allow plate to cool.
5. Remove supernatant and add 50 μ L ice-cold methanol. Incubate at room temperature for 15 minutes.
6. Remove methanol and add 50 μ L 0.5% glutaraldehyde in PBS. Incubate at room temperature for 15 minutes.
7. Wash in plate washer 4 times with 200 μ L PBS or equivalent.
8. Block with 200 μ L per well 2% BSA in PBS for 1 hour at room temperature on gentle rotator.
9. Remove blocking solution and add 50 μ L per well primary antibody (rabbit mAb anti-*p*-SMAD 1/5/8), diluted 1:400 in 2% BSA in PBS. Incubate overnight at 4°C with gentle rotation or rocking.
10. Wash in plate washer 3 times with 200 μ L PBS or equivalent
11. Add 50 μ L per well secondary antibody (HRP-anti-rabbit IgG, Cell Signaling), diluted 1:400 in 2% BSA in PBS. Incubate 1 hour at room temperature on rotator
12. Wash plate in plate washer 4 times with 200 μ L PBS or equivalent

⁷In vitro, dorsomorphin and other compounds will attenuate steady state levels of phosphorylated SMAD1/5/8 within minutes of addition to culture media. Given their solubility and high permeability, pre-treatment of cells for approximately 10–30 min. prior to the addition of ligand agonists is sufficient to abrogate effects of ligands in activating signaling. (21).

⁸Inhibitor compounds can be diluted in PBS. An 11-point 1/2-log scale of dilutions, i.e., 100 μ M, 31.3 μ M, 10 μ M, 3.13 μ M, 1 μ M, 313 nM, 100 nM, 31 nM, 10 nM, 3.1 nM, 1 nM, is sufficient to cover most biologically relevant concentrations of inhibitor within a standard row of a 96-well plate, allowing one control well with no inhibitor. Triplicate measurements are recommended.

13. Add 30 μ L ECL reagent and read plate in a sensitive microplate luminometer immediately. Do not let the plate develop more than 2–3 minutes prior to reading.

3.3 Determining kinetics of SMAD signaling and function in vivo in zebrafish (2, 19)

Goal: to inhibit SMAD signaling in zebrafish embryos to determine BMP signaling role in development

1. Transfer newly fertilized eggs to 96-well microplate in 200 μ L E3 buffer
2. Add inhibitor at 5–10 μ M at 4–8 hours post fertilization (h.p.f) and incubate at 28.5C (*see* Note 9)
3. At 12, 24, and 48 h.p.f. examine embryos for dorsalization or ventralization of embryonic axis

3.4 Protocol for murine in utero suppression of SMAD signaling (17)

Goal: to inhibit SMAD signaling during embryonic development

1. Day E-5 to E-1: check for morning plugs in female mice in breeding cages
2. Day E0 to E13⁹ remove pregnant dams to single cage housing and monitor pregnancy
3. Day E14 to P0: Begin daily treatment with LDN-193189 2.5 mg/kg i.p. to pregnant dams (*see* Note 10)
4. P0 to P15: Continue treating lactating females with LDN-193189 at a dose of 2.5mg/kg i.p.
5. P16: May euthanize pups for analysis or continue monitoring pups for phenotype

5. References

1. Wang RN, Green J, Wang Z, et al. (2014) Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes Dis* 1:87–105 [PubMed: 25401122]
2. Yu PB, Hong CC, Sachidanandan C, et al. (2008) Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nat Chem Biol* 4:33 [PubMed: 18026094]
3. Steinbeisser H, Fainsod A, Niehrs C, et al. (1995) The role of gsc and BMP-4 in dorsal-ventral patterning of the marginal zone in *Xenopus*: a loss-of-function study using antisense RNA. *EMBO J* 14:5230–5243 [PubMed: 7489713]
4. Biehs B, François V, and Bier E (1996) The *Drosophila* short gastrulation gene prevents Dpp from autoactivating and suppressing neurogenesis in the neuroectoderm. *Genes Dev* 10:2922–2934 [PubMed: 8918893]
5. Nagaso H, Suzuki A, Tada M, et al. (1999) Dual specificity of activin type II receptor ActRIIb in dorso-ventral patterning during zebrafish embryogenesis. *Dev Growth Differ* 41:119–133 [PubMed: 10223708]

⁹The effects of various inhibitors upon dorsalization in zebrafish embryos appears to be related to the time at which inhibitors are added to embryos, with most severe effects occurring at 3–4 h.p.f., and less severe effects occurring beyond 7–8 h.p.f. The desired degree of dorsalization can be tailored via the concentration to which embryos are exposed as well as the timing of exposure.

¹⁰LDN-193189 plasma levels decrease to 50% of peak levels within 4 hours after administration.

6. Chaikuad A, Alfano I, Kerr G, et al. (2012) Structure of the Bone Morphogenetic Protein Receptor ALK2 and Implications for Fibrodysplasia Ossificans Progressiva. *JBiolChem* 287:36990–36998
7. Yu PB, Deng DY, Lai CS, et al. (2008) BMP type I receptor inhibition reduces heterotopic [corrected] ossification. *Nat Med* 14:1363–1369 [PubMed: 19029982]
8. Mohedas AH, Xing X, Armstrong KA, et al. (2013) Development of an ALK2-biased BMP type I receptor kinase inhibitor. *ACS Chem Biol* 8:1291 [PubMed: 23547776]
9. Hao J, Ho JN, Lewis JA, et al. (2010) In Vivo Structure Activity Relationship Study of Dorsomorphin Analogs Identifies Selective VEGF and BMP Inhibitors. *ACS Chem Biol* 5:245 [PubMed: 20020776]
10. Mohedas AH, Wang Y, Sanvitale CE, et al. (2014) Structure–Activity Relationship of 3,5-Diaryl-2-aminopyridine ALK2 Inhibitors Reveals Unaltered Binding Affinity for Fibrodysplasia Ossificans Progressiva Causing Mutants. *J Med Chem* 57:7900 [PubMed: 25101911]
11. Yu PB, Deng DY, Beppu H, et al. (2008) Bone Morphogenetic Protein (BMP) Type II Receptor Is Required for BMP-mediated Growth Arrest and Differentiation in Pulmonary Artery Smooth Muscle Cells. *J Biol Chem* 283:3877–3888 [PubMed: 18042551]
12. Cunha SI, Pardali E, Thorikay M, et al. (2010) Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. *J Exp Med* 207:85–100 [PubMed: 20065063]
13. Derwall M, Malhotra R, Lai CS, et al. (2012) Inhibition of bone morphogenetic protein signaling reduces vascular calcification and atherosclerosis. *Arterioscler Thromb Vasc Biol* 32:613–622 [PubMed: 22223731]
14. Yu PB, Beppu H, Kawai N, et al. (2005) Bone morphogenetic protein (BMP) type II receptor deletion reveals BMP ligand-specific gain of signaling in pulmonary artery smooth muscle cells. *J Biol Chem* 280:24443–24450 [PubMed: 15883158]
15. Bagarova J, Vonner AJ, Armstrong KA, et al. (2013) Constitutively active ALK2 receptor mutants require type II receptor cooperation. *Mol Cell Biol* 33:2413–2424 [PubMed: 23572558]
16. Yung L-M, Sánchez-Duffhues G, Ten Dijke P, et al. (2015) Bone morphogenetic protein 6 and oxidized low-density lipoprotein synergistically recruit osteogenic differentiation in endothelial cells. *Cardiovasc Res* 108:278–287 [PubMed: 26410368]
17. Komatsu Y, Yu PB, Kamiya N, et al. (2013) Augmentation of Smad-dependent BMP signaling in neural crest cells causes craniosynostosis in mice. *J Bone Miner Res Off J Am Soc Bone Miner Res* 28:1422
18. Vogt J, Traynor R, and Sapkota GP (2011) The specificities of small molecule inhibitors of the TGF β and BMP pathways. *Cell Signal* 23:1831–1842 [PubMed: 21740966]
19. Williams CH and Hong CC (2015) High content screening for modulators of cardiovascular or global developmental pathways in zebrafish. *Methods Mol Biol Clifton NJ* 1263:167–174
20. Sanvitale CE, Kerr G, Chaikuad A, et al. (2013) A New Class of Small Molecule Inhibitor of BMP Signaling. *PLOS ONE* 8:e62721 [PubMed: 23646137]
21. Horbelt D, Boergemann JH, Chaikuad A, et al. (2015) Small Molecules Dorsomorphin and LDN-193189 Inhibit Myostatin/GDF8 Signaling and Promote Functional Myoblast Differentiation. *J Biol Chem* 290:3390 [PubMed: 25368322]
22. Inman GJ, Nicolás FJ, Callahan JF, et al. (2002) SB-431542 Is a Potent and Specific Inhibitor of Transforming Growth Factor- β Superfamily Type I Activin Receptor-Like Kinase (ALK) Receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 62:65–74 [PubMed: 12065756]
23. Dey D, Bagarova J, Hatsell SJ, et al. (2016) Two tissue-resident progenitor lineages drive distinct phenotypes of heterotopic ossification. *Sci Transl Med* 8:366ra163
24. Waghbi MC, Souza EM de, Oliveira GM de, et al. (2009) Pharmacological Inhibition of Transforming Growth Factor β Signaling Decreases Infection and Prevents Heart Damage in Acute Chagas' Disease. *Antimicrob Agents Chemother* 53:4694 [PubMed: 19738024]
25. Sheng Y, Sun B, Xie X, et al. (2015) DMH1 (4-[6-(4-isopropoxyphenyl)pyrazolo[1,5-a]pyrimidin-3-yl]quinoline) inhibits chemotherapeutic drug-induced autophagy. *Acta Pharm Sin B* 5:330–336 [PubMed: 26579463]

26. Hover LD, Owens P, Munden AL, et al. (2016) Bone morphogenetic protein signaling promotes tumorigenesis in a murine model of high-grade glioma. *Neuro-Oncol* 18:928–938 [PubMed: 26683138]
27. Windhausen T, Squifflet S, Renn J, et al. (2015) BMP Signaling Regulates Bone Morphogenesis in Zebrafish through Promoting Osteoblast Function as Assessed by Their Nitric Oxide Production. *Molecules* 20:7586–7601 [PubMed: 25919279]

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Figure 1. Structures of BMP inhibitor tool compounds. **A.** Dorsomorphin and pyrazolo[1,5-a]pyrimidine derivatives. Solvent-exposed domains of LDN-193189 and DMH1 are shown in red boxes. Deep hydrophobic pocket binding domains of LDN-193189 and LDN-212854 are shown in green. **B.** K02288 and derivative LDN-214117 shown. Solvent exposed domains are shown in red boxes, and putative hinge binding groups (2-amino versus 2-methyl) shown in green boxes.

Figure 2.

Typical In-Cell Western assay data. **A.** In-Cell Western data showing a high-throughput screen of dorsomorphin derivatives arranged by potency of inhibition of BMP4-mediated phosphorylation of SMAD1/5/8 in C2C12 cells measured in the presence of 100 nM of compounds and 10 ng/mL of BMP4, over 30' at 37 degrees C. LDN-212854 and LDN-193189 are indicated with their respective IC_{50} values measured in this same cellular assay. **B.** In-Cell Western is used to evaluate the inhibition of BMP9-mediated phosphorylation of SMAD1/5/8 in bovine aortic endothelial cells (BAEC) by varying concentrations of a BMP9 ligand trap, ALK1-Fc, measured in the presence of 1 ng/mL of BMP9 over 30' at 37 degrees C. The IC_{50} for the activity of BMP9 indicated by dotted lines is approximately 175 ng/mL.

Table 1.

BMP signaling modulators and basic information on commonly used concentrations of published data. Concentrations should be adapted for individual assays (*see* Note 3, Note 4). Please note that concentrations listed are not inclusive of all concentrations published, but are representative of the common ranges used.

Compound	Receptor for which compound has high specificity	Concentration in Vitro	Concentration in Vivo (mice)	Concentration in Vivo (zebrafish)
LDN-214117	ALK2	21nM-10µM (10)		
LDN-212854	BMP Type 1 receptors	1nM-10µM (8)	To prevent HO 6mg/kg, i.p. twice daily; 3mg/kg, s.c. twice daily (23)	
LDN-193189	BMP Type 1 receptors: ALK1, ALK2, ALK3, ALK6	1nM-10µM (8) 0.05µM-5µM (21) (<i>see</i> Note 8)	To prevent HO: 6mg/kg, i.p. twice daily; In utero: 2.5mg/kg i.p. daily	
SB-431452	TGF-β Type 1 receptors: ALK4, ALK5, ALK7	0.05µM-5µM (21)	10mg/kg i.p. daily (24)	
DMH1	ALK2	5µM-10µM (25) 3µM-100µM (26) (<i>see</i> Note 11)		0.2µM at 3 h.p.f. (9) (<i>see</i> Note 11)
K02288a	BMP Type 1 receptors: ALK1, ALK2, ALK3, ALK6	0.1nM-10µM (8)		8–10µM at 12 h.p.f. (20) 10uM-20µM (27)
Dorsomorphin (Compound C)	ALK2, ALK3, ALK6	5µM (21) 2µM (11)		100µM at 2 d.p.f. (27) 10µM at 48 h.p.f. (20)

¹¹The LDN-193189 derivative DMH1 has excellent selectivity when assayed in biochemical kinase assays, but loses some of this selectivity in cell assays apparently as a result of its reduced permeability in cells (8).

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