

Drug Discovery: Recent Progress and the Future

Review

ALK2: A Therapeutic Target for Fibrodysplasia Ossificans Progressiva and Diffuse Intrinsic Pontine Glioma

Katsuhiko Sekimata,^{*,a} Tomohiro Sato,^b and Naoki Sakai^c

^aDrug Discovery Chemistry Platform Unit, RIKEN Center for Sustainable Resource Science; 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; ^bDrug Discovery Computational Chemistry Platform Unit, RIKEN Center for Biosystems Dynamics Research; 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan; and ^cDrug Discovery Structural Biology Platform Unit, RIKEN Biosystems Dynamics Research; 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan.

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Fibrodysplasia ossificans progressiva (FOP) and diffuse intrinsic pontine glioma (DIPG) are diseases that typically manifest in childhood and are associated with severely reduced life expectancy. However, there are currently no effective therapies for these diseases, which remain incurable. Activin receptor-like kinase-2 (ALK2), encoded by the *ACVRI* gene, is a bone morphogenetic protein (BMP) type-I receptor subtype that plays an important physiological role in the development of bones, muscles, brain, and other organs. Constitutively active mutants of ALK2 have been identified as causative of FOP and involved in the tumorigenesis of DIPG owing to abnormal activation of BMP signaling, and therefore have emerged as promising treatment targets. Here, we describe these two diseases, along with the link to ALK2 signal transduction, and highlight potential ALK2 inhibitors that are under development to offer new hope for patients with FOP and DIPG.

Key words activin receptor-like kinase-2 (ALK2); drug discovery; rare disease; fibrodysplasia ossificans progressiva (FOP); diffuse intrinsic pontine glioma (DIPG)

1. Introduction

Fibrodysplasia ossificans progressiva (FOP) is a rare severe genetic musculoskeletal disorder with an incidence of about 1 in 2 million and typically occurs in children below the age of 10 years.¹⁻³ The disease is characterized by heterotopic ossification (HO), endochondral bone formation at extra-skeletal sites, and great toe malformation at birth, along with episodic HO in the tendons, fascia, ligaments, and muscle.¹⁻³ HO development in FOP patients can be triggered by muscle injury through accidental trauma, biopsies or surgical treatment, but is most often spontaneous and initiates with a local “flare-up” characterized by redness, swelling and pain.¹⁻³ HO formation causes several health problems and impediments, including skeletal deformities, chronic pain, growth impairment and joint ankyloses, which can also lead to breathing difficulties and premature death.^{2,3} Analysis of patient samples demonstrated that HO typically involves endochondral ossification in which progenitor cells undergo chondrogenesis and cartilage formation, and gradually become organized into growth plates that are eventually replaced by bone and marrow.³ Longitudinal X-ray monitoring of HO lesions has shown that the ectopic bone masses reach a threshold size after which growth ceases, likely following completion of the cartilage-to-bone transition.³

Surgical procedures are not suitable for the treatment of FOP because the resulting trauma causes a far more serious HO.^{2,4} Thus, there are limited therapeutic options for FOP patients,⁵ and there is a need for the development of pharma-

ceutical agents for prevention and treatment, which requires further explanation of the underlying molecular drivers and causes of the disease.

Some insight into this mechanism can be gained by findings of mutation profiles of a seemingly unrelated disease, diffuse intrinsic pontine glioma (DIPG), a pediatric brainstem glioma originating in the pons. DIPG has a yearly incidence of about 2 per million people in individuals aged 0–20 years,⁶ and it is histopathologically classified as a fibrillary astrocytoma.⁷ In the 2016 WHO Classification of Tumors of the Central Nervous System (WHO 2016), DIPG is defined as diffuse midline glioma with histone H3K27M mutation.⁸ Because the pons has essential life-sustaining functions, including those related to its role as pneumotaxic center, the tumor or its surgical removal can cause devastating neurological damage and can be fatal.^{6,7} The median age at diagnosis of DIPG is 6–7 years, with a survival rate <1%.⁶ Typically, patients present with a neurological triad of symptoms, including cranial nerve deficits (diplopia and facial asymmetry), cerebellar signs (ataxia, dysmetria, and dysarthria), and long tract signs (hyperreflexia, upward Babinski, and decreased strength) that have been experienced for less than 3 months.⁹ Although radiation therapy, including chemotherapy combinations, remains the standard of care at present, more than 250 clinical trials have failed to demonstrate an improvement in the poor prognosis of DIPG.^{7,10,11} Tumor samples from DIPG patients were rarely available for preclinical research, but now the acquisition of samples through biopsy and autopsy protocols has led to

* To whom correspondence should be addressed. e-mail: katsuhiko.sekimata@riken.jp

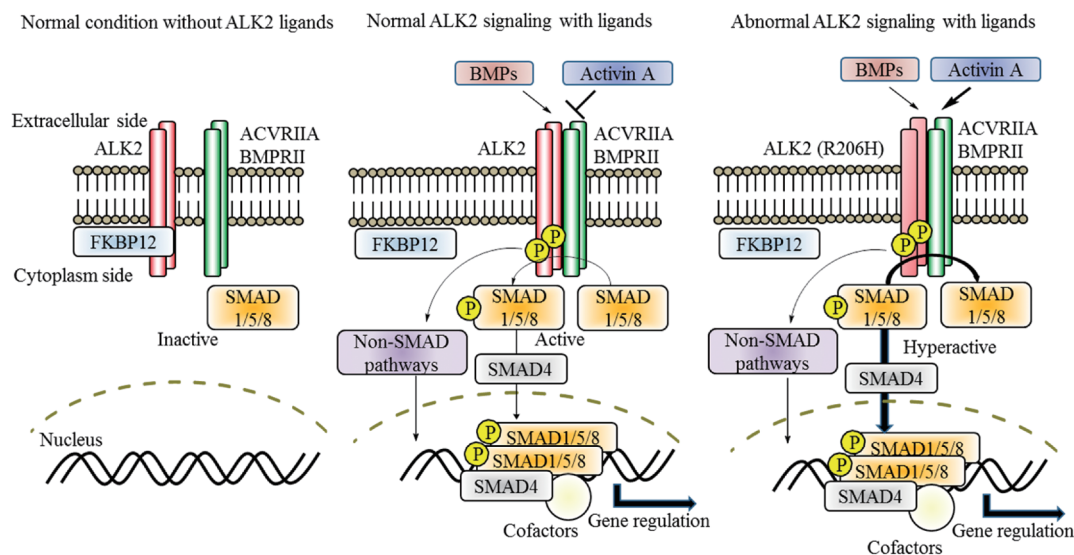


Fig. 1. Schematic Representation of ALK2 Signal Transduction

identification of the key oncogenic drivers implicated in DIPG development.^{12–15)}

Importantly, recent studies have indicated that constitutively activated activin receptor-like kinase 2 (ALK2) signaling may lead to the development of both FOP and DIPG, which bear common activating mutations in the ALK2 encoding gene *ACVR1*.^{16–18)}

In this review, we briefly summarize the known mutations of ALK2 that cause FOP and DIPG, and provide an overview of progress in the development of small-molecule inhibitors of ALK2 in drug discovery to provide new and promising treatment options for these currently incurable diseases.

2. ALK2 Signal Transduction

ALKs are type I receptors responsible for mediation of signal transduction of the transforming growth factor- β (TGF β) superfamily.^{19,20)} Of the seven known ALKs (ALK1–7), ALK1, ALK2, ALK3, and ALK6 form a group of bone morphogenetic protein (BMP) type I receptors,²⁰⁾ whereas the others (ALK4, ALK5, and ALK7) are TGF β type I receptors.²⁰⁾

ALK2, encoded by the *ACVR1* gene, is expressed ubiquitously^{21,22)} and shows the typical domain structure of ALK family receptors, which includes a ligand-binding extracellular domain, transmembrane domain, glycine-serine-rich (GS) domain, and serine/threonine kinase domain.^{3,23)} X-ray crystallographic studies show that the ALK2 kinase domain has a bilobal fold and the GS domain extends from the N-lobe into a helix-loop-helix motif that binds to the endogenous inhibitor FKBP12.^{3,23)} FKBP12 prevents access to the regulatory GS loop and inhibits movement of α C needed for kinase activation, and the inactive conformation of the GS loop region is stable even in the absence of FKBP12.^{3,24)} Several BMP ligands, such as BMP2, BMP4, BMP6, BMP7, BMP9, and BMP10, and activins, can bind to ALK2.^{25,26)} These BMP ligands can trigger the release of FKBP12 and the phosphorylation of SMAD1/5/8, which forms heteromeric complexes with Co-SMAD (SMAD4) and non-SMAD

signaling pathway including the p38 mitogen-activated protein kinase pathway²⁸⁾ (Fig. 1). These SMAD4-containing transcriptional complexes then translocate to the nucleus where they regulate expression of the inhibitor of DNA binding/differentiation genes (*ID1*, *ID2*, and *ID3*).^{20,29)} The other TGF β family ligands, *i.e.*, activins and TGF β , induce activation of SMAD2/3 on the formation of receptor membrane signaling complexes comprising ALK4 or ALK5 and ACVR1IA/B.²⁰⁾ This signaling pathway not only induces transcription of a different subset of genes, but also commonly counterbalances the SMAD1/5/8 pathway, for example by competing with the common mediator SMAD.²⁰⁾ Although activin A, an ALK4 ligand, can bind to ALK2, these interactions do not induce SMAD1/5/8 activation.³⁰⁾ In contrast, binding of activin A to ALK2 mutants is activated *via* the SMAD1/5/8 signaling pathway.³¹⁾ Therefore, in normal circumstances, activins prevent BMP signaling by competing for ALK2 at the ligand–receptor interaction level.

3. FOP and ALK2

In 2006, mutations in *ACVR1* were identified as the genetic cause of FOP.¹⁶⁾ In addition, 13 ALK2 mutations have been identified in FOP patients to date.³²⁾ Five mutations (L196P, P197_F198delinsL, R202I, R206H, and Q207E) occur in the GS domain and the remainder (R258G/S, G325A, G328R/W/E, G356D, and R375P) occur in the serine/threonine (Ser/Thr) kinase domain, with none reported in other domains.

More than 90% of patients with typical FOP have a gain-of-function mutation (c.617G > A; R206H) in the *ACVR1* gene.³²⁾ R206H mutations affect the cytosolic GS domain of the receptor, and result in its constitutive activation by interfering with binding of the negative regulator FKBP12 and/or by enhancing the response to certain BMP ligands and, especially activin A³¹⁾ (Fig. 1). Analysis of the crystal structure of the complex formed between ALK2 and FKBP12 revealed that the phenylalanine and leucine residues at positions 198 (F198) and 199 (L199) in ALK2 are the critical residues for FKBP12 binding.^{23,27)} The ALK2 mutations identified in FOP patients occur in the GS domain and the ATP adjacent pocket in the kinase domain.³²⁾ The affected residues function to maintain

the α -helical structure of the GS domain as well as critical bonds between the GS domain and the kinase *N*-lobe required to stabilize the inactive conformation of ALK2; thus, these mutations likely destabilize the protein conformation, altering its sensitivity to FKBP12 and eliciting enhanced ALK2 signaling activity.^{3,23)}

With regard to clinical characteristics, FOP patients with mutations other than ALK2 (R206H) show diverse phenotypes.¹⁾ Patients with the ALK2 (G328W) or ALK2 (G328E) mutation have early-onset HO, reduction of multiple digits, thumb malformations, mild cognitive impairment, and sparse scalp hair.¹⁾ Conversely, a case report of a patient with the ALK2 (G325A) mutation described late-onset HO at age 47 and a mild clinical phenotype, including typical malformation of the great toe.³³⁾ Most FOP cases are sporadic and thought to arise from germline mutations that would begin to exert their pathogenic effects starting from embryogenesis, resulting in evident malformation of the great toe at birth. However, it is also possible that some patients may have acquired the ACVR1 mutation at a later embryonic or neonatal stage and may thus be mosaic; this could potentially be the case for patients without the characteristic skeletal malformations or who have late-onset HO and/or mild forms of the disease.³⁾

4. DIPG and ALK2

Molecular profiling of DIPG patient samples has provided important insight into the drivers of DIPG tumorigenesis, including epigenetic changes, gene mutations, deletions or overexpression and chromosomal number changes.^{15,34)} Detailed molecular analyses have identified several mutations associated with tumorigenesis, such as histone H3, ACVR1, TP53, PDGFRA, PIK3CA, and MYC mutations.^{35–37)} The most highly recurrent and selective mutations in DIPG are the histone variants H3.1 and H3.3, encoded by the *HIST1H3B* and *H3F3A* genes, respectively.³⁸⁾ These mutations involve replacement of lysine with methionine at residue 27 (K27M), leading to the loss of histone trimethylation (H3K27me3), which in turn inhibits polycomb repressive complex 2 and promotes abnormal epigenetic silencing.^{39–41)} H3K27M has been reported to contribute to abnormal cell-cycle control, inhibition of autophagy, and tumor resistance to radiotherapy.^{12,42)} Although these mutations clearly represent a fundamental genetic driver in DIPG, the pathological function of H3K27M in tumor initiation remains unclear. However, the combination of H3K27M with additional mutational events, such as those affecting the cellular proliferation pathway gene *ACVR1/PI3KR1* or cell-cycle regulatory genes *TP53*, could synergistically enhance tumorigenesis, as a likely early transformational event in DIPG.^{38,43,44)}

Somatic ACVR1 mutations are almost completely limited to DIPG in the Catalogue of Somatic Mutations in Cancer database, suggesting that ACVR1 is a potential oncogenic driver of tumorigenesis.^{17,36,45)} Currently, seven ALK2 mutations have been identified in DIPG patients. Two mutations (R206H and Q207E) occur in the GS domain and the others (R258G, G328E, G328V, G328W, and G356D) in the Ser/Thr kinase domain.^{17,18,46,47)} These mutations have been identified in approximately 25% of DIPG tumors, tend to co-segregate with H3.1 mutations, and have been linked to an increased median overall survival.^{17,18,42)} Since FOP patients with similar germline mutations do not develop DIPG, these mutations

of ALK2 likely do not initiate tumorigenesis themselves but rather lead to DIPG when coexisting with other mutations.^{15,17,47)} ACVR1-HIST1H3B co-segregating tumors do not show *TP53* loss or *PDGFRA* amplifications, but approximately 60% of these tumors also have mutations in genes involved in the phosphatidylinositol 3-kinase signaling pathway.^{46,48)} Since ALK2 mutations facilitate tumor growth in conjunction with other molecular aberrations, they represent attractive targets for therapy.^{36,48)}

5. Chemotherapy and Small Molecule ALK2 Inhibitors

The current standard treatment for FOP is a brief 4-d course of high-dose corticosteroids such as prednisone that is initiated within 24h of a flare-up.⁵⁾ Although this treatment can relieve inflammation and pain, it does not reduce the frequency of progressive HO.⁴⁹⁾ As an alternative to corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs) and aminobisphosphonates can also be used.⁵⁾ NSAIDs inhibit prostaglandin biosynthesis, which induces resistant HO in animal models.⁵⁰⁾

Aminobisphosphonates, as bone-seeking agents, affect osteoclast function and survival, and thereby bone formation, and have strong antiangiogenic effects in the context of cancer.⁵¹⁾ Because angiogenesis is required during the transition from hypertrophic cartilage to bone,⁵²⁾ aminobisphosphonates can ameliorate FOP by blocking this essential developmental process in a similar transition that occurs during OH. Although systemic treatment with bisphosphonates inhibited HO in trauma-induced and BMP4-induced HO mouse models, the efficacy and safety of these drugs have not been established.

Exogenous retinoid agonists have been reported potent inhibitors of chondrogenesis and skeletal development.^{53,54)} Retinoic acid γ agonists were shown to inhibit HO in an animal model by reducing SMAD protein levels and BMP signaling.⁵⁵⁾ Palovarotene, a retinoic acid γ agonist, is currently in phase III clinical trial.

Chemical screening methods using pluripotent stem cells derived from FOP patients with ALK2 (R206H) mutation have been established, leading to the discovery of several types of chondrogenesis inhibitors. The mode of action of the mammalian target of rapamycin (mTOR) inhibitors, including rapamycin, is believed to consist of modulation of aberrant inflammatory signaling, FOP-ACVR1 signaling, and hypoxic signaling during the development of HO.⁵⁶⁾ Rapamycin has the potential to suppress HO in animal models and is currently in phase II for FOP patients.⁵⁷⁾

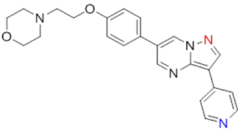
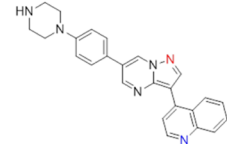
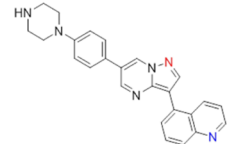
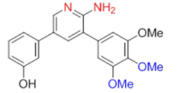
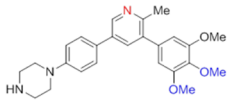
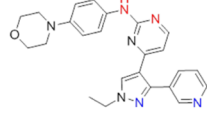
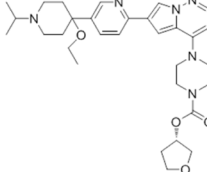
In addition to these several small chemical inhibitors of inflammation and/or chondrogenesis investigated for the treatment of FOP *via* clinical trials, a human anti-activin A-neutralizing antibody (REGN2477) has been reported to exhibit potential inhibition of HO in a mouse model of FOP.⁵⁸⁾ Currently, REGN2477 is in phase II. In addition, with increasing knowledge of the pathogenesis of FOP, various new targets are being discovered, including numerous ALK2 inhibitors, as shown in Table 1. Several years after its discovery as the small-molecule inhibitor of BMP signaling using phenotypic screening in zebrafish embryos, dorsomorphin was identified as the first small-molecule inhibitor of ALK2.⁵⁹⁾ X-Ray crystallography revealed that this compound directly binds to the ALK2-ATP binding pocket.²³⁾ Specifically, dorsomorphin

forms a hydrogen bond between the pyrazolo[1,5-*a*]pyrimidine moiety and the main chain amine of the His286 residue in the ATP hinge region of ALK2 (Fig. 2). Other hydrogen bonds are formed between the nitrogen of the 4-pyridyl ring of the compound, the amine group of the conserved Lys235 sidechain from the β 3 strand, and the carboxyl group of the conserved Glu248 sidechain from the α C helix *via* a conserved water molecule in a hydrophobic region. The piperidinyl-ethoxy group of dorsomorphin is then exposed to the solvent region. Dorsomorphin has thus far been reported as a non-selective inhibitor that acts on AMP-activated protein kinase and vascular endothelial growth factor receptors.⁶⁰ LDN-193189 was developed through chemical modifications of dorsomorphin possessing a pyrazolo[1,5-*a*]pyrimidine scaffold, which exhibited improved selectivity, potency, and pharmacokinetic properties and could inhibit HO in a mouse model of FOP.^{61,62} Moreover, the nitrogen position in the quinolone of LDN-193189 was further modified to yield LDN-212854, which showed im-

proved selectivity for ALK2 compared with that of the closely related BMP and TGF β type I receptor kinases.⁶³

K02288 was also discovered as a new scaffold ALK2 inhibitor through biochemical screening of a library of kinase-directed compounds.⁶⁴ X-ray crystallographic studies showed that K02288 binds to the ALK2 ATP-binding pocket in the same manner as dorsomorphin. K02288 forms two hydrogen bonds between the aminopyridine moiety, the main chain carbonyl of the His284 residue, and the amine of the His286 residue in the hinge region of the enzyme (Fig. 2). A third hydrogen bond is formed between the two oxygens of three methoxy groups of the compound and the amine group of the conserved Lys235 sidechain from the β 3 strand *via* a conserved water molecule. Another hydrogen bond is formed between the hydroxy group of the compound and the carboxylic group of Asp293. Based on this finding, a K02288 derivative, LDN-214117, was developed, which exhibited superior activity in cells, with more than 100-fold selectivity for ALK2 over

Table 1. Small-Molecule ALK2 Inhibitors

| Chemical name | chemical structure | IC ₅₀ ALK2 / ALK2 (R206H) (nM)* | crystal structure PDB ID | reference | clinical stage |
|---------------|---|--|--------------------------|------------|----------------|
| Dorsomorphin |  | - / - | 4C02 3H9R | 28, 59, 60 | - |
| LDN-193189 |  | 23 / 20 | 3Q4U | 61, 62, 66 | - |
| LDN-212854 |  | 80 / 14 | 5OXG | 63 | - |
| K02288 |  | - / 19 | 3MTF 6EIX | 64, 65 | - |
| LDN-214117 |  | - / 17 | - | 65, 66 | - |
| RK71836 |  | 112 / 26 | - | 68, 69 | - |
| BLU-782 |  | - / - | - | 67 | 2 |

Representative examples of small-molecule ALK2 inhibitors including those disclosed and under clinical evaluation. Atoms participating in binding to the hinge region and the hydrophobic region are shown in red and blue, respectively. *ALK2 and ALK2 (R206H) enzyme assays were conducted by Reaction Biology Corporation using the "HotSpot" assay platform and kinase Assay Protocol.⁶⁹ (Color figure can be accessed in the online version.)

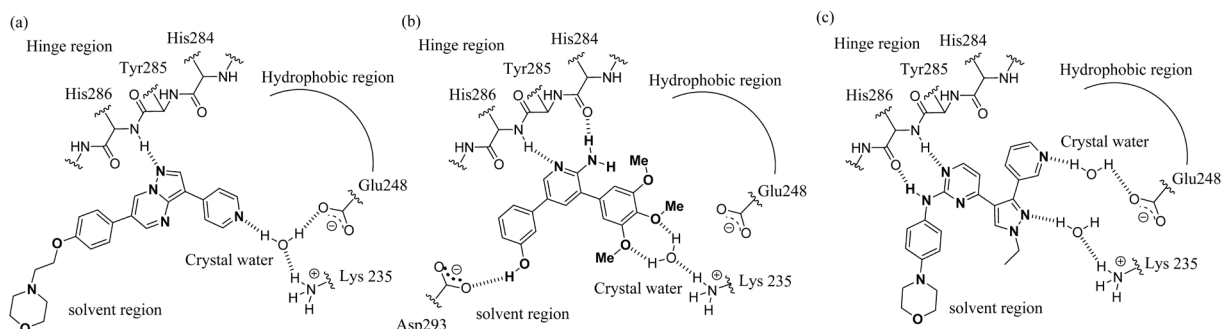


Fig. 2. Schematic 2D Representation of the Binding Mode of (a) Dorsomorphin in ALK2,²⁸⁾ (b) K02288 in ALK2,⁶⁴⁾ and (c) RK71836 in ALK2 (R206H)⁷⁰⁾

Hydrogen bonds are depicted as dashed lines.

ALK5.⁶⁵⁾ LDN-193189 and LDN-214117 exhibit oral bioavailability and were well-tolerated, with good brain penetration at potentially active concentrations.⁶⁶⁾ In an orthotopic xenograft mouse model bearing the H3K27M mutation, and in ACVR1 R206H mutant HSJDDIPG-007 cells, both compounds significantly extended survival compared with vehicle controls.⁶⁶⁾ Despite these several potent ALK2 kinase inhibitors in development, none of these compounds is currently in clinical trials. However, BLU-782 was reported as a new scaffold ALK2 inhibitor,⁶⁷⁾ which is currently in a phase II.

Our group has also sought to develop small-molecule ALK2 (R206H) inhibitors that are orally effective. To find “screening hit compounds” suitable for optimization, we adapted *in silico* approaches combining ligand-based drug discovery, employing a similarity search and machine learning using existing small-molecule inhibitors of the ALK kinase family in the ChEMBL database as query compounds, and structure-based drug discovery method, employing docking simulation with ALK2 crystal structures. From the careful assessment of ALK2 (R206H) kinase assay results for 976 compounds selected by two- and three-dimensional *in silico* approaches, we were able to identify a bis-heteroaryl pyrazole scaffold as a core structural motif for optimization. Structure–activity relationship analyses were conducted for assessing optimal enzyme inhibition, liver microsomal stability, rodent pharmacokinetics, and off-target activity profiles.^{68,69)} Based on the X-ray co-crystal structures, we were able to identify key structural motifs that interacted with the enzyme and are critical for its potency.

These studies revealed that the compounds directly bind to the ALK2 (R206H) ATP-binding pocket. For example, RK71836 forms two hydrogen bonds between the core structure, aminopyrimidine moiety, and the main chain amine and carbonyl of the His286 residue in the hinge region of the enzyme⁷⁰⁾ (Fig. 2). A third hydrogen bond is formed between the nitrogen of the 3-pyridyl group of the compound and the carboxyl group of the conserved Glu248 sidechain from the α C helix *via* a conserved water molecule. Another hydrogen bond is formed between the nitrogen of the pyrazole ring of the compound and the amino group of Lys235 from the β 3 strand *via* a conserved water molecule. Based on these observations, we were able to modify the rest of the structure for pharmacokinetic/pharmacodynamics optimization, especially for improved oral bioavailability.⁶⁹⁾ After studying more than 1300 newly synthesized compounds, we identified a prelini-

cal candidate that exhibits dose-dependent suppression of HO in a mouse model of FOP.⁷⁰⁾ A series of detailed biochemical analyses *in vivo* is in progress.

6. Conclusion and Prospects

Molecular profiling of FOP or DIPG patient samples has provided important insights into the disease-related genes. Molecular analyses led to the identification of common mutations including those of ALK2 as causative mutations of FOP and the driver of DIPG tumorigenesis. The development of an ALK2 inhibitor to suppress HO in FOP patients and tumor growth in DIPG patients will serve as a major step toward improving prognoses of FOP and DIPG patients. As reviewed, there are currently only a few drug candidates with ALK2 as the primary target, and only one such candidate can be considered as a small-molecule ALK2 inhibitor. Therefore further studies are needed to establish a specific ALK2 mutant inhibitor and validate the concept of ALK2 inhibition to improve treatment and establish a cure for these devastating diseases.

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Conflict of Interest Katsuhiko Sekimata and Tomohiro Sato are inventors of the following patents: WO 2018/124001 (A1). Katsuhiko Sekimata and Tomohiro Sato are employees of RIKEN.

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