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Structural basis for the potent and selective binding of LDN-212854 to the BMP receptor kinase ALK2

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ABSTRACT

Individuals with the rare developmental disorder fibrodysplasia ossificans progressiva (FOP) experience disabling heterotopic ossification caused by a gain of function mutation in the intracellular region of the BMP type I receptor kinase ALK2, encoded by the gene *ACVR1*. Small molecule BMP type I receptor inhibitors that block this ossification in FOP mouse models have been derived from the pyrazolo[1,5-*a*]pyrimidine scaffold of dorsomorphin. While the first derivative LDN-193189 exhibited pan inhibition of BMP receptors, the more recent compound LDN-212854 has shown increased selectivity for ALK2. Here we solved the crystal structure of ALK2 in complex with LDN-212854 to define how its binding interactions compare to previously reported BMP and TGFβ receptor inhibitors. LDN-212854 bound to the kinase hinge region as a typical type I ATP-competitive inhibitor with a single hydrogen bond to ALK2 His286. Specificity arising from the 5-quinoline moiety was associated with a distinct pattern of water-mediated hydrogen bonds involving Lys235 and Glu248 in the inactive conformation favoured by ALK2. The structure of this complex provides a template for the design of future ALK2 inhibitors under development for the treatment of FOP and other related conditions of heterotopic ossification.

1. Introduction

Bone morphogenetic proteins (BMPs) represent the largest subgroup in the TGFβ family of extracellular ligands [1,2]. Signal transduction by these ligands requires type I and type II transmembrane receptor serine/threonine kinases which form heterotetrameric complexes for ligand interaction. The identity of the ligand determines the specific recruitment of receptors and the downstream signalling [3]. Type I receptors phosphorylate SMAD family transcription factors, whereas type II receptors are required to activate the type I receptors by phosphorylation of their juxtamembrane GS (glycine-serine rich) domain. Type I receptors ALK1/*ACVRL1*, ALK2/*ACVR1*, ALK3/*BMPR1A* and ALK6/*BMPR1B* all participate in BMP signalling and phosphorylate SMAD1/5/8. In contrast, the Activin type I receptors ALK4/*ACVR1B* and ALK7/*ACVR1C* and the TGFβ type I receptor (ALK5/*TGFBRI*) all primarily signal through SMAD2/3. In addition, the receptor signalling complexes can activate a variety of non-canonical pathways, including the p38 MAPK and phosphoinositide 3-kinase (PI3K) signalling cascades. Characterisation of these receptors and their signalling pathways has been pivotal to our understanding of the effects of BMP signalling in processes such as embryonic patterning, tissue differentiation and homeostasis [4–6].

The importance of BMP signalling is evident from the many disease conditions that are genetically linked to the dysregulation of these pathways [6,7]. Most commonly these involve loss of function mutations, as exemplified by *ACVRL1* and *BMPR1A* mutations which predispose to hereditary haemorrhagic telangiectasia [8] and juvenile polyposis syndrome [9,10], respectively. By contrast, fibrodysplasia ossificans progressiva (FOP) is a rare monogenic condition in which a gain of function germline mutation in *ACVR1* leads to increased signalling through ALK2 in response to BMP ligands as well as neofunction in response to Activin A and the consequent formation of heterotopic bone in muscle and connective tissue [11,12]. Similar somatic mutations in *ACVR1* are also observed in 25% of cases of diffuse intrinsic pontine glioma (DIPG), a rare childhood brain tumour [13]. BMP signalling has also been linked to other human cancers. For example, BMP2 can promote the expansion of ovarian cancer stem cells [14], while BMP6 overexpression is associated with prostate cancer skeletal metastases [15,16]. Notably, BMP type I receptor inhibitors have demonstrated promising effects in DIPG patient cell lines [17], as well as a number of other cancer models [18–25]. BMP signalling has also been identified as a promising therapeutic target to normalize hepcidin expression in chronic anaemia of inflammation [26,27].

These data have stimulated interest in the development of small molecule BMP type I receptor inhibitors both as therapeutic agents and as chemical tools to probe cellular signalling mechanisms [7,28]. Dorsomorphin was discovered as the first small molecule BMP receptor inhibitor using a phenotypic screen to identify compounds capable of inducing the dorsalization of zebrafish embryos, as observed for the mutant BMP receptor *lost-a-fin* [26]. A crystal structure of the human

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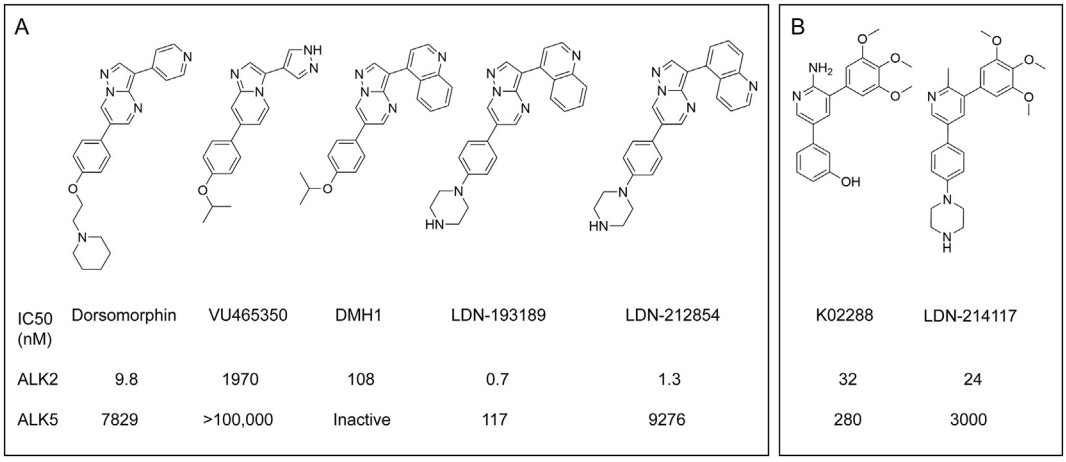

Fig. 1. Chemical scaffolds of selected BMP type I receptor inhibitors. (A) Selected inhibitors derived from the core pyrazolo[1,5-*a*]pyrimidine scaffold of dorsomorphin. (B) Inhibitors based on an alternative pyridine-based scaffold. IC_{50} data are those reviewed by Hopkins [38] except the data for LDN-214117 which are taken from Mohedas et al. [37].

orthologue ALK2 confirmed the direct binding of dorsomorphin to the ATP-binding pocket of the receptor's intracellular kinase domain [29]. Further development of the pyrazolo[1,5-*a*]pyrimidine-containing scaffold has subsequently produced a series of derivative compounds, including LDN-193189 [30], DMH1 [31], VU465350 [32], and LDN-212854 [33] (Fig. 1a). LDN-193189 was selected for its improved potency and pharmacokinetic properties [30], which have enabled its use in animal models of heterotopic ossification [34], vascular calcification [35] and anaemia of inflammation [27]. DMH1 was identified from further zebrafish screening to reduce off-target activity against the VEGF pathway [31]. Finally, VU465350 and LDN-212854 were recently reported as inhibitors harboring increased subfamily selectivity for ALK3 and ALK2, respectively [32,33]. In addition, a second series of pyridine-based small molecule BMP type I receptor inhibitors was identified from a biochemical screen against the purified ALK2 kinase domain (Fig. 1b). The initial screening hit K02288 showed remarkable selectivity for BMP receptors over a panel of some 250 human kinases [36]. Subsequent work yielded the compound LDN-214117, which displayed superior activity in cells, as well as enhanced selectivity for ALK2 over ALK5 [37].

The compound LDN-212854 is of particular interest for the development of inhibitors against heterotopic ossification. It has demonstrated notable efficacy in two different mouse models, including one using an inducible constitutively-active *ACVR1*^{Q207D} transgene [33] and another harboring an *Acvr1*^{R206H} knock-in allele that more faithfully recapitulates human FOP [39]. LDN-212854 was developed from LDN-193189 through the substitution of a 4-quinoline moiety for a 5-quinoline (Fig. 1a) [33]. Remarkably, this simple change increased the compound's selectivity for ALK2 over ALK3 from 21-fold (LDN-193189) to 66-fold (LDN-212854). In addition, the selectivity for ALK2 over ALK5 was increased from 175-fold to over 9000-fold [33]. Thus, the ALK2 bias of LDN-212854 is ideally suited to counter the *ACVR1*^{R206H} mutation that underlies FOP. Here we determined the crystal structure of ALK2 in complex with LDN-212854 revealing subtle differences in its binding compared to LDN-193189.

2. Materials and methods

2.1. Protein expression and purification

The recombinant ALK2 kinase domain was prepared with a Q207D mutation as previously described [36]. Briefly, ALK2 residues 201–499 were cloned into transfer vector pFB-LIC-Bse and baculovirus prepared in DH10Bac cells. Baculoviral expression was performed in Sf9 insect cells grown at 27 °C. Some 48 h post-infection, cells were harvested and lysed using ultrasonication. ALK2 protein was purified sequentially by nickel affinity and size-exclusion chromatography. The eluted protein was stored at −80 °C buffered in 50 mM HEPES, pH 7.5, 300 mM NaCl, 2 mM DTT, 50 mM arginine, 50 mM glutamate. The N-terminal hexahistidine tag used for purification was cleaved using tobacco etch virus (TEV) protease.

2.2. Crystallization

Crystallization was achieved at 4 °C using the sitting-drop vapor diffusion method. ALK2 was preincubated with 1 mM LDN-212854 at a protein concentration of 13.6 mg/mL and crystallized using a precipitant containing 18% PEG8000, 0.2 M calcium acetate, 0.1 M cacodylate pH 6.5. Viable crystals were obtained when the protein solution was mixed with the reservoir solution at 2:1 volume ratio. Crystals were cryoprotected with mother liquor plus 25% ethylene glycol, prior to vitrification in liquid nitrogen.

Table 1
Data collection and refinement statistics (molecular replacement).

	ALK2-LDN-212854 (PDB ID 5OXG)
Data collection	
Space group	*I*121
Cell dimensions	
a, *b*, *c* (Å)	85.9, 102.2, 177.3
α, β, γ (°)	90.0, 94.0, 90.0,
Resolution (Å)[a]	88.49–1.73 (1.80–1.73)
R_{merge}[a]	0.0681 (1.284)
$I/\sigma I$[a]	4.91 (0.56)
Completeness (%)[a]	99.30 (97.95)
Redundancy[a]	1.9 (1.8)
Refinement	
Resolution (Å)	2.13
No. reflections[a]	293,366 (27506)
R_{work}/R_{free}	0.22/0.25
No. atoms	9810
Protein	9326
Ligand/ion	139
Water	345
B-factors	46.00
Protein	45.90
Ligand/ion	47.10
Water	46.70
R.m.s deviations	
Bond lengths (Å)	0.012
Bond angles (°)	1.49

Data from a single crystal.
[a] Highest resolution shell is shown in parenthesis.

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