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Mini-Review

Recent Advances in ALK2 Inhibitors

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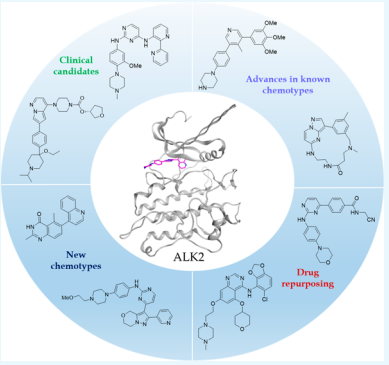
ABSTRACT: Activin receptor-like kinase-2 (ALK2) is a type I bone morphogenetic protein (BMP) receptor which has a role in biological processes that control the development of bone, heart, brain, and other tissue. Gain of function mutations in ALK2 have been identified in fibrodysplasia ossificans progressiva (FOP) and the childhood brain tumor, diffuse intrinsic pontine glioma (DIPG), which has given focus to the development of ALK2 inhibitors as targeted treatments. This review covers the structural features of ALK2 inhibitors which contribute to their ALK2 potency and selectivity, and the pharmacokinetic or *in vivo* efficacy data available to demonstrate their suitability for treating a peripheral or CNS disease.



INTRODUCTION

Activin receptor-like kinase 2 (ALK2) is one of seven activin receptor-like kinases (ALKs) involved in bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) family signaling.[1a,b] It plays an important role in biological processes controlling the development and regulation of bone, heart, and nervous and reproductive systems. Gain of function mutations in *ACVR1*, the gene encoding ALK2, have been identified in two currently untreatable rare diseases.[2] Germline mutations result in the congenital malformation syndrome fibrodysplasia ossificans progressiva (FOP) characterized by the ossification of soft tissue.[3] Somatically, these base changes are associated with diffuse intrinsic pontine glioma (DIPG), an invariably fatal childhood brainstem tumor.[4] Mutations occur in either the juxtamembrane GS domain (including R206H, comprising >95% cases of FOP) or the intracellular kinase domain[2] and result in enhanced SMAD1/5 signaling due to ligand-independent activation via disruption of inhibitory FKBP12 binding[5] and/or aberrant responsiveness to Activin-A and autophosphorylation.[6] In both diseases, the unmet clinical need has driven the development of small molecule inhibitors of ALK2.

INTRODUCTION TO ALK2 INHIBITORS

The first known inhibitor of ALK2, Dorsomorphin (Figure 1), was discovered from the *in vivo* screening of a diverse chemical library for their ability to dorsalize developing zebrafish embryos.[7] Dorsomorphin, containing the pyrazolo[1,5-*a*]-pyrimidine scaffold, possessed significant kinase off-target activity and a lack of metabolic stability. Expanding the 4-pyridyl ring to a 4-quinoline and replacing the ether with a piperazine improved potency and provided the metabolically

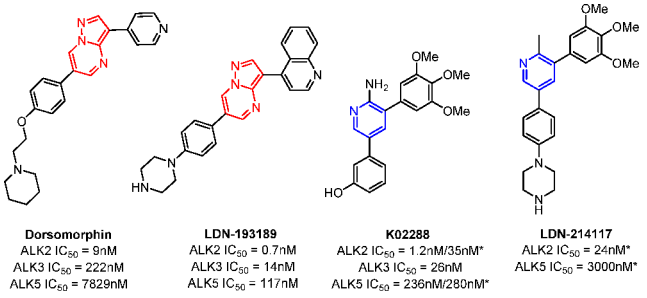


Figure 1. Early ALK2 inhibitors containing the pyrazolo[1,5-*a*]pyrimidine (red) or pyridine (blue) core and reported ALK IC_{50} data from ref 8b or ref 9b*.

stable LDN-193189.[8a] Further exploration discovered the 5-quinoline LDN-212854 which possessed greater selectivity for ALK2 over other ALK subtypes.[8b]

An *in vitro* screen of a kinase directed library identified the alternative scaffold in K02288 (Figure 1).[9a] Poor solubility limited the cell potency of K02288, which was addressed by replacement of the 3-phenol with the 4-phenylpiperazine.[9b] Selectivity for BMP versus TGF-β signaling (ALK4, ALK5) could be improved by replacing the 2-amino group of the

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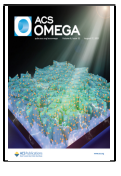

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pyridyl with either H- (LDN-213844) or Me- (LDN-214117) with minimal effect on the ALK2 potency.

These chemotypes have provided very useful *in vitro* and *in vivo* tools for establishing ALK2 as a therapeutic target in both DIPG and FOP. LDN-193189 and LDN-212854 are efficacious *in vivo* in preventing heterotopic ossification in an inducible transgenic ALK2^{Q207D} mouse model of FOP.[8b] For DIPG, examples from both series, LDN-193189 and LDN-214177, prolong the survival of ACVR1 R206H mutant HSJD-DIPG-007 bearing animals *in vivo*.[10] A number of recent publications describe further advances to these chemotypes as well as the identification of novel chemical series targeting ALK2. Here, we review the features of these chemotypes which are important for achieving ALK2 potency, selectivity, and a suitable pharmacokinetic profile for either a peripheral or CNS disease.

ALK2 STRUCTURE

The ALK2 receptor comprises an extracellular domain where BMP ligand binding occurs, a single transmembrane region, and an intracellular region containing the glycine-serine-rich (GS-domain) and the serine/threonine kinase. The development of ALK2 inhibitors has been aided by numerous X-ray crystallographic studies of the kinase domain showing the binding modes of several chemotypes. The ALK2 kinase is observed in the inactive conformation with the activation region folded toward the solvent-exposed region of the ATP binding pocket, preventing ATP binding (Figure 2A). The predominant activating mutations observed in FOP (R206H) and DIPG (G328V/G328E/G328W, R206H, R258G) are not in proximity to the ATP binding pocket, with the exception of G356D. Studies on pyridine compounds have demonstrated a similar ability to inhibit wild-type ALK2 compared to 7 mutants including R206H and G328E.[9b]

All current ALK2 inhibitors bind in the ATP pocket and form an H-bond interaction with the hinge amide of His286. The salt bridge between the catalytic Lys235 and Glu248 is predominantly broken, and these residues participate in direct or water-mediated interactions with inhibitors: for example, the water bridge with the 5-quinoline nitrogen of LDN-212854 (Figure 2B) or the trimethoxyphenyl ring of LDN-213844 (Figure 2C).

The central region of the ALK2 pocket is largely hydrophobic (Val214, Val222, Leu263, Leu343) and toward the solvent channel Asp293 is proximal to the charged amine groups to allow electrostatic interactions. Hydrophobic and water mediated interactions have been seen with the P-loop residue Tyr219 in FEP/H-REMD simulations.[11]

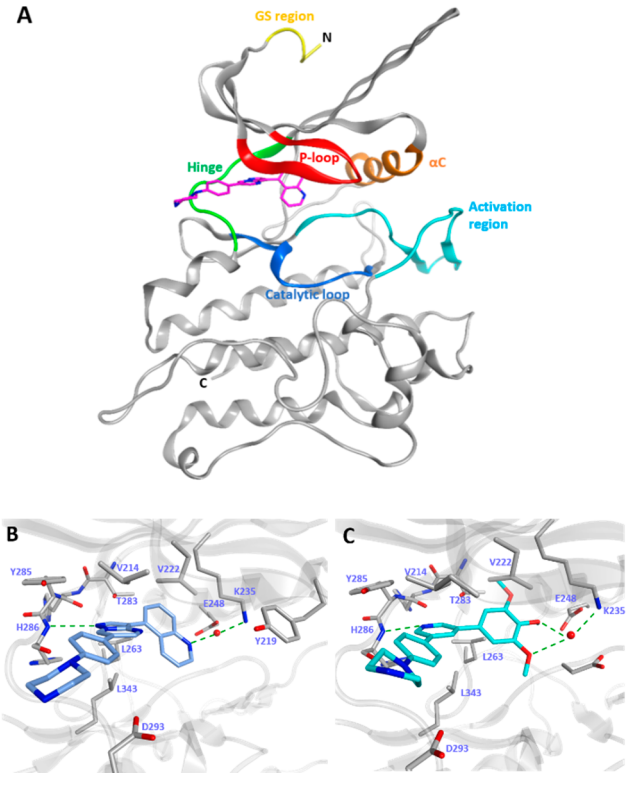

Figure 2. (A) Structure of the ALK2 kinase domain. (B) Binding of LDN-212854 to ALK2 (PDB ID: 5OXG). (C) Binding of LDN-213844 to ALK2 (PDB ID: 4BGG).

SELECTIVITY OVER ALK SUBTYPES

A high similarity of the kinase domain exists between the 7 ALK subtypes, with a high degree of identity (82−85%) of the residues found in the ATP binding pocket of ALK2 versus other ALK members. In the development of ALK2 inhibitors, most concern has been given to the selectivity over ALK5, with potent ALK5 inhibition linked to cardiac toxicity.[12] In the ALK5 crystal structures, the kinase domain is observed in an active conformation, with the position of the αC-helix positioned to allow the direct salt bridge between Lys232 and Glu245 (corresponding to Lys235 and Glu248 in ALK2). The Asp351 ALK5 side chain is not constrained by interaction with the activation loop (Figure 3). The back pocket of ALK5 is larger, due to the smaller gatekeeper Ser280 versus Thr283 in ALK2, and toward the solvent channel, the larger Ile211 replaces Val214. In the P-loop, wherein ALK2 Tyr219 has been shown to interact with inhibitors, is Phe216.

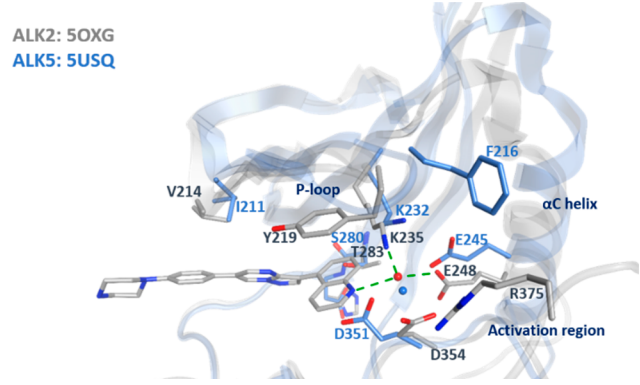

Figure 3. Binding of LDN-212854 to ALK2 (PDB ID: 5OXG, gray) overlay with ALK5 structure (PDB ID: 5USQ, blue, ligand not shown).

LDN-212854 has greater selectivity for ALK2 over ALK5 than LDN-193189 which has been attributed by Williams et al. to the shift in position of Lys232 and Glu245 (corresponding to Lys235 and Glu248 in ALK2) and the water network surrounding these residues.[13] The water bridged interactions formed from LDN-212854 to Lys235 and Glu248 in ALK2 (Figure 3) would not be favored by the conformation and water position observed in ALK5. Currently, there is no 3D structure for ALK4. ALK4 has a very high sequence homology

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to ALK5, and therefore, many ALK2 inhibitors possess a similar affinity to both these TGF-β receptors.

Achieving selectivity over BMP receptors ALK1, 2, 3, and 6 has been more challenging for ALK2 inhibitor development. Comparisons of the ALK2 binding pocket with ALK1 and ALK6 can be made to cocrystal structures of these subtypes with LDN-193189. The identity and positioning of side chains within the ALK1 pocket (PDB ID: 3MY0) is an excellent match for ALK2. Differences are only present in residues on the edge of the pocket, making it challenging to rationalize any selectivity observed. The ALK6 binding pocket is more distinct, due to differences in residues 234−239 (corresponding to ALK2 residues 238−243) leading into the αC helix. The αC helix is shifted (Figure 4); the P-loop is folded down, altering the position of the ALK6 Lys231. No 3D structure for ALK3 is available; however, ALK3 has a very high homology with ALK6.

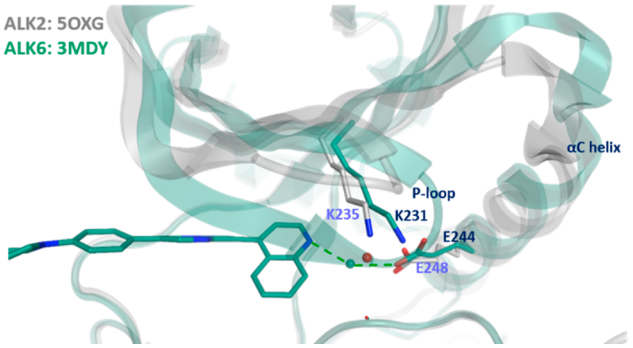

Figure 4. Binding of LDN-193189 to ALK6 (PDB ID: 3MDY, green) and comparison with ALK2 (PDB ID: 5OXG, gray, ligand not shown).

■ ADVANCES IN ALK2 INHIBITORS

Pyrazolo[1,5-*a*]Pyrimidines. The pyrazolo[1,5-*a*]-pyrimidine based compounds already described have moderate to excellent selectivity over the TGF-β receptors; however, they have limited selectivity over BMP receptors. To address this, Jiang et al. explored the replacement of the quinoline with sulfonyl containing moieties to identify the 4-sulfamoylnaphthyl **1** as a selective ALK2 inhibitor (Figure 5).[14] Computational

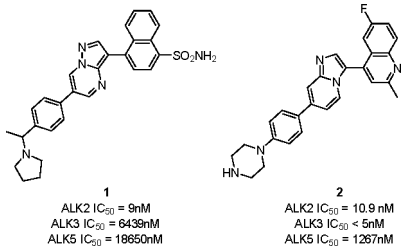

Figure 5. Structure and reported IC_{50} data of sulfamoylnaphthyl and imidazo[1,2-*a*]pyridine ALK2 inhibitors.[14,15]

modeling suggested the oxygen atoms of the sulfamoyl form direct interactions with the Lys235 side chain amino group and the N−H of the backbone amide of Asp354. High selectivity was achieved over all related BMP and TGF-β receptors except for ALK1, and **1** possessed an acceptable *in vivo* pharmacokinetic profile.

Scaffold replacement has been explored by Engers et al. to identify the imidazo[1,2-*a*]pyridine as a suitable alternative.[15] Exploration around the quinoline and amine groups focused on improving the metabolic stability. A number of heterocyclic replacements for the quinoline were investigated before finding that blockage of the 2-position of the quinoline reduced the metabolic liability. Compound **2** (Figure 5) demonstrated a similar selectivity profile for BMP/TGF-β receptors to LDN-193189 and is highly potent against ALK2 R206H. **2** is orally bioavailable in rats and showed a favorable brain:plasma ratio of 1.6.

Macrocyclization of the pyrazolo[1,5-*a*]pyrimidine core achieved higher kinome-wide selectivity demonstrated by OD-36 and OD-52 (Figure 6) in studies by Sánchez-Duffhues

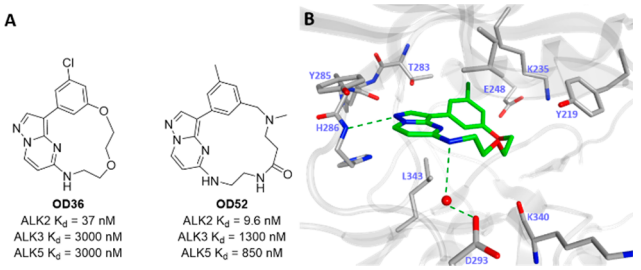

Figure 6. (A) Structures and reported K_d values for OD36 and OD52.[16] (B) ALK2 binding mode of OD36 (PDB ID: 5OY6).

et al.[16] This advance in selectivity also extended to kinase receptors in the BMP and TGF-β pathways, most notably for the BMP Type I receptor ALK3 and the Type II receptor ACVR2B. Both compounds showed enhanced *in vitro* activity at ALK2 (R206H) over the wild-type. OD-36 binds to ALK2 in a similar alignment of the core pyrazolo[1,5-*a*]pyrimidine to LDN-212854 to form the key hinge H-bond interaction to His286. The linker of OD-52 can provided additional interactions with Lys235 and Tyr219.

Pyridines. Pyridine derivatives have been progressed through an open science model to provide advanced preclinical compounds.[17a] Thorough investigation of substitution at the pyridyl 4-position established the methyl group to give the most favorable profile for potency and selectivity over ALK5 (M4K2009, Figure 7). Limited modification of the trimethoxyaryl group was tolerated. Replacement of the 4-methoxy with 4-fluoro (M4K2163) maintained ALK2 potency and reducing the polarity of the compound increased the brain:plasma ratio. More information on the brain penetration of this series was obtained through positron emission tomography (PET)

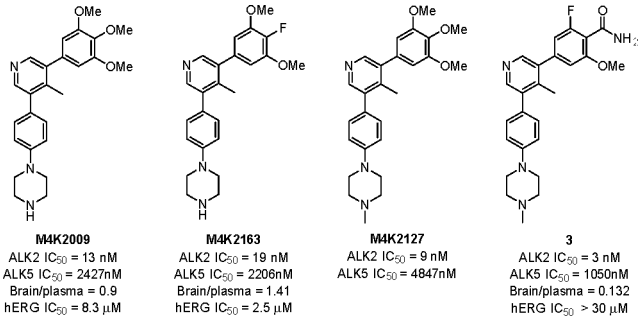

Figure 7. Structures of advanced pyridine analogues and reported IC_{50} data.[17a,c]

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neuroimaging of ^{11}C-radiolabeled M4K2009, M4K2163, and a related analogue M4K2127.[17b] Of these, M4K2127 showed good permeability into the brain as well as homogeneous distribution into the pons, the region of interest for DIPG, though low metabolic stability limits its further development.

Alternatively, the increased polarity of benzamide analogue **3** was able to reduce moderate off-target affinity for the hERG ion channel.[17c] Comparable potency was seen between wild-type ALK2 and ALK2 mutants for M4K2009 (R206H, G328V, R258G, G356D, Q207D) and **3** (R206H, G328V, and R258G). M4K2009 and M4K2163 also demonstrated significant growth inhibition of patient-derived *ACVR1* and wild-type cell lines HSJD-DIPG-007 and SU-DIPG-XXI. A cocrystal structure of a benzamide analogue with ALK2 (PDB ID: 6T6D) demonstrated direct binding of the amide carbonyl with Lys235.

Quinazolinones. Two divergent series of quinazolinones were developed by Hudson et al. from a single hit from *in vitro* screening of a focused kinase fragment library.[18] Exploration of 2-, 3-, and 5-substitution around the quinazolinone core of the hit showed a tolerance to substitution inconsistent with a single hinge binding mode. Expansion of the SAR at the 3-position showed a gain in potency in compound **4** (Figure 8A)

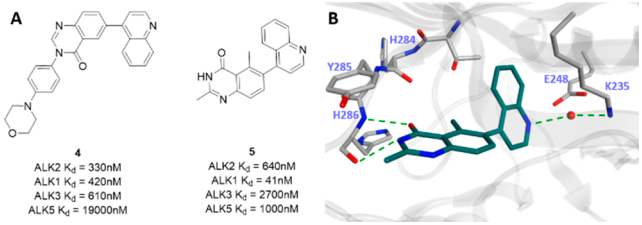

Figure 8. (A) Structures and reported K_d data of advanced quinazolinones.[18] (B) Binding mode of **5** to ALK2 (PDB ID: 6GIP).

by extending out toward the solvent channel with a 4-morpholinophenyl group. A cocrystal structure of **4** (PDB ID: 6GIN) confirmed a binding mode analogous to LDN-193189, and **4** has high selectivity over ALK4/5 but similar affinity for ALK1/3/6.

Compound **5** with substitution at the 2- and 5-positions of the quinazolinone had an alternative binding mode confirmed by cocrystal structure (Figure 8B). The quinazolinone core interacts with the hinge through both the amide donor and acceptor. The quinoline also flips to maintain a water bridged interaction with Lys235 and sits further into the back of the pocket, a region larger in ALK5. This may explain the reduced selectivity for **5** over ALK4, 5. Interestingly, **5** shows a distinction between ALK1 and ALK2 affinity and demonstrates that selectivity can be achieved between these highly similar binding pockets.

Pyrazoles. Pyrazole-based inhibitors have been developed from the hit RK-59638 (Figure 9A), identified from the screening of ligand and structure based *in silico* hits from the Drug Discovery initiative compound library at the University of Tokyo.[19a] RK-59638 interacts with ALK2 R206H via the aminopyrimidine forming hydrogen bond acceptor and donor interactions with His286 and through the water network surrounding Lys235/Glu248 to bind both the nitrogens of the pyridine and pyrazole rings. A fused morpholinopyrazole **6** was designed to make additional CH−π interactions with Tyr219 and water mediated hydrogen bonds across to Ser290 (Figure 9B).[19b] Addition of *N*-methoxyethylpiperazine group increased

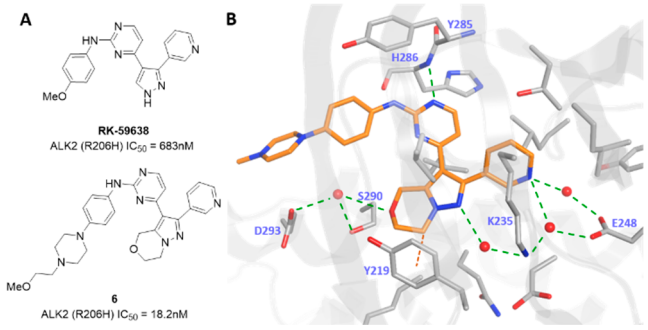

Figure 9. (A) Structure of pyrazole-based inhibitors and reported ALK2 IC_{50} data.[19b] (B) Binding of **6** to ALK2 (PDB ID: 7C3G).

potency without MDR1 efflux, and compound **6** was highly potent in cellular ALK2 assays. An earlier analogue of RK59638 was reported as nonselective over ALK4,5, and it has not been stated whether the morpholinopyrazole provides any selectivity.

Other Chemotypes. There is an emerging number of patents describing ALK2 inhibitors with Incyte[20a] and Keros[20b] containing a pyrazolo[1,5-*a*]pyrimidine or related core, and Novartis describing additional pyridine based compounds (Figure 10).[20c,d] Patents from Tolero,[20e] Merck,[20f] Biocryst,[20g]

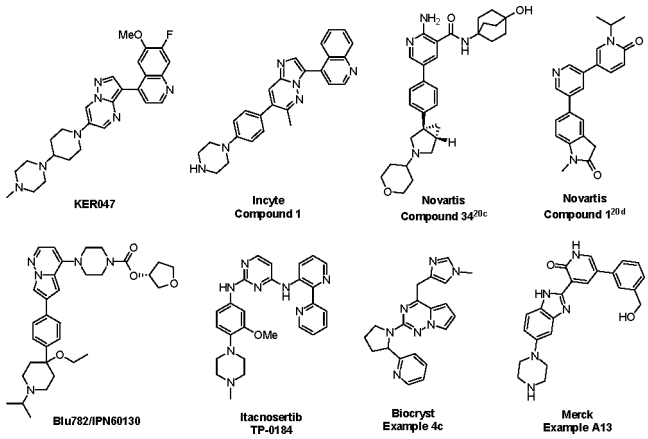

Figure 10. Examples of ALK2 inhibitors described in patents.

and Blueprint[20h] show greater diversity in chemotypes. This has translated into clinical research with Phase 1 clinical trials successfully completed for Blu782(IPN60130), KER047, and BCX9250 (structure unknown), and progressing for Itacnosertib (TP-0184) and INCB00928 (structure unknown).

Encouraging to the field is repurposing of kinase inhibitor drugs found to additionally inhibit ALK2. The covalent MEK1/2 inhibitor E6201 (Figure 11A/B) is a reversible inhibitor of ALK2 which binds to the hinge His286 via the phenol, and the adjacent carbonyl interacts with Thr283.[21] Selectivity over ALK subtypes has been attributed to its occupation of the region toward the P-loop. E6201 has demonstrated *in vivo* efficacy in prolonging survival of mice xenografted with ACVR1 R206H mutant HSJD-DIPG-007 or SU-DIPG-XXXVI.

The dual SRC/ABL inhibitor Saracatinib (Figure 11A) is a potent ALK2 inhibitor selective over ALKs 3, 4, 5, and 6.[22] The cocrystal structure of Saracatinib with ALK2 (PDB ID: 6ZGC) suggests opportunities to develop this chemotype for specific interactions with ALK2. Saracatinib is efficacious *in*

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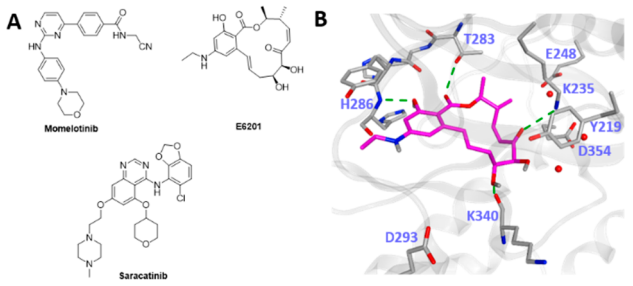

Figure 11. (A) Kinase inhibitor drugs with ALK2 inhibition. (B) Binding of E6201 to ALK2 (PDB ID: 6I1S).

vivo in an *ACVR1*^{Q207D}*-Tg* model of heterotopic bone formation and is progressing into a Phase II study for prophylaxis for FOP progression.

Momelotinib (Figure 11A), a JAK1/2 inhibitor, is equipotent at ALK2 and moderately selective over ALK subtypes.[23] Momelotinib (PDB ID: 7NNS) and a Blu782 analogue (PDB ID: 6T8N) bind in a similar manner to ALK2 through direct binding of the carbonyl moieties with Lys235 and preserving a network of four water molecules within the pocket. Both Momelotinib and Saracatanib however have shown only minimal effects on DIPG cells containing mutant or wild-type *ACVR1*.[10]

CONCLUSIONS

The past few years has seen an increase in the number of publications describing ALK2 inhibitors with the identification of a greater diversity of chemotypes. With this has come examples of compounds demonstrating that high selectivity can be gained across not only the kinome but the majority of similar BMP and TGF-β receptors. Within the series, properties can be modulated to ensure oral bioavailability and, where desired, CNS penetration. ALK2 inhibitors have demonstrated efficacy *in vivo* in preclinical models of FOP and DIPG. Now, as ALK2 inhibitors enter clinical studies a clearer understanding will be gained as to how this research translates into clinical efficacy.

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Author Contributions

The manuscript was written by L.R. with the introduction by C.J.

Notes

The authors declare no competing financial interest.

Biographies

Lisa Rooney received her Ph.D. in Organic Chemistry from the Australian National University in 2004. Following postdoctoral training with Professor Samir Zard at École Polytechnique, she worked in the field of medicinal chemistry at the Novartis Institutes for Biomedical Research for 8 years. She has lectured in medicinal chemistry at Queen Mary University of London and is currently a Daphne Jackson Fellow at the Institute of Cancer Research using computational methods to aid the design of small molecules to target paediatric brain tumours.

Chris Jones is Professor of Childhood Brain Tumour Biology at the Institute of Cancer Research in London. He received his PhD in Molecular Biology at University College London. His lab focuses on diffuse high-grade glioma in children, and is engaged in genomic profiling, model development and preclinical screening. He is founding Chair of the SIOP Europe High Grade Glioma Biology Group, Preclinical Lead of the international CONNECT Consortium, and Steering Committee Member for ITCC-Brain.

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ABBREVIATIONS

ALK2, activin receptor-like kinase 2; BMP, bone morphogenetic protein; CNS, central nervous system; DIPG, diffuse intrinsic pontine glioma; FEP/H-REMD, free energy perturbation coupled with Hamiltonian replica-exchange molecular dynamics; FOP, fibrodysplasia ossificans progressive; SAR, structure−activity relationship; TGF-β, transforming growth factor-β

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