

High-throughput screens for agonists of bone morphogenetic protein (BMP) signaling identify
potent benzoxazole compounds

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Running title: *Identification of BMP signaling agonists*

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ABSTRACT

Bone morphogenetic protein (BMP) signaling is critical in renal development and disease. In animal models of chronic kidney disease (CKD), re-activation of BMP signaling is reported to be protective by promoting renal repair and regeneration. Clinical use of recombinant BMPs, however, requires harmful doses to achieve efficacy and are costly because of BMPs complex synthesis. Therefore, alternative strategies are needed to harness the beneficial effects of BMP signaling in CKD. Key aspects of the BMP signaling pathway can be regulated by both extracellular and intracellular molecules. In particular, secreted proteins like Noggin and Chordin inhibit BMP activity, whereas Kielin/Chordin-like proteins (KCP) enhance it and attenuate kidney fibrosis or CKD. Clinical development of KCP, however, is precluded by its size and complexity. Therefore, we propose an alternative strategy to enhance BMP signaling by using small-molecules, which are simpler to synthesize and more cost-effective. To

address our objective, here we developed a small-molecule high-throughput screen (HTS) with human renal cells having an integrated luciferase construct highly responsive to BMPs. We demonstrate the activity of a potent benzoxazole compound, sb4, that rapidly stimulated BMP signaling in these cells. Activation of BMP signaling by sb4 increased the phosphorylation of key second messengers (SMADs-1/5/9) and also increased expression of direct target genes (inhibitors of DNA binding, ID1 and ID3) in canonical BMP signaling. Our results underscore the feasibility of utilizing HTS to identify compounds that mimic key downstream events of BMP signaling in renal cells and yielded a lead BMP agonist.

Bone Morphogenetic Proteins (BMPs) have essential roles in development, tissue homeostasis, and disease processes for a wide array of cell types. Genetically ablating key elements of the BMP signaling pathway causes

embryonic lethality or leads to development defects across organs such as the kidneys (1), heart (2), lungs (3) and central nervous system (4). Tissue homeostasis of these organs and others is also heavily BMP dependent, as disturbed BMP signaling is implicated in a multitude of diseases (5,6). Modulating BMP signaling has proved beneficial in several disease contexts. Specifically, in animal models of liver (7), kidney (8,9), cardiac (10), and pulmonary fibrosis and hypertension (11-13), restoration or enhancement of BMP signaling leads to reversal or attenuation of disease progression. In contrast, suppression of BMP signaling is beneficial in musculoskeletal and oncogenic diseases such as, Fibrodysplasia Ossificans Progressiva and potentially in brainstem gliomas that have overactive BMP signaling (6,14).

BMPs belong to the TGF- β superfamily of cytokines. In humans, a diverse set of BMP ligands have been identified that possess overlapping yet distinct functions. In particular, BMP4 and BMP7 ligands are critical in renal development (15,16), whereas BMP2 and BMP7 ligands function in bone and cartilage formation (17). Several human type I BMP receptors (Alk1/2/3/6) and constitutively active type II BMP receptors (BMPR2, ACVR2A/B, AMHR2) have been described (17). Cleaved and dimerized BMP ligands commence the BMP signaling cascade by engaging with a heterotetramer comprised of two type I BMP receptors and two type II BMP receptors. Type II BMP receptors then transphosphorylate the glycine/serine-rich domains of the type I BMP receptors. In turn, the activated type I BMP receptors recruit and activate intracellular receptor-activated SMADs (R-SMADs). Canonical BMP signaling is transduced by SMADs-1/5/8/9, which form a transcriptional complex with common-SMAD4 (co-SMAD4). Translocation of the SMAD heteromeric complex to the nucleus permits recruitment of tissue-specific transcription factors which drive the expression of direct BMP gene targets. Specifically, bona fide canonical BMP signaling gene targets include the Inhibitors of Differentiation or Inhibitors of DNA binding (ID) family of genes (18). Functionally, ID proteins sequester basic-helix-loop-helix transcription

factors to both positively regulate cell proliferation and negatively regulate cell differentiation (19).

To modulate the intensity and duration of BMP signaling, various steps of the pathway can be regulated at both intracellular and extracellular levels. For instance, inhibitory-SMADs-6/7 (I-SMADs) compete for co-SMAD4 and thus, negatively regulate BMP signaling intracellularly. Whereas, Noggin and Chordin regulate extracellular levels of BMP ligands via binding sequestration and thereby suppress BMP signaling (20,21). Secreted Kielin/Chordin-like proteins (KCP), on the other hand, enhance BMP signaling via stabilizing BMP ligand:receptor interactions (22). Notably, KCP overexpression leads to increased p-SMAD-1/5/9 levels and lessens the severity of acute and chronic kidney disease, non-alcoholic fatty liver disease, and diet-induced obesity in mouse models (22-25).

Several laboratories have identified small-molecules that stimulate both canonical and non-canonical BMP signaling. These agents have been characterized in a host of cell lines for potential applications toward bone repair (26-29), medulloblastoma (30,31), pulmonary hypertension (12), and stem cell differentiation (32,33). Immunosuppressants, Sirolimus (Rapamycin) and Tacrolimus (FK506), promote activation of type I BMP receptors by liberating the glycine/serine-rich domain of type I BMP receptors from FKBP12 inhibition (12,33). Potent and selective small-molecule inhibitors of BMP signaling have also been identified and include: dorsomorphin (34), dorsomorphin homolog 1 (35), LDN-193189 (36), and LDN-212854 (37), all of which compete with ATP for binding to the type I BMP receptor kinase.

In this report, we developed a cell based high-throughput screen for BMP agonists using human renal cells (HEK293s) carrying an integrated BMP reporter. After screening more than 63,000 small-molecules, we identified, sb4, a potent benzoxazole small-molecule that activates a BMP reporter by stabilizing intracellular p-SMAD-1/5/9. The increased levels of phosphorylated SMAD-1/5/9 observed with sb4 result in activation of BMP target genes such as ID1 and ID3. Significantly, sb4 mediated activation of the BMP pathway is resistant to

inhibition by Noggin or type I BMP kinase inhibitors, which may prove beneficial in disease contexts where BMP inhibitors are also expressed.

Results

Creation of BMP agonist reporter cells for High-Throughput Screening (HTS)

A modified BMP reporter plasmid (BRE-Luc) construct was designed by cloning an inverted repeat of conserved elements of the ID1 promoter that are necessary for p-SMAD-1/5/9 binding and transactivation (38) upstream of a minimal promoter and the firefly luciferase coding region (Fig. 1A). The BRE-Luc plasmid was linearized and stably integrated into the genome of human embryonic kidney cells (HEK-293). Individual clonal isolates were then screened for responsiveness to both BMP4 and BMP7 and multiple responders identified.

The inverted repeats of the p-SMAD-1/5/9 binding elements confer high sensitivity to rhBMP4 treatment and a wide dynamic range of detection of activated BMP signaling. In luciferase assays, we observed that rhBMP4 treatment caused a dose-dependent increase in luciferase activity in BRE-Lucs ($EC_{50} = 2.4$ ng/ml), whereas vehicle (DMSO) treatment had no effect (Fig. 1B). The BRE-Luc cell line displayed a highly specific response to BMP treatment, with 10 ng/ml of rhBMP4 resulting in a 10-fold increase in luciferase activity over DMSO (Fig. 1B). Furthermore, immunoblotting assays revealed that stimulation of BRE-Lucs with 0.4, 2, or 10 ng/ml of rhBMP4, yielded a dose-dependent induction of p-SMAD-1/5/9 (Fig. 1C). With 2 ng/ml of rhBMP4 yielding a 16-fold induction of p-SMAD-1/5/9 compared to untreated cells (Fig. 1D). In the presence of increasing doses of rhBMP4, basal levels of the TGF- β effector, p-SMAD-2, remained constant (supplemental Fig. S1A). Thus, the BRE-Luc reporter cells displayed the sensitivity and specificity needed to detect activated BMP signaling in a cell based HTS assay for small-molecule agonists of BMP signaling.

Identification of BMP signaling agonists via a cell based HTS

We next screened 63,608 small-molecules from the Center for Chemical Genomics library at

a single concentration (10 μ M) in the BRE-Lucs in our primary HTS (Fig. 2A, B). We identified small-molecules whose activity was greater than 18% activity of the positive control (25 ng/ml rhBMP4) or greater than 3 standard deviations above the negative control (0.1% DMSO) as a hit. To triage the primary screening hits, we applied various filters such as Pan Assay Interference Compounds (PAINS) (39), reactivity, aggregators, and solubility forecast index (SFI) (40) implemented in MScreen (41). The initial 1,453 hits from our primary screen were retested in triplicate for confirmation. After applying MScreen filters, 211 were deemed dose response candidates. Of these 211, 70 compounds showed specificity for the BMP response element in our counter screen using a luciferase reporter cell line that contained a PAX protein response element unresponsive to BMPs (42). Fresh powders of the top sixteen compounds were purchased to conduct dose response studies. Twelve of the sixteen small-molecules displayed dose response activity with relatively potent EC_{50} 's (Fig. 3). We determined that sb4 was the most promising compound because it displayed a sigmoidal dose response curve with the lowest EC_{50} (74nM) of the top twelve compounds. The Hill Slope of 1.24 indicates sb4 binds non-cooperatively (i.e. single ligand interaction) to its target. Luciferase activity after sb4 treatment was increased 2.5-fold compared to DMSO at the highest concentration of 1 μ M.

p-SMAD-1/5/9 is induced by BMP signaling agonists

To determine if phosphorylation of SMAD-1/5/9 was increased in the presence of our top twelve compounds, we treated HEK293 cells for 1hr with 10 μ M of each compound and assayed for p-SMAD-1/5/9 induction by immunoblotting. 2 ng/ml of rhBMP4 served as our positive control and induced p-SMAD-1/5/9 approximately 11-fold over DMSO (Fig. 4A, B). Additionally, we found that 1hr of treatment with several of our top twelve BMP signaling agonists significantly increased p-SMAD-1/5/9 levels at least 2-fold or more compared to DMSO treatment (Fig. 4A, B). The increased levels of p-SMAD-1/5/9 within 1hr suggests a direct stimulation of canonical BMP signaling. Our data support the feasibility of

identifying small-molecules using high-throughput screening that can mimic key downstream effects of endogenous BMP ligands.

To determine if sb4 can induce SMAD-1/5/9 phosphorylation in other cell types, we employed serum-starved primary mouse kidney epithelial cells (PRECs) for compound testing, which lack the BRE-Luc reporter construct. We observed induction of phosphorylated SMAD-1/5/9 in a dose-dependent manner (Fig. 4C, D), with sb4 at 100 and 300 nM yielding a 2-fold induction over DMSO after 24hrs of treatment. Although not as robust as rhBMP4, our results suggest that sb4 can significantly increase p-SMAD-1/5/9 abundance in PRECs under serum-starved conditions.

To ensure specificity, we examined p-SMAD-2 levels after treating cells with candidate BMP signaling agonists at 10 μ M for 1hr. No significant effects were observed on the TGF- β effectors p-SMAD-2 or p-SMAD-3 with any of the compounds tested (supplemental Fig. S1B). We also examined the activation of non-canonical BMP signaling by our top twelve compounds by immunoblotting for phosphorylation of mitogen-activated kinases p38, ERK1/2, and JNK. We found our BMP signaling agonists (sb7-sb11) decreased levels of p-ERK1/2 after 1hr treatment but had little effect on p-JNK or p-p38 (supplemental Fig. S2C, D). In addition, BMPs can stimulate the phosphorylation of Transforming growth factor beta-activated kinase 1 (p-TAK-1). However, sb12 decreased levels of p-TAK-1, while there was no significant increase of p-TAK-1 by the other compounds (supplemental Fig. S2B).

Noggin and type I BMP receptor inhibition is bypassed by sb4

To address the mechanism of sb4 mediated activation of BMP signaling, we conducted luciferase-based inhibition studies with both endogenous and chemical inhibitors of the BMP signaling pathway. Noggin is an endogenous inhibitor of BMP signaling that acts by sequestering dimerized BMP ligands away from BMP receptors and thus suppresses BMP signaling (21). Addition of 250 ng/ml of Noggin completely suppressed the effects of rhBMP4 in

the BRE-Luc cells (Fig. 5A). However, Noggin had no effects on the sb4 mediated activation of BRE-Luc (Fig. 5B). Additionally, we tested the chemical inhibitor, LDN-193189, which selectively inactivates type I BMP receptors (36,43). Inhibition of type I BMP receptors completely abolished the activation of luciferase by rhBMP4 (Fig. 5C). However, sb4 remained active even in the presence of 1 μ M LDN-193189 (Fig. 5D). Taken together, our results indicate that sb4 can activate BMP signaling independent of Noggin and type I BMP receptors, suggesting that it works through an alternate mechanism.

BMP signaling efficacy is enhanced and maintained with sb4 treatment

To determine if the efficacy of BMP signaling is enhanced in the presence of sb4 we assayed varying concentrations of rhBMP4 in the presence of sb4. We found that sb4 enhanced the efficacy of signaling at each concentration of rhBMP4 tested (Fig. 6A). This effect was most pronounced at low concentrations of rhBMP4, whereby sb4 increased BRE-luc expression 2-fold at 0.4 ng/ml of rhBMP4. These data suggest that sb4 may act to stabilize p-SMAD-1/5/9 to enhance the transcriptional response. To test this more directly, we examined the decay of p-SMAD-1/5/9 after rhBMP4 stimulation in the presence or absence of sb4. The BRE-Luc cells were stimulated with 2 ng/ml rhBMP4 for one hour, after which the media were removed and replaced with fresh media containing either DMSO or 1 μ M sb4. p-SMAD-1/5/9 levels were then examined after 0, 5, 15, 30, 45, and 60 minutes of sb4 treatment and compared to DMSO controls (Fig. 6C, D). At each time point, cells treated with sb4 had higher levels of p-SMAD-1/5/9. Furthermore, the decay of p-SMAD-1/5/9 was attenuated, suggesting that sb4 works primarily by increasing the half-life of p-SMAD-1/5/9.

Enhancement of BMP signaling efficacy in our luciferase and western blot studies prompted us to ask if endogenous BMP target genes are also upregulated after sb4 dosing. Since our compounds mimic the activity of low-dose rhBMP4, we conducted a transcriptomic analysis in BRE-Luc cells treated for 4hrs with 2 ng/ml

rhBMP4. We identified thirty-one genes upregulated between 1.5-3.7-fold above untreated cells ($p < 0.05$) (fig. 7A, B). We assayed two of the top rhBMP4 target genes by quantitative RT-PCR to determine if sb4 could affect the expression of these genes. At 24 hours, sb4 significantly increased the expression of ID1 and ID3 (figure 7C, D) but had no effect on COL2A1, which also did not respond to rhBMP4. These data demonstrate that at least some endogenous BMP target genes show increased expression in cells treated with sb4.

Analogs of sb4 increase BMP signaling dose-dependently

Testing of structural analogs of sb4 could identify compounds with increased efficacy and also reveal structure activity relationships (SARs). Thus, we compared 10 related compounds to sb4 and sb3 in our cell based luciferase reporter assay and determined the EC_{50} 's (Fig. 8). Of these 10 compounds, 4 were inactive and 6 were active compounds with EC_{50} 's ranging from 16.2 nM to 274.4 nM. For these benzyl-thio-benzo-oxazole compounds, the substitution at the benzene ring of the benzyl group seems to be important. The data shows very tight structure-activity relationships. For example, benzyl or pyridyl for R_1 makes compounds inactive as in sb4.a1 and sb4.a4 but a methyl substituted benzyl at the *para* position makes the most active compound with EC_{50} of 16.16 nM as in sb4.a2. Different substitutions of benzyl with fluorine, chlorine, and bromine at the *para* position as in sb4.a5, sb4.a3, and sb4, respectively also make compounds active but not as active as the methyl group. For *para* substitution, methyl is the best (sb4.a2: 16.16), followed by fluorine (sb4.a5: 60.14), bromine (sb4: 73.62), then by chlorine (sb4.a3: 77.05). Compared to substitution at the *para* position of the benzyl as in sb4.a5, substitution at the *ortho* position with F as in sb3 reduces activity. Compared to substitution at the *para* position of the benzyl as in sb4.a3, substitution at the *meta* position with Cl as in sb4.a6 makes compound 5 times less active. Substitution at the *ortho* position of the benzyl with methoxy is 2 times less active than fluorine. The fluorine-substituted compound at the *ortho* position is 10% more active when chlorine is substituted at the other *ortho* position at

the same time as in sb4.a10. The inactivity of sb4.a7 could be either due to the steric effect of the substitution of methyl at the benzo-oxazole or due to the lack of aromatic ring at the right-hand side, or both. The oxazolo-pyridine in place of the benzo-oxazole as in sb4.a9 kills the activity. Overall, the substitutions at the *para* or *ortho* position of benzyl group is preferred to at the *meta* position. These data suggest that modification of benzo-oxazole is not tolerated.

Discussion

In order to identify chemical compounds that could be the basis for developing therapeutic BMP agonists, we designed a cell based HTS and identified multiple specific small-molecules that enhance and/or stabilize the BMP effectors p-SMAD-1/5/9. Activation of BMP signaling is reported to be protective in a variety of disease contexts, including animal models of acute and chronic kidney disease. Our unbiased HTS identified a novel compound, sb4, that rapidly activates BMP signaling in renal cell cultures. We found that sb4 increased levels of p-SMAD-1/5/9 within 1hr and increased the expression of direct key BMP4 target genes (ID1 and ID3). Additionally, our experiments revealed that sb4 acts downstream of type I BMP receptors and bypasses negative regulation by Noggin, an extracellular inhibitor of BMP signaling. Moreover, in the presence of rhBMP4, sb4 enhances BMP signaling efficacy by slowing the turnover or decay of p-SMAD-1/5/9.

In contrast to animal models of CKD, human CKD patient samples have elevated levels of BMP expression (44) along with elevated levels of negative regulators of BMPs similar to Noggin such as Gremlin (45,46) and connective tissue growth factor (CTGF, 47). Hence, a mechanism such as that demonstrated by sb4, which bypasses negative regulation by Noggin and similar negative regulators of BMP signaling, could be beneficial in specific clinical situations where these negative regulators are elevated. Further, the ability to activate BMP signaling when type I BMP receptors are inhibited could also be a useful mechanism in the event receptor status is low due to diseased states (48,49). Moreover, compounds

like sb4 could synergize with endogenous levels of BMPs and potentially abrogate the need for harmful doses of rhBMPs that lead to unwanted side effects (50-52).

Several other laboratories have also reported the identification of activators/sensitizers of BMP signaling in other cell types. Of note, some of these previously published BMP activators such as Isoliquiritigenin (31), FK506 (12), PD-407824 (32), were tested in our primary HTS screen. However, they did not meet our cut off for further analysis due to either having low activity in our primary screen or not meeting the criteria to pass our counter screen. This may underscore the potential of diverse compound structures to induce cell type specific responses. In a broader sense, it stresses the importance of identifying direct targets and determining the mechanism of action of these diverse compounds. Since, each molecule may target different levels/aspects of canonical or non-canonical BMP signaling. And therefore, induce varying intensities of activation of BMP signaling culminating in diverse biological effects.

Canonical R-SMAD-1/5/8/9 activation occurs via phosphorylation by type I BMP receptors. Phosphorylation at the c-terminal transcriptional domain of SMADs-1/5/8/9 facilitates co-SMAD4 docking and heteromeric SMAD transcriptional complex formation. In addition, the linker interdomain of SMADs-1/5/8/9 undergo phosphorylation by cyclin dependent kinases (CDK8/9) and glycogen synthase 3 (GSK3), which renders R-SMADs fully functional (53). To control activated BMP/SMAD-1/5/8/9 signaling duration and intensity, fundamental turnover mechanisms, such as dephosphorylation and ubiquitination, are necessary. In particular, small c-terminal domain ser/thr phosphatases (SCPs) have been identified that dephosphorylate the c-terminal transcriptional domain of SMAD-1 after type I BMP receptor phosphorylation (54,55) and the linker interdomain after CDK8/9 phosphorylation (55). Experimental siRNA mediated knockdown of SCPs-1/2 conferred increased BMP signaling and ID1 expression in human keratinocytes and osteosarcoma cell lines (54). Thus, small molecules such as sb4 could potentially inhibit

such phosphatases and prove beneficial in disease states such as CKD.

The Smurf proteins (SMAD ubiquitination regulatory factors) also negatively regulate BMP/SMAD activity (56). GSK3 phosphorylation recruits Smurfs to the BMP/SMAD linker interdomain to ubiquitinate activated BMP/SMADs for proteasomal degradation (57,58). Increased BMP signaling is also observed following siRNA mediated knockdown of Smurf (59). Yes associated proteins (YAPs) are also recruited to the linker region of SMAD-1, but instead, function to enhance BMP/SMAD transcriptional activity and therefore signaling (59). Because sb4 activates BMP signaling independent of Noggin or type I BMP receptor inhibition, it's plausible that increased levels of p-SMAD-1/5/9 observed in this study can be potentially attributed to inhibiting SCP or Smurf activity or promotion of YAP function. Further studies are needed to identify the direct targets of sb4 and its analogs to unravel the precise mechanism of action.

Clinical trials have been conducted to assess the clinical use of peptide BMP agonist (THR-184) in acute kidney injury (60), as well as, the use of a humanized, neutralizing TGF- β 1 monoclonal antibody in CKD (61). Both clinical trials yielded futile results due to lack of efficacy. This may suggest the need for combination treatments that not only repress TGF- β activity but simultaneously activate BMP signaling while bypassing negative regulators such as Noggin. Compounds with a benzoxazole moiety similar to sb4, have been reported to have important clinical applications. Pharmacological activities include working as anti-inflammatory (62) and anti-diabetic agents (63). Our studies suggest that these agents could also possess anti-fibrotic pharmacological activity due to their ability (sb4 and its analogs) to biologically activate and enhance BMP signaling. Therefore, and in conclusion, identification of the renal BMP signaling agonists reported here provides a novel tool compound that can be used to interrogate BMP signaling pre-clinically in animal models of kidney disease.

Experimental procedures

Construction of BMP reporter cells and cell culture.

Human embryonic kidney cells (HEK293) were transfected with a linearized plasmid containing two inverted repeats of a BMP responsive element containing SMAD binding sites and parts of the ID1 promoter as described (38). Transformants were selected in 800 mg/ml of geneticin (G418) and clones were derived by single cell dilution. Individual BRE-Luc containing HEK-293 clones were tested for responsiveness to BMP4 and BMP7. For testing of small-molecules, BRE-Lucs were maintained in complete medium (Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose and 584 mg/L L-glutamine supplemented with 10% fetal bovine serum and 1% Pen/Strep) and cultured to sub-confluency at 37°C and 5% CO₂. Primary Mouse Renal Epithelial Cells (PRECs) were immortalized by using temperature sensitive T-antigen transformation and cultured to sub-confluency in complete medium containing 800 µg/ml Geneticin (G418) at 33°C and 5% CO₂.

Small-molecule screening library

63,608 drug/lead-like structures were screened in the Center for Chemical Genomics (CCG) small-molecule library housed at the Life Science Institute at the University of Michigan. Small-molecules were selected for their drug/lead like physicochemical properties which fell within the Lipinski's space for bioavailable/orally active drugs having molecular weights < 500, hydrogen-bond donors < 5, hydrogen-acceptors < 10 and cLogP's < 5 (64). The libraries utilized at the CCG consisted of compounds from the ChemDiv 100K diversity set in which 61,120 structures were screened returning a hit ratio 17.32%. 1,280 compounds from the Library of Pharmacologically Active Compounds (Sigma) returned a hit ratio of 9.06%. 1,280 compounds from the Prestwick library returned a hit ratio of 8.2% and screening 320 compounds from the NIH Clinical Compound library returned a 7.81% hit ratio. Instant JChem was used for structure database management, search and prediction (Instant JChem 5.9.0, ChemAxon, <http://www.chemaxon.com>).

sb4 analog similarity search

Analogs similar to sb4 were obtained as fresh powders after doing a structure similarity search at the Aldrich Market Select website (<https://www.aldrichmarketselect.com/>). A 70% similarity search type was used that included product filters to select for compounds with molecular weights less than 500 and cLogP less than 5. Purity of compounds ranged from 85-90% and were synthesized by AMS suppliers including: AMS Private Supplier I (sb4.A7), ChemBridge (sb4, 85% purity), ChemDiv (sb4.A9, 90% purity), Labotest (sb3, sb4.A2-A6, sb4.A8, sb4.A10, 90% purity), and Pharmeks (sb4.A1, 90% purity).

Immunoblotting

BRE-Lucs were passaged in DMEM supplemented with 5% FBS (vol/vol) for both HTS and 6-well plate studies. Compounds were added in the presence of 5% FBS. PRECs were serum starved 1hr before addition of factors in Serum Free Medium (SFM). Sub-confluent BRE-Luc or PREC cultures were seeded into 6-well plates at 3.0×10^5 or 2.0×10^5 cells/well respectively, using an automated cell counter (Countess™ II). Cells were treated with rh-BMP4 (R & D Systems; 314-BP), vehicle (DMSO-D6), or compound (10 µM) for 1hr or 24hrs. After the indicated time points, treatment medium was removed, adhered cells were washed once in 1X PBS and subsequently harvested in 1X PBS and pelleted by low centrifugation. The cell pellet was then suspended in PK lysis buffer (50mM HEPES pH 7.5, 150mM NaCl, 1.5mM MgCl₂, 1mM EGTA, 10% Glycerol, 1% Triton X-100, 1mM Na₃VO₄ 50mM NaF) containing both phosphatase and protease inhibitors (PhosSTOP and cOmplete Mini; Roche) and allowed to lyse on ice for 30 minutes with intermittent vortexing. To pellet the insoluble fraction, the suspended pellet was centrifuged at 15,000 rpm for 15 minutes at 4°C. Supernatant was transferred to clean Eppendorf tubes and 6X sodium dodecyl sulfate was added 1:5 and heated for 5 minutes at 95°C. Equal amounts of total protein lysates were resolved on hand-casted 8% SDS-polyacrylamide gels. Separated proteins were then electrophoretically transferred onto Immobilon®-FL polyvinylidene difluoride membranes. Membranes were dried and

reactivated in methanol rinsed in 1X TBS and blocked for 1hr in 5% bovine serum albumin or 5% milk dissolved in 1X TBS-T (1X TBS + 0.1% Tween[®] 20) and then incubated overnight with primary antibodies consisting of one of the following: anti-Rb phospho-SMAD-1/5/9 1:1,000 (CST[®]; 13820), anti-Rb total-SMAD-1 1:2,000 (CST[®]; 9734), anti-Rb phospho-SMAD-2 1:1000 (CST[®]; 3104), anti-Rb phospho-SMAD-3 1:1000 (CST[®]; 9520) anti-Rb total-SMAD-2/3 1:1000 (CST[®]; 3102) anti-Rb-phospho-TAK1 (CST[®]; 9339), anti-Rb-phospho-MAPK Family Sampler kit (CST[®]; 9910T), anti- β -Actin 1:10,000 (Proteintech[®]; 60008-1-Ig) The next day, membranes were washed in 1% non-fat dry milk prepared in 1X TBS-T and incubated for 1 hour at room temperature in anti-Rb HRP or anti-Ms-HRP diluted in 1% non-fat dry milk (1X TBS-T). Membranes were washed in TBS-T and rinsed twice with 1X TBS and incubated with ECL western blotting substrate (Pierce[™]; 32106). Finally, membranes were incubated with HyBlot ES[™] autoradiography film (Denville Scientific) and developed on the Kodak X-OMAT 2000A processor and quantified using ImageJ (Version 2.0.0)

Luciferase Assays

Compounds were dissolved in MagniSolv[™] dimethyl sulfoxide-D6 (DMSO-D6, EMD Millipore; 2206-27-1) and then diluted in 5% FBS medium and added to 96-well plates. Recombinant proteins (rhBMP4 or rhNoggin, R&D Systems; 314-BP or 6057-NG) were reconstituted per manufactures' instructions and diluted in 5% FBS medium. BRE-Lucs were passaged in 5% FBS medium and seeded into factor containing 96-well plates at 3.0×10^3 cells/well using an automated cell counter (Countess[™] II). BRE-Lucs were incubated overnight at 37°C and 5% CO₂. The next day, 85% of the culture medium was aspirated using a microplate washer (BioTek[®] ELx405[™]) and an equal volume of luciferase reagent (Steady-Glo[®] Promega) was added to the remaining culture medium in each well using a Multidrop[™] dispenser. Following Steady-Glo[®] cell lysis, luminescence activity was measured on a luminometer (PHEARstar[®] BMG Labtech).

BRE-Luc cell Transcriptome profiling

To identify direct gene targets of low dose rhBMP4 (2ng/ml) specifically in BRE-Lucs, cells were seeded at 3.0×10^5 cells in 6-well plates in triplicate for each sample. Cells were cultured in 5% FBS medium overnight and treated the next day with or without 2ng/ml rhBMP4 for 4 hours. After treatment, BRE-Lucs were harvested to isolate total RNA using the RNeasy[®] Mini Kit (Qiagen) by following the manufactures' protocol. Transcriptome profiles of low-dose rhBMP4 (2ng/ml) (treated) and 5% FBS medium only (untreated) cells were assessed via Affymetrix[™] microarray analysis performed by the Microarray Core housed within University of Michigan DNA Sequencing core. Concisely, the integrity and yield of total RNA was assessed by using the RNA 6000 Nano kit on the 2100 Bioanalyzer (Agilent Technologies). Reported RNA integrity numbers were above 9 for the two samples submitted in triplicate. 400ng of total RNA was used to generate fragmented and biotin conjugated single-stranded cDNA (ss-cDNA) using the GeneChip[™] Whole Transcript PLUS reagent kit (Applied Biosystems). ss-cDNA was then hybridized to the Affymetrix[®] GeneAtlas Human Gene 2.1 Sense Target microarray. Following hybridization—wash, stain, and imaging procedures were carried out according to the manufactures protocol. The robust multi-array average method (65) was used to correct for variations between microarrays. Oligo and Limma packages of Bioconductor/R were used to identify differentially expressed genes by fitting normalized gene expression data to weighted linear models (66). Probe sets with a variance above 0.025 and fold changes greater than 1.5 were selected. Multiplicity assessment was carried out by using the false discovery rate method to adjust p-values (67). Final data was loaded into iPathwayGuide to generate volcano plot (Advaita Bioinformatics).

qRT-PCR

To assess the capacity of HTS compounds to induce the expression of endogenous direct gene targets of BMP signaling, BRE-Lucs were seeded in 6-well plates at 3.0×10^5 cells/well and cultured in 5% FBS medium overnight. The following day, BRE-Lucs were treated with low dose rhBMP4 (2ng/ml), 0.04% DMSO-D6, or increasing

concentrations of compounds (all with 0.04% DMSO-D6). After 24hrs of treatment, BRE-Lucs were harvested for total RNA isolation using the RNeasy[®] Mini Kit (Qiagen) by following the manufactures protocol. Synthesis of first-strand cDNA was prepared by using 1µg of total RNA, Superscript[™] III (Invitrogen) reverse transcriptase, and 100ng of random primers. Amplified cDNA templates were diluted 1:50 and SYBR[®] green Master Mix was used to monitor cDNA template amplification on the Applied Biosystems[®] 7500 real-time PCR system. HPRT was used to normalize gene expression. For each sample, fold change of gene expression over 0.04% DMSO-D6 was calculated. Human primer pair sequences used were obtained from PrimerBank (68) (except for HPRT) and include the following:

ID1-(f)-CTGCTCTACGACATGAACGG,
 (r)-GAAGGTCCCTGATGTAGTCGAT
ID3-(f)-GAGAGGCACTCAGCTTAGCC,
 (r)-TCCTTTTGTGCGTTGGAGATGAC
COL2A1-(f)-CCAGATGACCTTCCTACGCC,
 (r)-TTCAGGGCAGTGACGTGAAC
HPRT-(f)-ATGGACAGGACTGAACGTCTT,
 (r)-TCCAGCAGGTCAGCAAAGAA

Statistical Analysis

The following statistical test were used to assess significance and derive p-values, for each test, alpha = 0.05%: one-way ANOVA with post-hoc Dunnett's multiple comparison test, unpaired two-tailed t-test, and multiple t-test's (one unpaired t-test per row) by using GraphPad Prism7 version 7.0d

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Conflicts of Interest:

None.

Author Contributions:

Conceptualization and design of research project, GRD. Performed experiments, STJB, EJ, EG, GRD. Analyzed and interpreted data, STJB, PHL, and GRD. Drafted and edited original manuscript and prepared figures, STJB and GRD.

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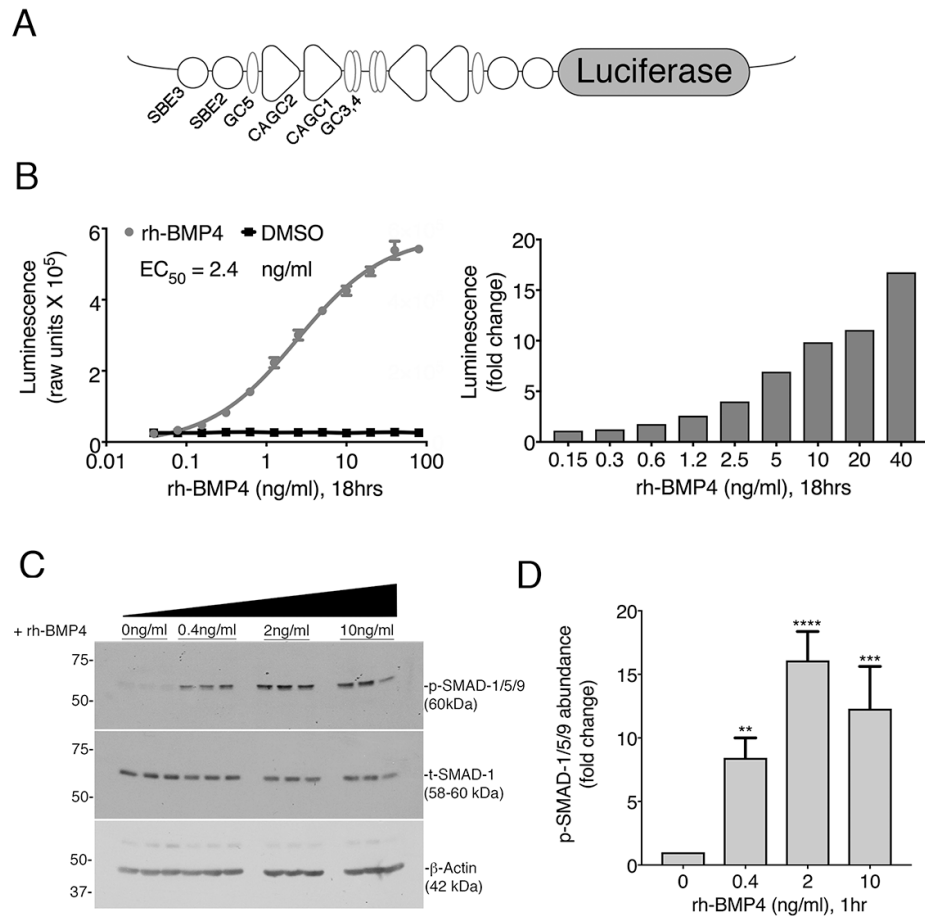


Figure 1. Characterization and Validation of BRE-Luc Cells for HTS. A) The BRE-Luc reporter construct contains an inverted repeat of BMP responsive elements driving luciferase. Elements of the ID1 promoter and SMAD binding sites are marked as originally identified by Korchynskyi *et al.* (38). B) A dose response of BRE-Luc cells to increasing concentrations of rhBMP4 performed in triplicate. C) Western blot of lysates from BRE-Luc cells treated with increasing concentrations of rhBMP4. Membranes were probed with p-SMAD-1/5/9 and total-SMAD-1; β -Actin was used as an additional loading control. D) p-SMAD-1/5/9 protein levels were quantified by densitometry. The signal of p-SMAD-1/5/9 was normalized to total-SMAD-1 to control for loading variability. Data are expressed as fold change relative to the media alone, which was set to 1. Error bars represent one standard deviation from the mean of 3 independent biological replicates. ANOVA, Dunnett's post-hoc multiple comparisons test generated P values of: ** $p < 0.01$ *** $p < 0.001$, **** $p < 0.0001$ relative to controls.

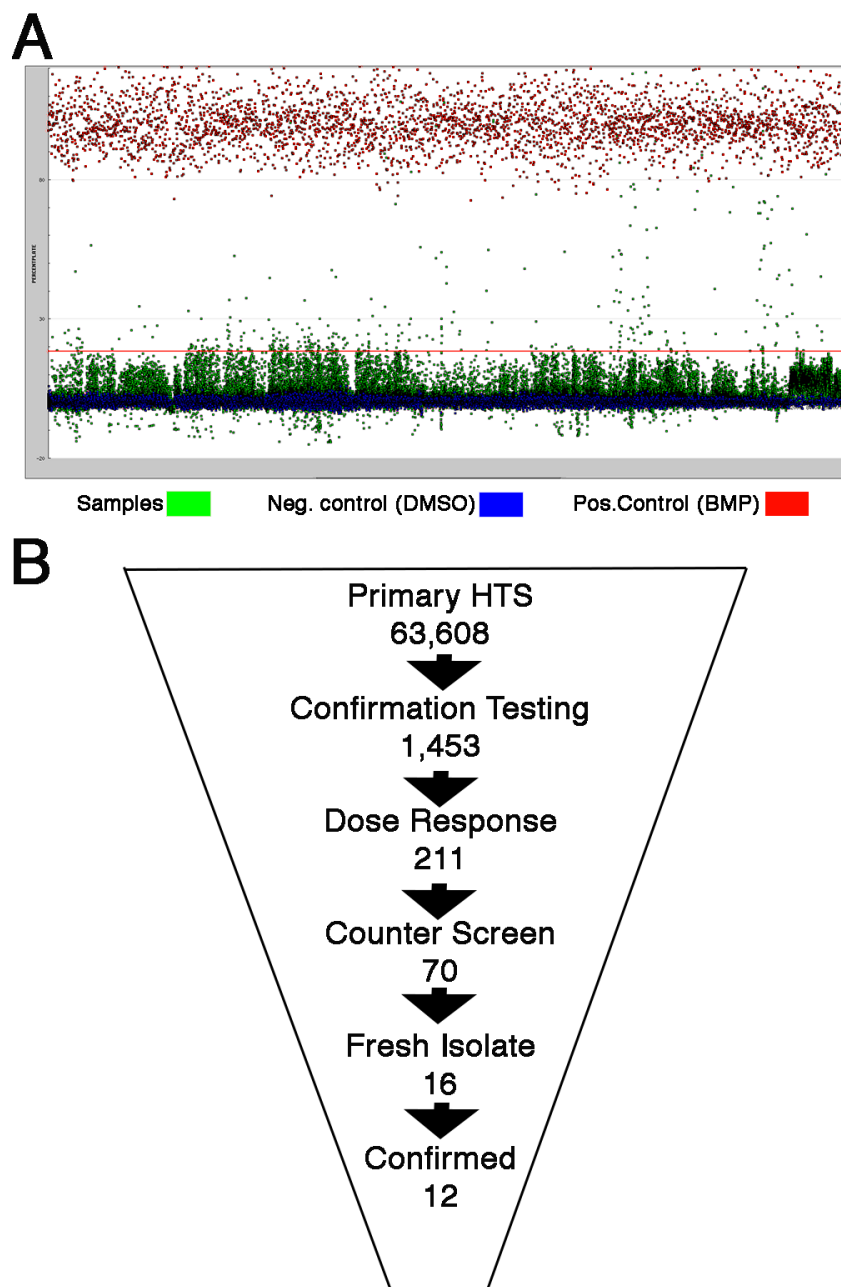


Figure 2. High-Throughput Screening Strategy in BRE-Luc Cells. A) A snapshot of the primary screen of 63,608 small-molecules (green dots) at a single concentration (10 μ M) in renal BRE-Luc cells. Positive controls were 25 ng/ml of rhBMP4 (red dots) whereas negative controls were 0.1% DMSO (blue dots). The red line represents 3 standard deviations above the DMSO. The average luciferase induction for 25 ng/ml of rhBMP4 was $355,000 \pm 34,000$, DMSO $44,000 \pm 4,300$, and for the small-molecules hits $127,000$. The average Z' score for all the plates screened was 0.71, indicating a high-quality screening assay. B) Triage strategy used to winnow down the starting 63,608 small-molecules from the Primary HTS campaign which allowed identification of the top twelve BMP signaling agonists based on dose response curves and structures.

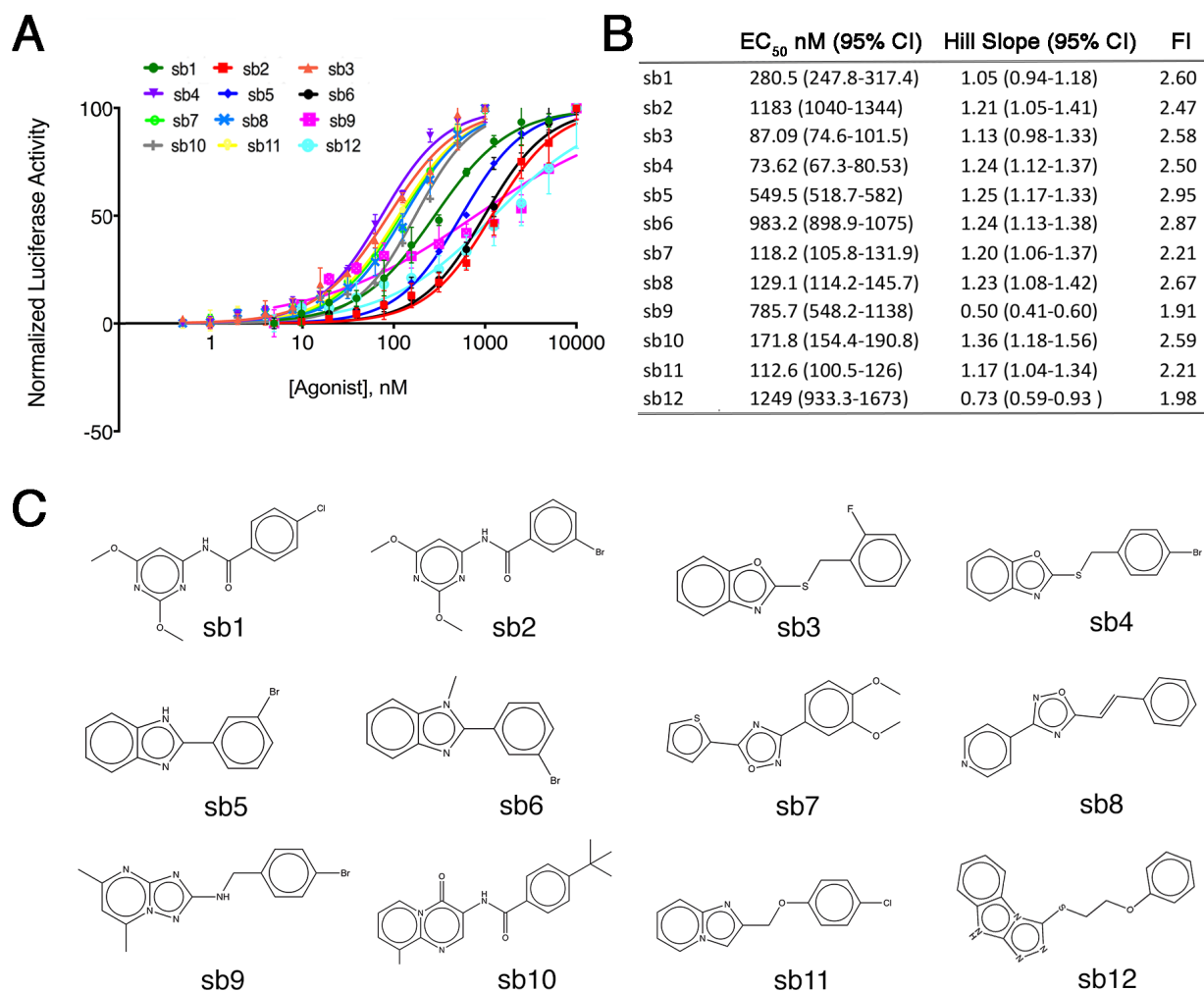


Figure 3. Dose Responses of the Top Twelve Potential BMP Agonists. A) 12 compounds were tested for BRE-Luc responses at two-fold increasing concentrations from 0.5 nM to 10 μ M in triplicate. B) Effective concentrations at 50% maximum response (EC₅₀) and the Hill Slopes were calculated for each compound. Maximum fold induction (FI) above DMSO controls is reported. C) Chemical structures of the 12 candidate BMP agonists are shown schematically.

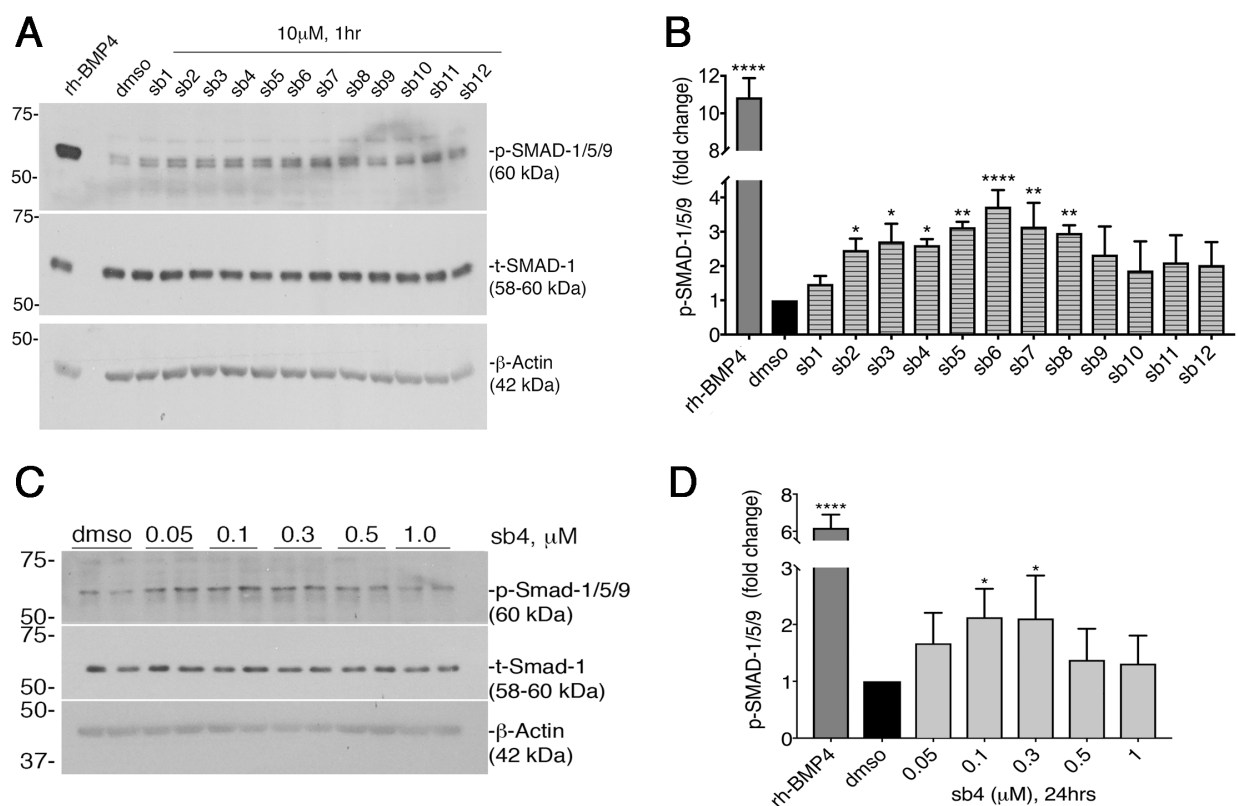


Figure 4. Activation of p-SMAD-1/5/9 by Small-Molecules. A) Immunoblot of whole cell protein lysates from BRE-Luc cells treated with either 2 ng/ml rhBMP4, 0.04% DMSO, or the top twelve BMP candidate agonists at 10 μ M for 1 hour in BRE-Luc cells. Membranes were probed with anti-p-SMAD-1/5/9 and anti-total-SMAD-1. β -Actin served as an additional loading control. B) Quantitation of p-SMAD-1/5/9 protein levels as determined by densitometry of three independently derived western blots. The p-SMAD-1/5/9 levels were normalized to total-SMAD-1. Final data are expressed as fold change relative to the mean negative control signal, with error bars representing one standard deviation (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ relative to control). ANOVA, Dunnett's post-hoc multiple comparisons test was used to determine significance. C) Immunoblot of lysates from serum-starved PRECs treated for 24hrs with increasing concentrations of sb4 in serum free medium. Total-SMAD-1 and β -actin serve as loading controls. D) Quantitation of p-SMAD-1/5/9 levels are shown. Statistical analyses of replicates were done as in B.

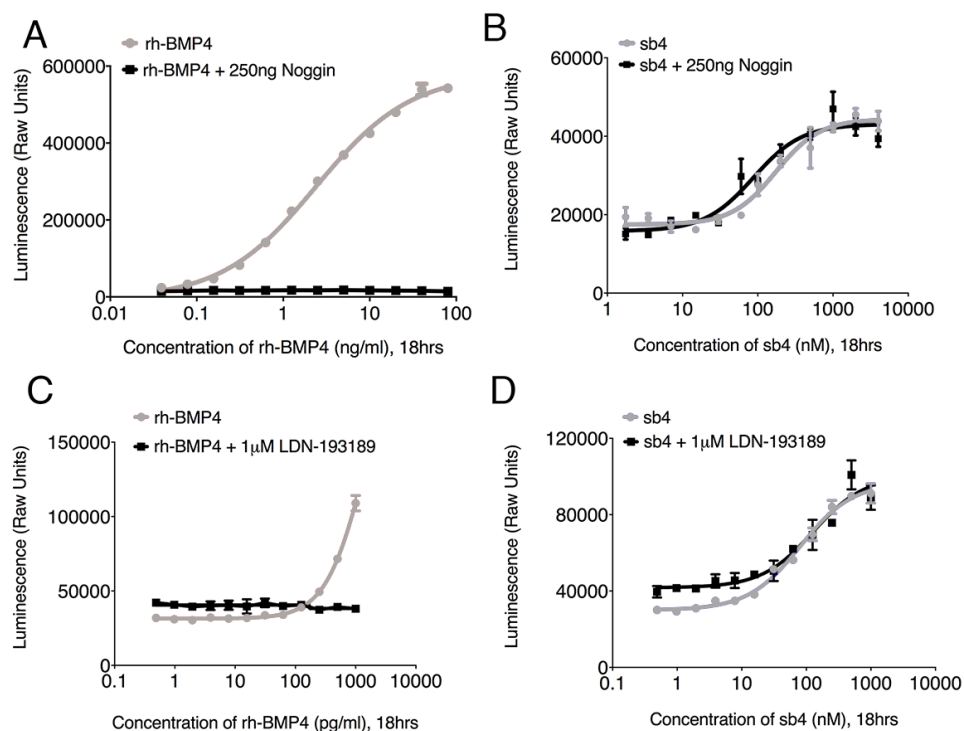


Figure 5. Effects of BMP Inhibitors on sb4 Activity. A) BRE-Luc cells were subjected to increasing concentrations of rhBMP4 with (black) or without (red) 250 ng/ml of rhNoggin. Maximum concentrations of 80 ng/ml for rhBMP4 were used and cells were incubated for 18hrs before measuring luminescence. B) A dose response for sb4 was measured in BRE-Luc cells alone (blue) or in the presence of 250 ng/ml rhNoggin (black). A maximum concentration of 4 μ M of sb4 was used. C) Activity of increasing concentrations of rhBMP4 in BRE-Luc cells alone (red) or with a constant dose of 1 μ M type I BMP receptor inhibitor (LDN-193189, black). For both curves a maximum concentration of 1000 pg/ml rhBMP4 was used and cells were incubated for 18hrs before measuring luminescence. D) Increasing concentrations of sb4 were added to BRE-Luc cells alone (blue) or in the presence of 1 μ M LDN-193189 (black). A maximum of 1000 nM of sb4 was used. Experiments were conducted in triplicate.

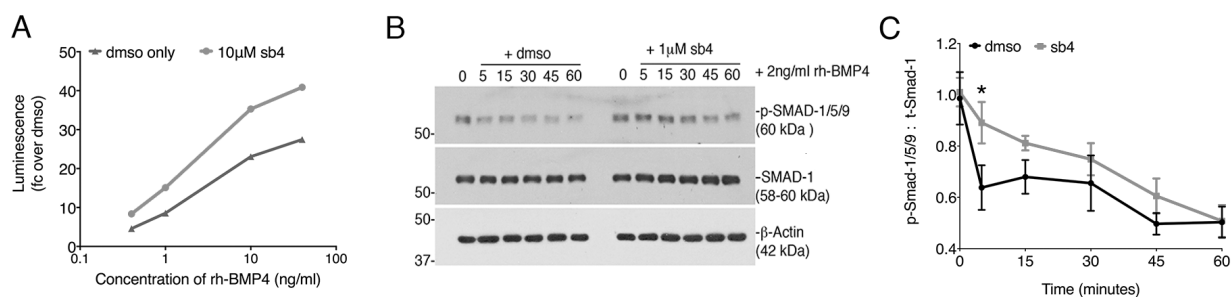


Figure 6. BMP signaling agonists enhance the efficacy of BMPs. A) BRE-Luc cells were treated with DMSO (black) or sb4 (grey) at a constant concentration of 10 μ M in the presence of increasing concentrations of rhBMP4 (0.4, 1, 10 and 40 ng/ml) for 18 hours. Representative experiment conducted in triplicate, reported as fold change over DMSO. B) Western blot of protein lysates from BRE-luc cells treated for 1 hour with rhBMP4 (2 ng/ml) followed by fresh media containing either vehicle (DMSO) or 1 μ M sb4. Samples were harvested at 0, 5, 15, 30, 45, and 60 minutes after sb4 addition. A representative western blot for p-SMAD-1/5/9 from three independent biological experiments is shown. C) Ratio of p-SMAD-1/5/9 to total-SMAD-1 as quantified by densitometry is shown for experiments outlined in C. Ratio of p-SMAD-1/5/9 to total-SMAD-1 over time was compared by unpaired multiple t-tests. *p-value = 0.02. Note the increased stability of p-SMAD-1/5/9 in sb4 treated cells.

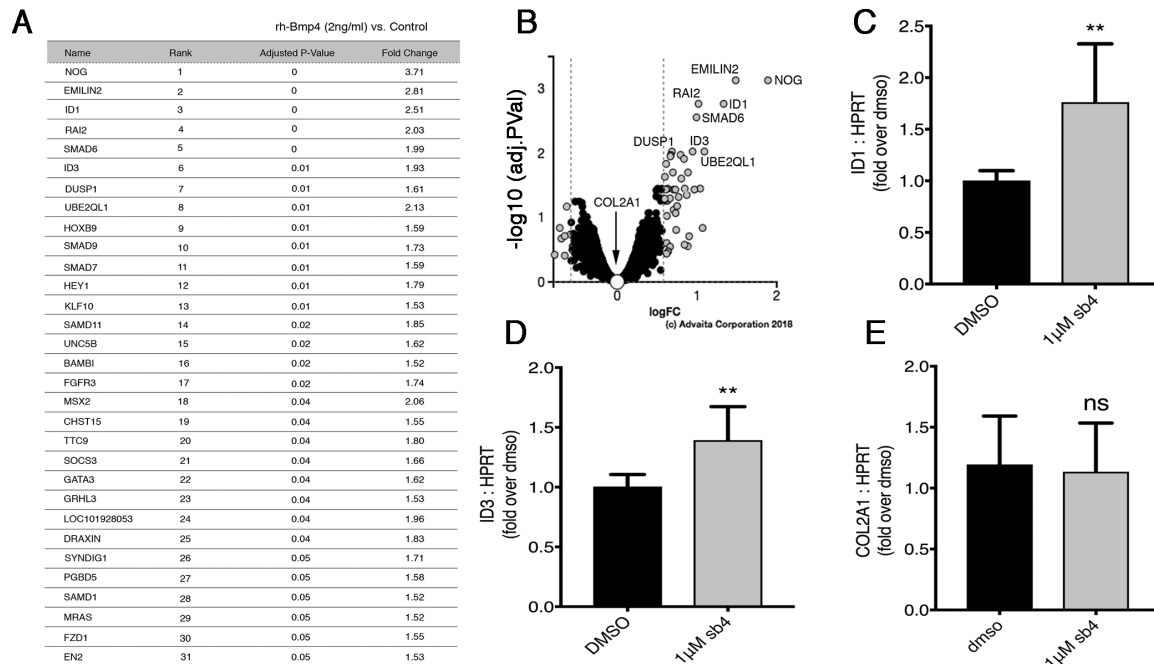


Figure 7. sb4 can Activate Endogenous BMP4 target genes. A) The top 31 genes with increased transcriptional activation after 4 hours of rhBMP4 (2 ng/ml) as determined by Affymetrix microarrays. Triplicate biological replicates were performed for treated and untreated groups. The p-values ≤ 0.05 and fold changes ≥ 1.5 are reported for treated vs. untreated groups. B) Volcano plot of 6,643 genes that were measured for expression. 51 genes were differentially expressed between treated and untreated groups. Significant differentially expressed genes are shown in grey dots, black and selected white dots represent non-significant genes. The volcano plot was generated by uploading transcriptomic data into iPathwayGuide, with cut off fold change and p-values set to 0.58 and 1 respectively. C-E) Quantitative RT-PCR analysis was used to determine the relative mRNA levels of endogenous rhBMP4 target genes after 1 μ M sb4 treatment. The ID1 (C) and ID3 (D) genes show significant upregulation, whereas COL2A1 (E) remains unchanged. Data represent the mean and one standard deviation from three independent biological experiments performed in triplicate (**p-value < 0.01 , ns = not significant: unpaired t-test).

$$\text{R}_1-\text{S}-\text{R}_2$$

	R1	R2	EC ₅₀
Sb3 3295002			87.1
Sb4 731364			73.6
Sb4.a1 497455			NA
Sb4.a2 708046			16.2
Sb4.a3 708044			77.1
Sb4.a4 3012531			NA
Sb4.a5 900994			60.1
Sb4.a6 4450488			274.4
Sb4.a7 117059117			NA
Sb4.a8 4292218			184.0
Sb4.a9 20948899			NA
Sb4.a10 4645992			78.9

Figure 8. Structure Activity Relationships Among 11 sb4 like Compounds. Dose response curves for analog structures of sb4 were tested in BRE-luc cells. The effective concentration at 50% maximum activity (EC₅₀) was calculated for each structure as shown. The PubChem ID numbers are listed below the designated name.

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identify potent benzoxazole compounds**

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