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Structure—Activity Relationship of 3,5-Diaryl-2-aminopyridine ALK2 Inhibitors Reveals Unaltered Binding Affinity for Fibrodysplasia Ossificans Progressiva Causing Mutants

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ABSTRACT: There are currently no effective therapies for fibrodysplasia ossificans progressiva (FOP), a debilitating and progressive heterotopic ossification disease caused by activating mutations of ACVR1 encoding the BMP type I receptor kinase ALK2. Recently, a subset of these same mutations of ACVR1 have been identified in diffuse intrinsic pontine glioma (DIPG) tumors. Here we describe the structure—activity relationship for a series of novel ALK2 inhibitors based on the 2-aminopyridine compound K02288. Several modifications increased potency in kinase, thermal shift, or cell-based assays of BMP signaling and transcription, as well as selectivity for ALK2 versus closely related BMP and TGF-β type I receptor kinases. Compounds in this series exhibited a wide range of in vitro cytotoxicity that was not correlated with potency or selectivity, suggesting mechanisms independent of BMP or TGF-β inhibition. The study also highlights a potent 2-methylpyridine derivative 10 (LDN-214117) with a high degree of selectivity for ALK2 and low cytotoxicity that could provide a template for preclinical development. Contrary to the notion that activating mutations of ALK2 might alter inhibitor efficacy due to potential conformational changes in the ATP-binding site, the compounds demonstrated consistent binding to a panel of mutant and wild-type ALK2 proteins. Thus, BMP inhibitors identified via activity against wild-type ALK2 signaling are likely to be of clinical relevance for the diverse ALK2 mutant proteins associated with FOP and DIPG.

INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-beta (TGF-β) signaling family, which includes over 30 different ligands.1 BMP signaling is essential for numerous processes, including cell fate determination, embryonic patterning, and iron homeostasis.2,3 The BMP signaling cascade parallels that of TGF-β signaling. BMP ligand dimers bind to transmembrane receptor complexes consisting of two constitutively active type II receptor kinases (BMPRII, ACTRIIA, or ACTRIIB), which transphosphorylate and activate two type I receptor kinases (ALK1, ALK2, ALK3, or ALK6).4 Activated type I receptors phosphorylate effector proteins (SMAD1/5/8) that complex with SMAD4, translocate to the nucleus, and activate BMP responsive genes such as the inhibitor of differentiation (ID) gene family. Functional and anatomic specificity of BMP signaling is regulated by the spatiotemporal expression of ligands and their cognate receptors as well as the expression of endogenous BMP antagonists such as noggin.5,6

Inappropriate BMP signaling has been shown to contribute to the pathophysiology of various disease processes.7 One of the most striking examples of BMP signaling-related disease is seen in fibrodysplasia ossificans progressiva (FOP), a rare and disabling genetic disease affecting approximately 2500 people worldwide.8 While individuals with the classical form of FOP...
are nearly normal at birth except for cervical and hallux joint deformities, during early life they develop progressive formation of endochondral bone in muscles, fascia, and ligaments, leading to severe immobility, pain, and premature mortality. A highly conserved gain-of-function mutation in the glycine–serine (GS) rich domain of the BMP type-I receptor ALK2 (c.617G>A; p.R206H) accounts for more than 98% of cases of classic FOP. Several other FOP-causing gain-of-function mutations in both the GS and kinase domains of ALK2 have also been described in nonclassic or variant forms of FOP.

Recently, several of the mutations identified in classic and nonclassic forms of FOP have been observed to arise in a proportion of tumors in diffuse intrinsic pontine glioma, a deadly childhood tumor also without effective therapies. The consistency of this finding across diverse patient cohorts by several independent groups suggests an important role of somatic activating mutations of ACVR1 in this disease, however, the pathogenetic role of these mutant proteins is currently under investigation.

We and others have previously reported the discovery and development of small molecule inhibitors of BMP type-I receptors such as dorsomorphin, LDN-193189, LDN-212854, and DMH1, all of which are based on the pyrazolo[1,5-a]pyrimidine scaffold (Figure 1). These compounds have proven to be useful chemical reagents for the study of in vitro phenomenon, and several have demonstrated in vivo efficacy in a mouse model of FOP. More recently we described a structurally distinct BMP type-I receptor inhibitor, K02288, which is based on a 2-aminopyridine scaffold and demonstrated greater kinase-wide selectivity than LDN-193189. The 2-aminopyridine scaffold is also found in crizotinib, which was recently approved by the FDA for the treatment of nonsmall cell lung cancer in patients with activating mutations in the anaplastic lymphoma kinase. Despite the high affinity and selectivity of K02288 for BMP receptors in thermal shift and in vitro kinase assays, it has comparatively weak potency in cell-based assays.

In this article, we describe a structure–activity relationship (SAR) study of K02288 with respect to ALK2 binding affinity, BMP and TGF-β signaling inhibition in biochemical and cellular assays, selectivity, and cytotoxicity. These studies were pursued as part of an effort to elucidate the BMP type I receptor inhibitor pharmacophore, while producing a set of compounds with greater utility as physiologic probes. This SAR provides unique insights into features of 2-aminopyridine derivatives that are required for potent and selective inhibition of ALK2 versus closely related BMP and TGF-β receptors. We found that substitution of the 3-phenol with 4-phenylpiperazine greatly increased potency in cells, yielding a series of compounds more likely to be useful as probes of ALK2 function. These included a 2-methylpyridine derivative that exhibited potent and relatively selective inhibition of ALK2 activity in cell-based and in vitro kinase assays, high selectivity across the kinome, and low cytotoxicity. Additionally, we used this novel set of derivatives to demonstrate for the first time that FOP-causing mutations do not affect inhibitor binding affinity as compared to wild-type ALK2. This finding strongly suggests that ATP-competitive kinase inhibitors identified on the basis of their activity against endogenous BMP signaling, such as dorsomorphin and its derivatives, or by their affinity for wild-type ALK2, as in the case of K02288, will inhibit with equal potency the mutant ALK2R206H found in classical FOP as well as the other GS- and kinase-domain mutants of ALK2 that have been described in nonclassical or variant FOP or DIPG. These results describe a novel series of specific and potent probe compounds for the interrogation of BMP signaling that may have therapeutic potential for FOP and other diseases of maladaptive or inappropriate BMP signaling.

### RESULTS AND DISCUSSION

**Chemistry.** A series of 2-amino-3-(3,4,5-trimethoxyphenyl)-pyridine derivatives were synthesized according to the procedures outlined in Scheme 1. Commercially available 2-amino-5-bromo-3-iodopyridine was coupled with 3,4,5-trimethoxyphenylboronic acid under Suzuki reaction conditions to give 2 in 80% yield. This intermediate was subjected to a second Suzuki reaction with a range of boronic acids to furnish the target compounds in 40–85% yield.

A variety of 2-substituted 3-aryl-5-(piperazinylphenyl)-pyridine derivatives were synthesized according to the method outlined in Scheme 2. An aryl group was first introduced at the 3-position of pyridine 1 or 4 via a Suzuki coupling to provide 5 in 65–85% yield. This was followed by a second Suzuki reaction using [(N-Boc)piperazin-1-yl]phenylboronic acid pinacol esters to generate 6, which was deprotected using trifluoroacetic acid (TFA) in dichloromethane at room temperature to give amines 7. Starting material 4 (R = NHMe or NMe2) was prepared by reductive amination of 1 in

![Figure 1. Previously described BMP inhibitors.](image-url)
Here, several functional groups were used as replacements for the 3-phenol (Figure 2a,b), chosen either to mimic hydrogen bonding of the phenol with Asp293 or to introduce an electropositive charge (e.g., a protonated amine) to mediate an ionic interaction with Asp293, thus maintaining a potentially important interaction. To gain insight into the potency and selectivity for BMP vs TGF-β signaling, derivatives were tested for their ability to bind BMP type I receptor ALK2 and TGF-β type I receptor ALKS, using an in vitro thermal shift kinase assay (Figure 2c). This type of assay has been previously shown by us and others to be highly predictive of biochemical kinase inhibition activity,31 which was also measured in a selected subset of the derivatives (Figures 2–5). Tm shift data were found to correlate highly (\( r^2 \geq 0.8 \)) with biochemical inhibition data (Supporting Information, Figure 2). To assess the potency and selectivity of these compounds in cells, inhibition of BMP6-induced transcriptional activity (BRE-Luciferase) and TGF-β1-induced transcriptional activity (CAGA-Luciferase) was measured for each of the compounds (Figure 2c), using cell lines (C2C12 for BMP6 and HEK293T for TGF-β1) previously shown to express a complement of BMP or TGF-β receptors required for ligand-mediated signaling.32 In general, the magnitude of \( \Delta T_m \) for ALK2 and ALKS correlated inversely with the log IC\(_{50} \) for inhibition of BMP and TGF-β1-induced transcriptional activity (Figure 1d) but with some minor exceptions. Notably, K02288 exhibited a large thermal shift for ALK2 kinase protein (\( \Delta T_m = 13.2 \) °C), consistent with potent inhibition of ALK2 activity by biochemical assay (IC\(_{50} = 34 \) nM) but was substantially weaker in the cell-based assay of BMP6 activity (IC\(_{50} = 421 \) nM, Figure 2c). Of the variants at the 3-phenol position, compound 13 exhibited the best in vitro inhibition of ALK2, whereas compound 15 demonstrated the best cell-based activity. The occasional discordance between biochemical (\( \Delta T_m \) and enzymatic) and functional assays (ligand-induced transcription) highlighted the need for multiple assays in an SAR aimed at identifying physiologic probes with useful potency and selectivity.

In addition to altering potency, modifications to the solvent-exposed 3-phenol showed significant alterations in selectivity. Replacing 3-phenol with 4-phenol (11) increased potency against BMP6 signaling by ~20-fold compared to K02288 while retaining a modest degree of selectivity for BMP6 versus TGF-β1 signaling (28-fold, Figure 2c). Adding a 3-methoxy group to the 4-phenol (12) reduced BMP6 inhibition modestly, with similar selectivity. Replacing the 3-phenol with a bioisosteric methylsulfonamide (13) improved BMP6 inhibition compared to K02288 but decreased selectivity. The largest increase in potency occurred with the replacement of the 3-phenol with 3- or 4-phenylpiperazine, as previously done with LDN-193189, likely due to the increased polarity of this

Scheme 2. General Procedure for the Synthesis of Various 2-Substituted 3-Aryl-5-(piperazinylphenyl)pyridine Derivatives

1 or 4 R = Cl, OMe, H, NH₂, NHMe, N(Me)₂

Reagents and conditions: (a) 3-phenylboronic acid, MeCN/DMF, NaN\(_2\)CO₃ (aqueous, 1 M), 10 mol % Pd(PPh₃)₄, 90 °C, 8 h, 65–85%; (b) [(N-Boc)piperazin-1-yl]phenylboronic acid pinacol ester, DME, Na₂CO₃ (aqueous, 1 M), 10 mol % Pd(PPh₃)₄, 90 °C, 8 h, 70–75%; (c) TFA, DCM, rt, 12 h, 100%.

dichloromethane using various amounts of paraformaldehyde in the presence of NaBH(OAc)$_2$.

3-Aryl-5-(4-piperazinylphenyl)pyridine derivatives containing a methyl substituent in the 2-position of the pyridine were prepared using the method outlined in Scheme 3. The 2-chloropyridine 8 was treated with trimethylboroxine in the presence of a palladium catalyst to generate 2-methylpyridine 9 in 90% yield.28 The carbamate protecting group was again removed with TFA to generate 10.

On the basis of several physiochemical properties, including calculated octanol–water partition coefficient (cLogD), distribution coefficient (cLogD₂₅), topological polar surface area (tPSA in Å²), and number of hydrogen bond donors, acceptors (HBD and HBA) and rotatable bonds (Supporting Information, Table 1), the prepared compounds are predicted to be cell permeable and orally absorbed.25,30

SAR of Solvent Exposed Position. We previously demonstrated that K02288 exhibits similar potency to LDN-193189 in biochemical kinase assays for inhibiting ALK2 and related BMP type I receptor kinases but was surprisingly less active in cell-based reporter assays using constitutively active BMP type I receptors.21,23 We speculated that the relatively weaker activity of K02288 in cells might be due to poor solubility or impaired interactions with solvent water molecules that might be addressed via modifications in the solvent-exposed domain. We created six derivatives of K02288 by modifying the 3-phenol substituent, which in the co-crystal structure of ALK2 occupied the solvent-exposed hydrophobic channel at the entrance of the ATP pocket (Supporting Information, Figure 1). Here, several functional groups were used as replacements of the 3-phenol (Figure 2a,b), chosen either to mimic hydrogen bonding of the phenol with Asp293 or to introduce an electropositive charge (e.g., a protonated amine) to mediate an ionic interaction with Asp293, thus maintaining a potentially important interaction. To gain insight into the potency and selectivity for BMP vs TGF-β signaling, derivatives were tested for their ability to bind BMP type I receptor ALK2 and TGF-β type I receptor ALKS, using an in vitro thermal shift kinase assay (Figure 2c). This type of assay has been previously shown by us and others to be highly predictive of biochemical kinase inhibition activity,31 which was also measured in a selected subset of the derivatives (Figures 2–5). Tm shift data were found to correlate highly (\( r^2 \geq 0.8 \)) with biochemical inhibition data (Supporting Information, Figure 2). To assess the potency and selectivity of these compounds in cells, inhibition of BMP6-induced transcriptional activity (BRE-Luciferase) and TGF-β1-induced transcriptional activity (CAGA-Luciferase) was measured for each of the compounds (Figure 2c), using cell lines (C2C12 for BMP6 and HEK293T for TGF-β1) previously shown to express a complement of BMP or TGF-β receptors required for ligand-mediated signaling.32 In general, the magnitude of \( \Delta T_m \) for ALK2 and ALKS correlated inversely with the log IC\(_{50} \) for inhibition of BMP and TGF-β1-induced transcriptional activity (Figure 1d) but with some minor exceptions. Notably, K02288 exhibited a large thermal shift for ALK2 kinase protein (\( \Delta T_m = 13.2 \) °C), consistent with potent inhibition of ALK2 activity by biochemical assay (IC\(_{50} = 34 \) nM) but was substantially weaker in the cell-based assay of BMP6 activity (IC\(_{50} = 421 \) nM, Figure 2c). Of the variants at the 3-phenol position, compound 13 exhibited the best in vitro inhibition of ALK2, whereas compound 15 demonstrated the best cell-based activity. The occasional discordance between biochemical (\( \Delta T_m \) and enzymatic) and functional assays (ligand-induced transcription) highlighted the need for multiple assays in an SAR aimed at identifying physiologic probes with useful potency and selectivity.

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Scheme 3. Synthesis of a 2-Methyl 3-Aryl-5-(piperazinylphenyl)pyridine Derivatives

Reagents and conditions: (a) trimethylboroxine, 1,4-dioxane, K₂CO₃ (2 equiv), 20 mol % Pd(PPh₃)₄, 110 °C, 8 h, 90%; (b) TFA, DCM, rt, 12 h, 100%.
substituent resulting in both improved inhibitor aqueous solubility and increased enthalpic interactions with solvent water molecules. Compounds 14 and 15 demonstrated 70–100-fold increases in BMP6 inhibition (IC$_{50}$ = 6 nM, and 4 nM) compared to K02288, with modest improvements in selectivity. Compound 15 is structurally similar to previously disclosed aminopyridine inhibitors of interleukin-2-inducible T-cell kinase (ITK) and pyridine benzamide inhibitors of protein kinase D (PKD). To further investigate the type I receptor selectivity of 15, cells were transfected with adenoviruses expressing constitutively active BMP type I receptors (caALK1, caALK2, and caALK3) and constitutively active activin or TGF-$\beta$ type I receptors (caALK4 and caALK5) in low serum conditions and in the absence of exogenous ligand (Supporting Information, Figure 3). Derivative 15 was most potent against BMP receptors caALK2 and caALK3 with IC$_{50}$ measurements of $\sim$3.5 nM, whereas the activin/TGF-$\beta$ type I receptors and caALK1 were inhibited with an IC$_{50}$ measurements of $\sim$20 nM, with nearly complete extinction of BMP and TGF-$\beta$ type I receptor signaling at approximately 250 nM. Taken together, these data demonstrate that replacing the 3-phenol in the solvent exposed region of K02288 with 4-phenylpiperazine dramatically improved its potency in cells but with relatively poor selectivity for BMP versus TGF-$\beta$ signaling. These results prompted us to explore structural variants at other positions that might refine selectivity while retaining gains in potency afforded by modification of the solvent-exposed 3-phenol with 4-phenylpiperazine in 15.

SAR of Hydrophobic Pocket Position. Further modifications of potent compound 15 were performed to develop an SAR for the 3,4,5-trimethoxyphenyl group (R$_2$) (Figure 3a,b) to identify the role of each methoxy group on potency and selectivity. The trimethoxyphenyl group has previously been shown to interact with the hydrophobic back pocket in ALK2 where it forms water-mediated hydrogen bonds with the catalytic lysine residue (K235) (Supporting Information, Figure 1). Compounds were again probed in thermal shift, biochemical enzyme inhibition, and cell-based luciferase reporter assays (Figure 3b,c). Removal of any of the methoxy groups resulted in a significant decrease in BMP inhibition. However, particular methoxy groups were more crucial than others. For example, removing one of the 3-methoxy groups (16) resulted in a 35-fold loss in potency compared to 15, although selectivity for BMP6 inhibition versus TGF-$\beta$ increased. Removing the 4-methoxy group (17) decreased activity 10-fold but did not improve selectivity. Combining these changes (18) demonstrated that the 4-methoxy group contributed less significantly to BMP6 inhibition as compared to either meta-methoxy group. As expected, removal of both meta-methoxy groups, while
retaining the para-methoxy group (20) drastically reduced potency by almost 500-fold. Incorporating the 3,4-dimethoxy groups into a benzo-1,4-dioxane (19) resulted in decreased activity compared to 16, perhaps reflecting disruption of the hydrogen bond with ALK2 residue K235. In addition, increasing the steric bulk of the 3-alkoxy group (21) or replacing the 4-methoxy with a chlorine (22) or a methyl (23) were also not productive. In conclusion, of the compounds studied the 3,4,5-trimethoxyphenyl group resulted in the best balance between BMP6 inhibition and selectivity over TGF-β.

This is likely due to its greater molecular volume for occupying the hydrophobic pocket in ALK2 while retaining hydrogen bond acceptors in the meta-positions of the phenyl ring. Future studies will seek to optimize binding in the hydrophobic pocket by replacing the 3,4,5-trimethoxyphenyl entirely with a diverse set of aryl and heteroaryl moieties. **SAR of Hinge Binding Position.** To further explore the SAR of 15, we modified the primary amine residue of the 2-aminopyridine (R₃, Figure 4a,b), a region that was previously shown to interact with the hinge region of the ALK2 kinase domain. Here, the amine was within hydrogen bonding distance to the backbone carbonyl of H284, as well as the gatekeeper residue T283 of ALK2 (Supporting Information, Figure 1). Both residues are changed in ALK5 (D281 and S280, respectively). Secondary and tertiary amines such as 24 and 25, respectively, exhibited reduced potency in both the thermal shift and cell-based assays. Similarly, 28, in which the primary amine is replaced with a methoxy substituent, exhibited decreased potency, suggesting that bulky substituents are not well tolerated at this position. Notably, these compounds exhibited negligible thermal shift despite detectable albeit low activity in cell-based assays (Figure 4c). Despite the high degree of correlation between the thermal shift and cell-based assays (Supporting Information, Figure 4), compounds that exhibit very low ΔTₘ may exhibit measurable inhibition in cells at moderately high concentrations, potentially due to cytotoxicity.

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<th>ALK5 ΔTₘ(°C)</th>
<th>ΔTₘ Diff.</th>
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Figure 3. Potency and selectivity of compound 15 derivatives based on thermal shift, biochemical kinase activity, and ligand induced transcriptional activity assays. (a) The 2-aminopyridine scaffold of 15. (b) Modifications to the ATP-binding pocket hydrophobic domain (R₂) of compound 15. (c) Thermal shift (ΔTₘ), biochemical enzymatic inhibition (IC₅₀) for ALK2 and ALK5 kinase proteins, and inhibition of cell-based BMP6 and TGF-β1-induced transcriptional activity (IC₅₀) by compound 15 derivatives (nd = not determined). (d) Correlation of thermal shift and cell-based BMP/TGF-β inhibition assays.

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at high concentrations (>50 μM, Supporting Information, Figure 5). Replacing the primary amine with hydrogen (26) resulted in only a modest decline in potency and significantly increased selectivity for BMP6 versus TGF-β signaling, suggesting that the primary amine hydrogen bond to the hinge backbone is more critical for binding to ALK5 (D281) than ALK2 (H284). Finally, we used two other small substituents, e.g., chlorine (27) and methyl (10) groups to explore the possibility that ALK2 is less sensitive than ALK5 to substituents at this position. Although both compounds lost potency relative to 26, there was a significant increase in selectivity for BMP over TGF-β signaling, with both showing greater than 150-fold selectivity in cell-based assays. In particular, 10 remained relatively potent with a biochemical IC50 of 24 nM for ALK2, a cell-based IC50 for BMP6 of approximately 100 nM, and 164-fold selectivity for BMP6 versus TGF-β1. The activities of compounds 15, 26, and 10 in the thermal shift kinase assay as well as two different cell-based assays (ligand induced transcription and constitutively active type I receptor transcriptional activity) are summarized in Supporting Information, Table 2. In each of the various assays, compound 10 (LDN-214117) exhibited improved selectivity for ALK2 over ALK5, consistent with a high degree of selectivity for BMP versus TGF-β signaling. In fact, when the activity of 10 was measured against closely related BMP type I receptors ALK1−3 via kinase assays, it appeared to inhibit ALK2 more potently than ALK3 by more than 40-fold (Figure 6a), a degree of selectivity which rivals previously reported compound LDN-212854.21 We tested whether or not compounds with improved receptor selectivity such as 10 or 26 might also exhibit more selective inhibition of BMP ligands.

We found that both of these compounds impacted the transcriptional activity of a BMP-responsive luciferase reporter (BRE-Luc) in response to BMP6, a known ALK2 ligand, more potently than that of BMP2 or BMP4, classic ligands of ALK3 (Figure 6b−d).35 In fact, compound 10 inhibited BMP6 preferentially to BMP2 and BMP4 by approximately 10-fold, whereas K02288 and compound 15 inhibited BMP2, -4, and -6 with equal potency. These data support the concept that increased selectivity of 10 and 26 for ALK2 translates into increased selectivity for the activity of ligands which signal primarily through ALK2, a class which include BMP6 and BMP7.36 These results further highlight that the 2-position of the pyridine in the K02288 series can be exploited to achieve reasonably potent and highly selective BMP inhibitors, presumably via optimization of hinge domain interactions.

SAR of K02288 and LDN-193189 Hybrid Molecules. We previously described a highly selective pyrazolo[1,5-a]-pyrimidine based BMP type I receptor kinase inhibitor LDN-212854 that exhibited biased activity for ALK2. This selectivity was achieved by a 5-quinoline moiety that interacts with the same hydrophobic pocket as the 3,4,5-trimethoxy group of K02288. With this in mind, we synthesized several derivatives of 15 that replaced the 3,4,5-trimethoxyphenyl with 4- or 5-
喹啉（图5a）。如预期，5-喹啉（31）显著地提高了对BMP与TGF-β抑制的选择性（图5b）。然而，所有这些化合物的效力都远远低于15。模型这些5-喹啉的替代化合物在ATP结合口袋中表明，通过2-氨基吡啶模板约束的喹啉基团可能在替代的pyrazolo[1,5-a]pyrimidine模板中处于不太优的位置（补充信息，图6）。相反，用LDN-212854的5-喹啉部分替代K02288的3,4,5-三甲氧基苯基形成32，其对BMP6抑制活性具有潜在性，但选择性较低。最后，我们假设两个单独的改变可能具有协同效应，我们结合了2-氨基与氢气和3,5-二甲氧基的替换，分别在26和17中形成33。虽然这个分子在整体选择性上已有显著改进，但它效力不大。

图5. K02288衍生物的效力和选择性基于热移位、生物化学激酶活性和配体诱导的转录活性检测。 (a) 杂合衍生物的结构。 (b) 热移位（△Tm）、生物化学激酶抑制（IC50）和细胞基质BMP6和TGF-β1诱导的转录活性抑制（IC50）（未确定）。 (c) 热移位和细胞基质BMP/TGF-β抑制活性检测的相关性。
which could manifest as differences in affinity for ATP and altered $K_m$ with implications for their cellular activity and susceptibility to inhibitors. We tested this directly by measuring the $K_m$ for ATP of wild-type ALK2 and four FOP-causing ALK2 mutants (L196P, Q207E, G328E, and R258S). The $K_m$ values for wild type and mutant ALK2 were between 16 and 48 μM (Supporting Information, Table 4). Importantly, none of the FOP-causing mutants exhibited enhanced affinity for ATP as compared with wild-type ALK2. Because ATP concentrations within cells vary from $1^{-10}$ mM,42 far in excess of the calculated $K_m$ values, these slight differences in $K_m$ would likely be inconsequential in cells.

A related, long-standing, and clinically relevant question in the FOP field has been whether mutant ALK2 proteins might exhibit differential inhibition by, or distinct affinity for, particular kinase inhibitors, and if so, whether highly specific inhibitors could be engineered to target selectively the activity of these activated mutant proteins. We sought to answer this question by probing a panel of seven representative mutant ALK2 proteins with the library of K02288 derivatives displaying varying potency against wild-type ALK2 using a thermal shift kinase assay. We found a highly linear correlation ($r^2 = 0.94^{-0.99}$) between the thermal shift induced by these derivatives with wild-type vs mutant ALK2 proteins (Figure 8).

Figure 6. LDN-214117 exhibits selectivity for ALK2-mediated BMP signaling. (a) 10 (LDN-214117) demonstrates selective inhibition of ALK2 and ALK1 in preference to ALK3 kinase activity. (b) In a cell-based assay measuring BMP-mediated transcription (BRE-Luciferase), K02288 (b) and 15 (c) exhibit relatively limited selectivity for diverse BMP ligands, whereas 26 (d) and 10 (e) exhibit relatively selective inhibition of BMP6 versus BMP2 or BMP4, consistent with selective inhibition of ALK2- versus ALK3-mediated signaling, respectively.
These results suggest that inhibitors engineered or identified against wild-type or mutant ALK2 proteins will have interchangeable activity against diverse mutant proteins found in FOP or DIPG. Conversely, these results would preclude the development of ATP-competitive ALK2 inhibitors which selectively target mutant proteins. The pathogenicity of activating mutations of ACVR1 has been well-established for FOP, and thus the development of highly selective inhibitors is an important step in validating ALK2 as a feasible clinical target. While the presence of ACVR1 mutations appears to be associated with increased downstream BMP signaling and transcriptional activity in DIPG tumors, further work will need to be performed to determine whether or not these mutations contribute to the initiation or progression of tumors in DIPG. The identification of selective inhibitors of ALK2 may help to elucidate the mechanisms of DIPG tumorigenicity and could potentially be therapeutic if a contribution of BMP signaling is confirmed.

Cytotoxicity of Kinase Inhibitors. We next sought to determine the cytotoxicity of these derivatives and to compare them to many of the current FDA approved kinase inhibitors (Figure 9). Because hepatotoxicity is one of the most common reasons for withdrawal of approved drugs, we used HEPG2 cells for evaluation of cytotoxicity. Compounds were tested in a large concentration range (1−100 μM) for 4 and 24 h. Upon the basis of residual cell viability after treatment, compounds were categorized as having low (>75%), medium (25−75%), or high (<25%) cytotoxicity. Of the 12 approved kinase inhibitors tested, only one exhibited high cytotoxicity at 100 μM after 4 h of incubation, whereas six of the 28 derivatives in our K02288 library exhibited high cytotoxicity after 4 h. Over a 24 h period four of the 12 approved kinase inhibitors tested, only one exhibited high cytotoxicity at 100 μM after 4 h of incubation, whereas six of the 28 derivatives demonstrated good potency and high BMP selectivity, exhibited very low cytotoxicity. In fact, cytotoxicity was not correlated with BMP signaling inhibition, TGF-β inhibition, nor selectivity for BMP versus TGF-β signaling (Supporting Information, Figure 5). For example, the highly potent BMP inhibitor 11 was also nontoxic, suggesting that the mechanisms of cytotoxicity within this series of compounds do not result from effects on BMP or TGF-β signaling.

Figure 7. Compound 10 exhibits increased kinome selectivity. Kinome dendrogram plot for compound 15 (LDN-212838) (a) and compound 10 (LDN-214117) (b) showing an improved selectivity profile for 10 for BMP type I receptor kinases.

Figure 8. FOP-causing ALK2 mutations do not affect inhibitor binding. (a) Strong correlation of thermal shift data for ATP competitive kinase inhibitors binding to wild-type ALK2 versus known FOP causing GS-domain mutations of ALK2 and (b) known FOP causing kinase domain mutations suggests the potency of ATP competitive inhibitors are not affected by these disease causing mutations. m = slope, R² = correlation coefficient.
Structural Basis of Inhibitor Binding. A number of the most promising derivatives were tested for co-crystallization with ALK2 to further understand the binding mode and SAR. Diffraction quality crystals were obtained in the presence of 26, and the structure of the complex was solved at 2.6 Å resolution (Figure 9; see Supporting Information, Table 5 for data collection and refinement statistics).

In the co-crystal structure, 26 was bound to the kinase hinge region as shown previously for the parent molecule K02288 (Figure 10). Both molecules established an ATP-mimetic hydrogen bond between the pyridine nitrogen and the amide of H286. Replacement of the 3-phenol and primary amine with 4-phenylpiperazine and hydrogen, respectively, did not alter the overall position of 26 but resulted in the loss of the hinge hydrogen bond interaction between the primary amine and the carbonyl of H284. The conserved 3,4,5-trimethoxyphenyl provided hydrophobic interaction as well as a water-mediated hydrogen bond to the catalytic lysine K235. Docking of 10 produced a similar binding mode, with no significant change resulting from the introduction of the methyl group. Overall, the ATP pocket occupied by these 3,5-diarylpyridines was similar to the pyrazolo[1,5-a]pyrimidine scaffold of LDN-193189. However, the two series differed slightly in their hinge binding orientation resulting in shifts in the position of their respective hydrophobic pocket groups as well as the shared 4-phenylpiperazine (Supporting Information, Figure 6).

The selectivity of these molecules for ALK2 over ALK5 likely results from dynamic conformational differences between these kinases as well as the modest number of sequence changes in the ATP pocket. Perhaps as a result of its smaller serine gatekeeper residue, the ATP pocket in many ALK5 co-crystal structures shows a more open conformation than those of ALK2 with a noticeable movement of the N-lobe away from the C-lobe (Supporting Information, Figure 1). Such conformational differences are expected to change the shape, volume, and dynamics of the ATP pocket to impact inhibitor binding.

**CONCLUSION**

We have developed a library of BMP receptor kinase inhibitors based on the 2-aminopyridine scaffold of K02288. This library allowed us to explore the SAR of various functional groups and resulted in the creation of several potent derivatives. Several of these compounds demonstrated improved activity, selectivity, or both, measured using a thermal shift assay, an enzymatic assay, and cellular assays of BMP/TGF-β-induced transcription,
thus overcoming the limited potency of the parent compound in cells. We determined that the solvent-exposed 3-phenol substituent of K02288 was responsible for its unexpectedly low activity in cells as compared to kinase assay IC_{50}. By replacing this group with either a 4-phenol or 4-phenylpiperezine, we were able to improve potency in cellular assays compared to K02288 by 20- and 100-fold, respectively. We previously reported that the 3,4,5-trimethoxyphenyl occupies the rear hydrophobic pocket to provide excellent shape complementarity and forms water-mediated hydrogen bonds to the catalytic lysine residue (K235).

Here we found that the 4-methoxy group was largely dispensable, while the 3- or 5-methoxy groups were more critical for maintaining potency. The balance of selectivity and potency found in the 3,5-dimethoxy derivative 17 suggests further medicinal chemistry optimization is possible and could yield further insights into the determinants of activity in the hydrophobic pocket. Within the 2-aminoypyridinone core, we found that the primary amine was more critical for TGF-β than BMP binding affinity and could be replaced with a nonpolar methyl group to generate a highly BMP selective compound 10 (LDN-214117), which is significantly biased toward ALK2 and its cognate ligands including BMP6 and also demonstrates a high degree of kinome selectivity and low cytotoxicity. Finally, we concluded that replacing the 3,4,5-trimethoxyphenyl with quinolines as previously described for cyclohexane/EtOAc to give products 3.

**General Synthesis of 2-Amino-5-aryl-3-(3,4,5-trimethoxyphenyl)-pyridine (2).** A mixture of 5-bromo-3-iodopyridin-2-amine (386 mg, 1.30 mmol), 3,4,5-trimethoxyphenylboronic acid (275 mg, 1.30 mmol), and Pd(PPh3)4 (180 mg, 0.156 mmol) were added to a sealed tube. The tube was evacuated and backfilled with argon (3 cycles). Acetonitrile (6.0 mL) and DMF (2.5 mL) were added by syringe at room temperature, followed by (1 M) aqueous Na2CO3 (2.6 mL, 260 mmol). After being stirred at 90 °C for about 8 h, the reaction mixture was filtered and concentrated. The residue was purified by flash column chromatography to give 2 as white solid (325 mg, 80%). 

**General Synthesis of 3-(6-Amino-5-(3,4,5-trimethoxyphenyl)pyridin-3-yl)phenol (K02288).** Yield: 40%. 1H NMR (500 MHz, CDCl3) δ 8.48 (d, J = 2.0 Hz, 1H), 7.69 (d, J = 2.0 Hz, 1H), 7.34 (t, J = 7.5 Hz, 1H), 7.20 (d, J = 2.0 Hz, 1H), 7.08 (d, J = 8.0 Hz, 1H), 6.90 (d, J = 2.0, 7.0 Hz, 1H), 6.68 (s, 2H), 4.81 (br, 2H), 3.91 (s, 3H), 3.89 (s, 6H). MS (ESI) calcd for C21H24N3O5S 430.1437 [M + H]+; found 430.1412; HRMS (ESI) calcd for C21H24N3O5S 430.1437 [M + H]+; found 430.1412; purity 95.6% (t, 1.35 min).

**General Synthesis of 2-Amino-5-aryl-3-(3,4,5-trimethoxyphenyl)-pyridines (3).** To a solution of 2 (1.0 equiv), an aryl boronic acid (1.1 equiv), and Pd(PPh3)4 (0.12 equiv) in DME, (1 M) aqueous Na2CO3 (2.0 equiv) was added. The reaction mixture was stirred under an argon atmosphere at 90 °C for 8 h. The reaction mixture was filtered and then concentrated. The residue was purified by flash column chromatography, eluting with a mixture of cyclohexane/ EtOAc to give products 3.

**General Synthesis of 3-(6-Amino-5-(3,4,5-trimethoxyphenyl)pyridin-3-yl)phenol (11).** Yield: 42%. 1H NMR (500 MHz, CDCl3) δ 8.27 (d, J = 2.5 Hz, 1H), 7.57 (d, J = 2.5 Hz, 1H), 7.43–7.41 (m, 2H), 6.92–6.90 (m, 2H), 6.90 (d, J = 2.0, 7.0 Hz, 1H), 6.69 (s, 2H), 4.64 (br, 2H), 3.91 (s, 3H), 3.89 (s, 6H). MS (ESI) calcd for C20H21N2O4 353.1501 [M + H]+; found 353.1462; purity 95.6% (t, 1.35 min).

**General Synthesis of 3-(6-Amino-5-(3,4,5-trimethoxyphenyl)pyridin-3-yl)-2-methoxyphenol (12).** Yield: 70%. 1H NMR (500 MHz, CDCl3) δ 8.27 (d, J = 2.5 Hz, 1H), 7.56 (d, J = 2.5 Hz, 1H), 7.06–6.98 (m, 3H), 6.70 (s, 2H), 4.65 (br, 2H), 3.95 (s, 3H), 3.91 (s, 3H), 3.89 (s, 6H). HRMS (ESI) calcd for C15H15N2O4 347.1052 [M + H]+; found 347.1049; purity 100.0% (t, 1.32 min).

**General Synthesis of 3-(6-Amino-5-(3,4,5-trimethoxyphenyl)pyridin-3-yl)-2-methoxyphenol (13).** Yield: 85%. 1H NMR (500 MHz, CDCl3) δ 8.89 (br, 1H), 8.39 (d, J = 2.5 Hz, 1H), 7.61 (d, J = 1.5 Hz, 1H), 7.47–7.40 (m, 3H), 7.34 (dt, J = 1.5, 7.5 Hz, 1H), 6.68 (s, 2H), 5.19 (br, 2H), 3.91 (s, 3H), 3.90 (s, 6H). MS (ESI) calcd for C16H15N2O4 363.1607 [M + H]+; found 363.1603; purity 98.3% (t, 1.34 min).

**General Synthesis of 3-Aryl-5-bromopyridines (5).** A mixture of 5-bromo-3-iodopyridin-2-amine (1.0 equiv), arylboronic acid (1.0 equiv), and Pd(PPh3)4 (0.12 equiv) was added to a sealed tube. The tube was evacuated and backfilled with argon (3 cycles). Acetonitrile and DMF (3:1 mL) were added by syringe at room temperature, followed by (1 M) aqueous Na2CO3 (2.0 equiv). After being stirred at 90 °C for about 8 h, the reaction mixture was filtered and concentrated. The residue was purified by flash column chromatography, eluting with a mixture of cyclohexane/EtOAc to give products 5.

**General Synthesis of 3-Aryl-5-bromopyridines (6).** To a solution of 5 (1.0 equiv), [N-Boc]piperazinylphenylboronic acid pinacol ester (1.1 equiv), and Pd(PPh3)4 (0.12 equiv) in DME, (1 M) aqueous Na2CO3 (2.0 equiv) was added. The reaction mixture was stirred under argon atmosphere at 90 °C for 8 h. The reaction mixture was filtered and concentrated. The residue was purified by flash column chromatography, eluting with a mixture of cyclohexane/EtOAc to give 6.

## EXPERIMENTAL SECTION

**Chemistry Material and Methods.** Unless otherwise noted, all reagents and solvents were purchased from commercial sources and used without further purification. The NMR spectra were obtained using a 300 or 500 MHz spectrometer. All 1H NMR spectra are reported in δ ppm units (ppm) and were recorded in CDCl3 and referenced to the peak for tetramethylsilane (TMS) or in DMSO. Coupling constants (J) are reported in hertz. Column chromatography was performed utilizing a CombiFlash 5g 100cc separation system with RediSep disposable silica gel columns. High-resolution mass spectra were obtained by using AccuTOF with a DART source. All test compounds reported in this manuscript had a purity ≥ 95% as determined by high-performance liquid chromatography (HPLC).
**General Synthesis of 3-Aryl-5-(piperazinyl)phenylpyridines**

(7) To a stirring solution of the 6 (0.01 mmol) in dry CHCl₃ (2 mL) at 0 °C, trifluoroacetic acid (0.2 mL) was slowly added and the reaction mixture was stirred overnight at room temperature. The mixture was concentrated under vacuum. The residue was suspended in ethyl acetate (10 mL), and then a saturated NaHCO₃ solution was added to adjust the pH to 7 at 0 °C. The mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic layer was dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The remaining residue was subjected to column chromatography to furnish 7 as a white to light-yellow foam.

![Image of 3-Aryl-5-(piperazinyl)phenylpyridine](image)

**N-Methyl-5-(4-(piperazin-1-yl)phenyl)-3-(4,5-trimethoxyphenyl)pyridin-2-amine** (24). Yield: 92%. H NMR (500 MHz, CDCl₃) δ 8.39 (d, J = 2.0 Hz, 1H), 7.50 (d, J = 2.5 Hz, 1H), 7.46−7.45 (m, 2H), 7.00−6.98 (m, 2H), 6.63 (s, 2H), 4.67 (q, J = 5.0 Hz, 1H), 3.91 (s, 3H), 3.88 (s, 6H), 3.25−3.32 (m, 0.6Hz) due to rotamer, 3.20−3.18 (m, 3.4Hz) due to rotamer, 3.08−3.06 (m, 3.6Hz) due to rotamer, 2.72−2.70 (m, 0.6Hz) due to rotamer. HRMS (ESI) calcd for C₂₃H₂₇N₄O₂ 391.2185 [M + H]+; found 391.2189; purity 100.0% (tₕ 1.16 min).

**5-(3-Piperazin-1-yl)phenyl-3-(4,5-trimethoxyphenyl)pyridin-2-amine** (24). Yield: 92%. H NMR (500 MHz, CDCl₃) δ 8.28 (d, J = 2.4 Hz, 1H), 7.58 (d, J = 2.4 Hz, 1H), 7.47−7.44 (m, 2H), 7.24−7.21 (m, 1H), 7.00−6.97 (m, 3H), 6.93 (d, J = 1.5 Hz, 1H), 4.65 (br, 2H), 3.85 (s, 3H), 3.20−3.16 (m, 4H), 3.07−3.03 (m, 4H), 2.67 (s, 2H). HRMS (ESI) calcd for C₂₃H₂₇N₄O₂ 385.2185 [M + H]+; found 385.2189; purity 100.0% (tₕ 1.18 min).

**3-(4-Chloro-3-methoxyphenyl)-5-(4-(piperazin-1-yl)phenyl)pyridin-2-amine** (22). Yield: 82%. 1H NMR (300 MHz, CDCl₃) δ 8.27 (d, J = 2.1 Hz, 1H), 7.57 (d, J = 2.4 Hz, 1H), 7.48−7.44 (m, 3H), 7.04−6.98 (m, 4H), 4.83 (br, 2H), 3.94 (s, 3H), 3.32−3.29 (m, 3.6Hz) and 3.26−3.22 (m, 0.4Hz) due to rotamer, 3.18−3.15 (m, 3.6Hz) and 2.74−2.69 (m, 0.4Hz) due to rotamer. HRMS (ESI) calcd for C₂₃H₂₇ClNO₃ 395.1639 [M + H]+; found 395.1647; purity 96.6% (tₕ 1.18 min).

**3-(Methoxy-4-methylphenyl)-5-(4-(piperazin-1-yl)phenyl)pyridin-2-amine** (23). Yield: 84%. 1H NMR (300 MHz, CDCl₃) δ 8.28 (d, J = 2.4 Hz, 1H), 7.58 (d, J = 2.4 Hz, 1H), 7.47−7.44 (m, 2H), 7.24−7.21 (m, 1H), 7.00−6.97 (m, 3H), 6.93 (d, J = 1.5 Hz, 1H), 4.65 (br, 2H), 3.85 (s, 3H), 3.20−3.16 (m, 4H), 3.07−3.03 (m, 4H), 2.67 (s, 2H). HRMS (ESI) calcd for C₂₃H₂₇NO₃ 375.2185 [M + H]+; found 375.2189; purity 100.0% (tₕ 1.16 min).
5-(4-(Piperazin-1-yl)phenyl)-3-(quinolin-5-yl)pyridin-2-amine (31). Yield: 84%. \(^\d\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.98 (dd, \(J = 2.0, 4.5\) Hz, 1H), 8.44 (d, \(J = 2.5\) Hz, 1H), 8.21 (d, \(J = 9.0\) Hz, 1H), 8.06–8.04 (m, 1H), 7.84–7.81 (m, 1H), 7.63 (d, \(J = 2.5\) Hz, 1H), 7.59–7.58 (m, 1H), 7.46–7.46 (m, 2H), 7.41–7.39 (m, 1H), 7.00–6.99 (m, 2H), 4.29 (br, 2 H), 3.25–3.23 (m, 0.4H) and 3.20–3.18 (m, 3.6H) due to rotamer. 3.06–3.04 (m, 3.6H) and 2.71–2.69 (m, 0.4H) due to rotamer. HRMS (ESI) calc. for C\(_{24}\)H\(_{24}\)N\(_5\) 382.2032 [M + H]+; found 382.2039; purity 95.2% (tq, 0.97 min).

1-(4-(5-(3,5-Dimethoxyphenyl)pyridin-3-yl)-pyridin-2-yl) piperazine (33). Yield: 98%. \(^\d\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 8.79 (d, \(J = 2.4\) Hz, 1H), 8.73 (d, \(J = 2.4\) Hz, 1H), 7.99 (t, \(J = 2.1\) Hz, 1H), 7.57–7.54 (m, 2H), 7.04–7.02 (m, 2H), 6.70 (d, \(J = 1.8\) Hz, 2H), 6.53 (t, \(J = 2.1\) Hz, 1H), 3.86 (s, 6H), 3.32–3.31 (m, 4H), 3.06–3.04 (m, 4H). HRMS (ESI) calc. for C\(_{23}\)H\(_{26}\)N\(_3\)O\(_2\) 376.2023; purity 100.0% (tq, 1.26 min).

Synthesis of 1-(4-(6-Methyl-3-(3,4,5-trimethoxyphenyl)- pyridin-3-yl)piperazine (10). A mixture of N-Boc-4-(4-(6-chloro-5-(3,4,5-trimethoxyphenyl)pyridin-3-yl)piperazin-4-yl) piperazine (43 mg, 0.080 mmol), trimethylboroxine (46 \(\mu\)L, 0.32 mmol), Pd(PPh\(_3\))\(_4\) (19 mg, 0.016 mmol) and K\(_2\)CO\(_3\) (22 mg, 0.16 mmol) were added to a sealed tube. The tube was evacuated and back filled with argon (3 cycles). 1,4-Dioxane (1.0 mL) was added by syringe at room temperature. The mixture was stirred for 10 min and a column chromatography was performed on silica gel and the analytically pure residue purified by flash column chromatography to give 9 (40 mg, 96%). \(^\d\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 8.70 (d, \(J = 2.4\) Hz, 1H), 7.69 (d, \(J = 2.4\) Hz, 1H), 7.54–7.51 (m, 2H), 7.02–6.99 (m, 2H), 6.55 (s, 2H), 3.91 (s, 3H), 3.88 (s, 6H), 3.61–3.58 (m, 4H), 3.21–3.18 (m, 4H), 2.55 (s, 3H), 1.48 (s, 9H). MS (ESI): 519.5 [M]+. The carbamate protecting group of 3.61 was also removed using the general method previously described using TFA to furnish 10 (49 mg, 93%). \(^\d\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.77 (s, 1H), 7.03–7.00 (m, 2H), 6.56 (s, 2H), 3.92 (s, 3H), 3.89 (s, 6H), 3.24–3.20 (m, 4H), 3.08–3.05 (m, 4H), 2.55 (s, 3H). HRMS (ESI) calc. for C\(_{24}\)H\(_{30}\)N\(_3\)O\(_3\) 420.2287 [M + H]+; found 420.2285; purity 100% (tq, 1.13 min).

Synthesis of 3-(3,4,5-Trimethoxyphenyl)-6-(4-(1- piperazinyl)phenyl)piprazol[1,5-alpyrimidine (32). This compound was prepared using a reported methodology. \(^\d\)H NMR (500 MHz, DMSO) \(\delta\) 9.38 (d, \(J = 2.0\) Hz, 1H), 9.04 (d, \(J = 2.0\) Hz, 1H), 8.80 (s, 1H), 7.75 (d, \(J = 9.0\) Hz, 2H), 7.51 (s, 2H), 7.07 (d, \(J = 9.0\) Hz, 1H), 3.88 (s, 6H), 3.70 (s, 3H), 3.25–3.18 (m, 4H), 2.92–2.90 (m, 4H). HRMS (ESI) calc. for C\(_{26}\)H\(_{36}\)N\(_4\)O\(_2\) 446.2192 [M + H]+; found 446.2188; purity 100% (tq, 1.45 min).

Thermal Shift Kinase Assay. Thermal melting experiments were performed using a Real Time PCR machine Mx3005p (Stratagene) with a protein concentration of 1 \(\mu\)M for the determination of \(K_m\) and \(V_{\text{max}}\) for each ALK2 mutant. Positive control samples lacking inhibitor compounds, and negative controls lacking recombinant kinase, were also measured in triplicate. The mixture was reacted at RT for 45 min, quenched with a final concentration of 2% phosphoric acid. The reaction mixture was transferred to 96-well P81 phosphocellulose filter plates (Millipore) and bound for 5 min. The plates were washed 20 times with 150 \(\mu\)L of 1% phosphoric acid solution per well by vacuum manifold. Plates were dried at RT for 1 h, sealed, and assayed with Microscint 20 scintillation fluid (PerkinElmer) using a Spectramax L luminometer ( Molecular Devices) using the photon counting setting with an integration time of one second per well. Data was normalized to positive controls at 100% enzyme activity, with negative controls being subtracted as background. GraphPad (Prism software) was used for graphing and regression analysis by sigmoidal dose–response with variable Hill coefficient, or by Michaelis–Menten analysis for the determination of \(K_m\) and \(V_{\text{max}}\).

Luciferase Reporter Assay. C2C12 myoblasts cells stably transfected with BMP responsive element from the Id1 promoter fused to luciferase reporter gene (BRE-Luc) were generously provided by Dr. Peter ten Dijke (Leiden University Medical Center, NL). Human embryonic kidney 293T cells stably transfected with the TGF-\(\beta\) responsive element from the PAI-1 promoter fused to luciferase reporter gene (CAGA-Luc) were generously provided by Dr. Howard Weiner (Brigham and Women’s Hospital, Boston, MA). C2C12 Bre-Luc and 293T CAGA-Luc cells were seeded in DMEM supplemented with 2% FBS at 20000 cells per well in tissue culture treated 96-well plates (Costar 3610; Corning). The cells were incubated for 1 h (37 °C and 10% CO\(_2\)) and allowed to settle and attach. Compounds of interest or DMso were diluted in DMEM and added at final compound concentrations of 1 mM to 10 \(\mu\)M. Cells were then incubated for 30 min. Adenosine expressing constitutively active BMP and TGF-\(\beta\) type I receptors (Ad.cAALK1–S), generously provided by Dr. Akiko Hata (University of California at San Francisco), were added to achieve a multiplicity of infection (MOI) of 100. Plates were incubated overnight at 37 °C. Cell viability was assayed with an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Promega) per the manufacturer’s instructions. Media was discarded, and firefly luciferase activity was measured (Promega) according to manufacturer’s protocol. Light output was measured using a Spectramax L luminometer ( Molecular Devices) with an...
integration time of one second per well. Data was normalized to 100% of incremental BRE-Luc activity due to adenoviruses specifying caALK1,-2, or -3, or the incremental CAGA-Luc activity due to adenoviruses specifying caALK4 or -5. Graphing and regression analysis by sigmoidal dose–response with variable Hill coefficient was performed using GraphPad Prism software.

Cell Viability Assay. HepG2 hepatocarcinoma cells were seeded in DMEM supplemented with 10% FBS to 25,000 cells per well in tissue culture treated 96-well plates (Costar 3610; Corning). The cells were incubated for 2 h (37 °C and 5% CO2) and allowed to settle and attach. Compounds of interest or DMSO were diluted in DMEM and added at final compound concentrations of 1, 10, and 100 μM. Cells were incubated for 4 and 24 h, after which the media was discarded. Cells were lysed by adding 30 μL of passive lysis buffer (Promega) and shaken at RT for 15 min. Cell viability was determined by quantifying the ATP present in each well by adding 10 μL of Cell Titer Glo (Promega) per well and measuring the light output Spectramax L luminometer (Molecular Devices) with an integration time of one second per well. Data was normalized to 100% viability for cells receiving only DMSO without any concurrent compound.

**ASSOCIATED CONTENT**

Additional data describing the cell-based activity of 15, the correlation of IC50 and cytotoxicity, the docking model of 10, diffraction data and refinement statistics, kinome selectivity data, and calculated physiochemical properties. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

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**Author Contributions**

A.H.M. and Y.W. contributed equally. Compounds were conceived by A.H.M., Y.W., S.C., G.D.C., and P.B.Y. Compounds were synthesized by Y.W., S.C., and X.X. Biological experiments were conceived and designed by A.H.M., C.E.S., A.N.B., G.D.C., and P.B.Y. These experiments were conducted by A.H.M. Crystallography experiments were conducted by C.E.S. and P.C. The manuscript was written and revised by A.H.M., Y.W., C.E.S., A.N.B., G.D.C., and P.B.Y.

**Notes**

Brigham and Women’s Hospital has applied for patents related to the use of these compounds for the treatment of FOP, DIPG and other disorders, and inventors (A.H.M., G.D.C., P.B.Y.) may be entitled to royalties. LDN-214117 will be available from Sigma-Aldrich (product SML1119) under a non-exclusive license for research use only.

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**ABBREVIATIONS USED**

BMP, bone morphogenetic protein; TGF-β, transforming growth factor β; FOP, fibrodysplasia ossificans progressive

**REFERENCES**


