Pharmacology & Therapeutics 191 (2018) 123–134

Contents lists available at ScienceDirect

Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/pharmthera

Structural and clinical consequences of activation loop mutations in class III receptor tyrosine kinases

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ARTICLE INFO

Available online 30 June 2018

Keywords:
KIT
PDGFRA
Kinase inhibitor
Activation loop mutation
Type I kinase inhibitor
Kinase inhibitor resistance

ABSTRACT

Mutations within the activation loop of members of the class III receptor tyrosine kinase (RTK) subfamily, which includes KIT, PDGFRA, and FLT3, have been observed in multiple human tumor types. These mutations confer constitutive activation as well as resistance to the type II tyrosine kinase inhibitors (TKI) that are currently clinically available, such as imatinib and sunitinib. It is now understood that activation loop mutations in class III RTKs shift the activation state equilibrium away from inactive states, to which type II TKIs bind, to the active state by destabilizing the inactive conformation. Recently, type I TKIs, which can bind to active kinase conformations, have been developed with specificity for class III RTK members. Some type I TKIs, such as crenolanib and avapritinib (BLU-285), have entered clinical studies for patients with activation loop mutations in KIT, PDGFRA, or FLT3. Preliminary results suggest that these type I TKIs show activity in these patient populations that previously lacked effective treatments. This article reviews the inactive and active structures of KIT, PDGFRA, and FLT3, how the mutations seen in human cancers affect kinase structure, and the clinical implications of these mutations in terms of type I vs. type II TKI binding.

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Contents

1. Introduction/overview

The class III receptor tyrosine kinase (RTK) subfamily includes KIT, platelet derived growth factor receptor α (PDGFRA) and β (PDGFRB), colony stimulating factor 1 receptor (CSF1R), and fms-like tyrosine kinase 3 (FLT3). Activating mutations in multiple members of the class III RTK family have been shown to drive human tumors, most prominently, KIT, PDGFRA, and FLT3. KIT mutations drive the majority of mastocytosis and GIST (Bannon, Klug, Corless, & Heinrich, 2017; Valent et al., 2017), but also subsets of seminoma, melanoma, and AML (Beadling et al., 2008; Care et al., 2003; Kemmer et al., 2004). PDGFRA point mutations are seen in a minority of GIST cases (Bannon et al., 2017) and are rarely seen in hematologic malignancies. Some cases of hypereosinophilia are associated with PDGFRA fusion translocation events, most notably FIP1L1-PDGFRA (Gotlib et al., 2004). Activating mutations in FLT3 are commonly observed in patients with leukemia, particularly acute myeloid leukemia (AML) (Care et al., 2003; Lim, Dubielecka, & Raghunathan, 2017).

Abbreviations: JM, juxtamembrane; GIST, gastrointestinal stromal tumor; RTK, receptor tyrosine kinase; TKI, tyrosine kinase inhibitor; PDGFRA, platelet derived growth factor receptor α; PDGFRB, platelet derived growth factor receptor β; FLT3, fms-like tyrosine kinase 3; CSF1R, colony stimulating factor 1 receptor; AML, acute myeloid leukemia; SM, systemic mastocytosis; DFG, Asp-Phe-Gly protein motif; Aspβ9, Asp residue within the β9 sheet of the activation loop; PFS, Progression-free survival.

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https://doi.org/10.1016/j.pharmthera.2018.06.016
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The cellular dependence on the constitutive activity of these enzymes can be exploited for the treatment of cancer patients. The majority of FDA-approved small molecule kinase inhibitors are classified as type I or type II, which bind to the catalytic site and compete with a kinase's natural co-substrate, ATP (Wu, Nielsen, & Clausen, 2015, 2016). There are two types of these molecules that differ in their ability to bind particular kinase domain conformations, which can change dramatically between inactive and active states. Type I kinase inhibitors can bind the active conformation of a kinase, whereas type II kinase inhibitors only bind inactive conformations (Wu et al., 2015; Zuccotto, Ardini, Casale, & Angiolini, 2010). Activating mutations of KIT, PDGFRA/B, or FLT3, or secondary mutations that confer drug resistance seen in human tumors can affect kinase domain conformations, and thus, the efficiency of small molecule kinase inhibitors binding.

Understanding of how recurrent mutations affect kinase domain structure is important for designing effective cancer treatments. This review focuses on the structural activation of these RTKs and how structure modulates kinase inhibitor binding. In addition, we also review how each activation loop mutation in this class of RTKs causes kinase inhibitor resistance and recent advances in drug design guided by structural activation state information to overcome these mutations.

2. Class III RTKs

2.1. Function

The class III RTK subfamily includes KIT (Besmer et al., 1986; Yarden et al., 1987), PDGFRA (Claesson-Welsh, Eriksson, Westermark, & Heldin, 1989) and PDGFRB (Yarden et al., 1986), CSF1R, also known as c-fms (Sherr et al., 1985), and FLT3 (Matthews, Jordan, Wiegand, Pardoll, & Lemischka, 1991; Rosnet, Mattei, Marchetto, & Birnbaum, 1991). These transmembrane receptors and their ligands play important roles in growth and maintenance of cells and tissues. FLT3 and KIT are both expressed by and maintain hematopoietic progenitor and stem cells (Matthews et al., 1991; Okada et al., 1991). KIT is also important for the development and differentiation of mast cells (Nocka, Buck, Levi, & Besmer, 1990), the interstitial cells of Cajal (ICC, the pacemakers of the gut) (Maeda et al., 1992), melanocytes (Natali et al., 1992), and primordial germ cells (Dolci et al., 1991). CSF1R primarily supports the development of the monocyte/macrophage lineage (Stanley & Chitu, 2014). PDGFRA is critical for the development of various mesenchymal cell populations, supporting and maintaining mesenchymal precursors in kidney, intestine, skin, and lung alveolar smooth muscle, among others (Tallquist & Kazlauskas, 2004). PDGFRB is important primarily for the development and physiology of vascular smooth muscle cells and pericytes (Tallquist & Kazlauskas, 2004).

Each of the class III RTKs binds a dimeric ligand, which induces receptor dimerization and initiates signaling to downstream pathways that support cell proliferation and survival. KIT and FLT3 each have exclusive homo-dimeric ligands, stem cell factor (SCF) and FLT3 ligand (FLT3L), respectively (Lev, Yarden, & Givol, 1992; Lyman et al., 1994), and the receptors themselves also homo-dimerize (**Fig. 1**). CSF1R is also thought to exclusively homo-dimerize, but can bind two independent homo-dimeric ligands, CSF1 or IL-34 (Ma et al., 2012; Yeung & Stanley, 2003). Upon ligand binding, PDGFRA and PDGFRB are each capable of homo-dimerization or hetero-dimerization with one another. PDGFRA and PDGFRB can bind various dimeric ligands made up of PDGF-A, -B, -C or -D monomers. PDGF-A and PDGF-B can form homo- or hetero-dimeric ligands, while PDGF-C and PDGF-D only form homo-dimeric ligands (Claesson-Welsh, 1994). These PDGF ligand dimers bind PDGFR complexes differently depending on the dimerization partners comprising the receptor complex (see **Fig. 1**).

After ligand binding and dimerization, signal transduction is initiated through phospho-tyrosine-dependent interactions between the receptor and effector scaffold proteins. Auto-phosphorylation of tyrosine residues on the receptor, serve as docking sites for phospho-binding proteins, such as PI3K, GRB2, SHC, SRC, and/or PLCγ (Claesson-Welsh, 1994; Dosil, Wang, & Lemischka, 1993; Lennartsson & Ronnstrand, 2012; Lev, Givol, & Yarden, 1991, 1992). These effectors then activate downstream signaling cascades through AKT, JAK/STAT, and MEK/ERK, which ultimately drive cell-specific proliferative transcriptional programs and support growth and survival (Claesson-Welsh, 1994; Dosil et al., 1993; Lennartsson & Ronnstrand, 2012).

2.2. Structure

Because of their role in initiating and rapidly amplifying cell signaling pathways that drive proliferation, the kinase activity of class III RTKs activity is tightly controlled, in large part by regulation of their protein structure. Class III RTKs have a shared overall structure; they are single-pass membrane receptors with an extracellular ligand-binding domain and an intracellular kinase domain. These RTKs are all between 950 and 1100 amino acids in length, with ~550 extracellular residues and 400-550 intracellular residues. The overall protein identity within this subfamily is 25-40%. However, there is greater identity within the intracellular region (45-55%), especially within the kinase domain (63-85%) (Schwab & SpringerLink, 2011) (**Fig. 2**). The solved kinase domain crystal structures demonstrate a high level of three-dimensional homology within this family and indicate that the transition from inactive to active conformation progresses by a common mechanism (see **Fig. 3**) (Griffith et al., 2004; Liang, Yan, Yin, & Yun, 2016; Mol et al., 2003; Mol et al., 2004; Schubert et al., 2007).

The extracellular domain of class III RTKs is made up of five immunoglobulin (Ig)-like domains with cysteine residues that form paired intramolecular disulfide bonds (**Fig. 1**) (Lokker et al., 1997; Majumder, Brown, Qiu, & Besmer, 1988; Yarden et al., 1986). The N-terminal Ig-like motifs (domains 2-3) participate in binding of ligands and C-terminal motifs (domains 4-5) mediate extracellular receptor dimerization (Verstraete & Savvides, 2012).

The intracellular region of class III RTKs comprises the cis-regulatory juxtamembrane (JM) domain and the kinase domain, which includes the catalytic site (where ATP and substrate bind) and the activation loop (**Fig. 1**). Together, these structures and their relative conformations control kinase activity. The kinase domain of class III RTKs produces a typical kinase fold seen in the majority of kinases; this fold is made up of a larger, mostly helical carboxy-terminal (C) lobe and the smaller amino-terminal (N) lobe, which is primarily made up of beta sheets (**Fig. 3**). The cleft formed between these lobes defines the catalytic site in all kinases, where ATP binds a hydrophobic pocket to orient its γ phosphate towards the substrate (Huse & Kuriyan, 2002).

The JM domain (~40 residues) serves an autoinhibitory function in class III RTKs by looping to insert into the catalytic pocket between the N and C lobes in the inactive form, preventing active state conformational changes and substrate binding (red loop, **Fig. 3**). The activation loop is another critical regulatory domain. The conformation of this domain controls access to the catalytic site and activity of the kinase. The activation loop of class III RTKs spans ~27 residues from the conserved aspartic acid-phenylalanine-glycine (DFG) sequence to the alanine-proline-glutamic acid (APE) sequence (Dibb, Dilworth, & Mol, 2004; Huse & Kuriyan, 2002; Johnson, Noble, & Owen, 1996). Amino acid sequence similarity of the activation loop in class III RTKs is greater than 70% (**Fig. 2B**). Moreover, the protein structure of this region is almost identical between KIT, PDGFRA, and FLT3 (blue loop, **Fig. 3**).

2.3. Kinase Activation

Kinases have two extreme states: on or off. Multiple layers of autoinhibition serve as barriers to prevent aberrant kinase activity. Wild type isoforms of class III RTKs exist at equilibrium between conformations (**Fig. 4**). Ligand binding, ATP binding, and phosphorylation events drive transitions between these states. The primary structures that determine kinase activation state are the JM domain and the activation

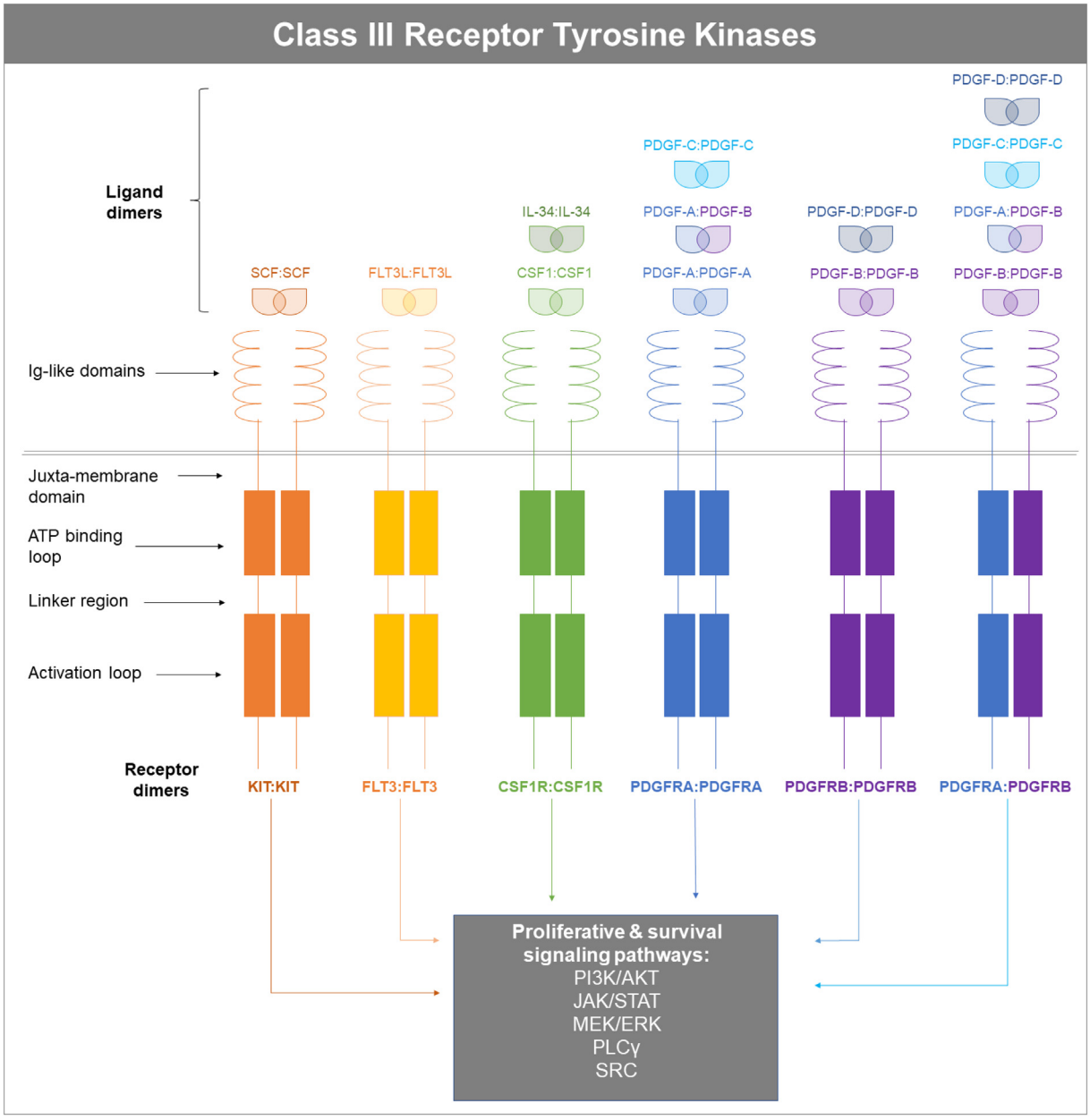

Fig. 1. Basic structure of the members of class III RTK family and their respective ligands. Dimeric ligand is bound via extracellular Ig-like domains to induce receptor dimerization, which activates the intracellular split kinase domain (solid rectangles) to then transmit signal downstream to various pathways that promote proliferation and survival in the cell. Ligands bound by members of the class III RTK family are dimeric. KIT receptor binds SCF and FLT3 receptor binds FLT3L to induce homodimerization and kinase activity. CSF1R homodimerization can be induced by binding CSF1 or IL-34. Four PDGF monomers can dimerize as homo-, or heterodimers in the case of PDGF-A and PDGF-B, to bind homo- or heterodimeric PDGFRA and PDGFRB receptors. The kinase domain of class III RTKs is distinct from most kinases in that the kinase domain is interrupted by a linker region (50-70 amino acids), which extends from the C lobe and is important for binding and signaling through downstream effectors, but does not greatly impact kinase domain structure (Gajiwala et al., 2009).

loop. The auto-inhibited (state I, **Fig. 4**) and non-auto-inhibited (state II, **Fig. 4**) conformations are both considered inactivated states, but differ in phosphorylation status, and thus, conformation of the JM domain. The two DFG-in, activated states (states III-IV, **Fig. 4**) differ in the overall stability of the activation loop, which can be "locked", by tyrosyl-phosphorylation within the activation loop. However, unlike other kinases, activation loop phosphorylation is not required for kinase activation (DiNitto et al., 2010; Foster, Griffith, Ferrao, & Ashman, 2004).

In an auto-inhibited, inactive state, class III RTKs are unphosphorylated at all tyrosine residues, the JM domain is docked, and the activation loop is in the DFG-out conformation (state I, **Fig. 4**). The entrance to the catalytic site is occluded, impeding ATP binding within the pocket (Gajiwala et al., 2009; Mol et al., 2004). This is predominantly achieved by docking of the JM domain within the cleft between the kinase domain lobes. The JM domain makes multiple contacts with the activation loop and the αC helix (the largest helix in the N lobe) when docked serves to stabilize the inactive form (Dibb et al., 2004). Multiple conserved residues within this domain are critical for making the hydrophobic interactions when docked (**Fig. 2A**). These multiple interactions which dock the JM in the inactive auto-inhibited conformation are energetically favored and require the greatest activation energy to overcome, preventing spontaneous activation of monomeric or unbound wild type receptors (Wodicka et al., 2010).

The activation process of a wild type class III receptor at the cell surface is initiated upon binding of dimeric ligand, which induces receptor dimerization. Receptor dimerization leads to low level kinase activity of each receptor allowing tyrosine phosphorylation of the JM domain in trans (**Fig. 2A**) (DiNitto et al., 2010). Phosphorylation at four tyrosine

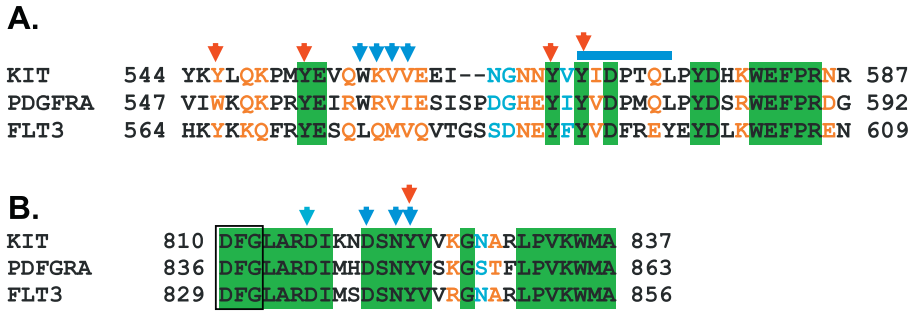

Fig. 2. Protein alignment of critical cis-inhibitory regions of class III RTKs. **A.** Juxtamembrane domain protein alignment. **B.** Activation loop protein alignment. Green highlighting indicates complete conservation. Orange text indicates conservation between groups of strongly similar properties. Blue text indicates conservation between groups of weakly similar properties. Red arrows indicate tyrosine phosphorylation sites. Blue arrows indicate common mutation sites. Blue line in A. indicates most common residues involved in FLT3 ITD. Light blue arrow in B. marks Aspβ9, and black box denotes the activation loop DFG sequence.

residues disrupts the contacts that stabilize the docking of JM domain, removing it from hindering the catalytic site (resulting in a non-auto-inhibited, inactive kinase conformation, state II, **Fig. 4A**). Auto-phosphorylation of JM occurs in trans, as the phosphorylation of the JM tyrosine residues in cis has been shown to be impossible given the distance between these residues and the catalytic domain in KIT crystallized structures (Mol et al., 2003). Phosphorylation of the JM in class III RTKs is a major step in kinase activation, but in isolation is not sufficient for activation.

The remaining steps to full activation are controlled by the activation loop, a flexible structure of the kinase domain that is highly conserved within the class III RTK family (**Fig. 2B**). The conformation of this loop is primarily controlled by the orientation of the side chains of the DFG triad. In the inactive state, the activation loop resides in the DFG-out conformation with the phenylalanine oriented into the ATP-binding pocket, and the aspartic acid of the DFG oriented away (**Fig. 5A**) (Gajiwala et al., 2009; Liang et al., 2016). This conformation is stabilized by hydrogen bonding between activation loop residues with those in the JM domain, the αC helix, and within the activation loop itself.

The aspartic acid six residues C-terminal from the DFG, termed Aspβ9 (it lies within β sheet 9 in the active conformation), plays a critical role in stabilizing each conformation of the activation loop (**Fig. 2B**). The Aspβ9 residue (KIT D816, PDGFRA D842, FLT3 D835) is exposed to solvent on the exterior of the protein in the inactive conformation; its charge stabilizes the dipole moment of an adjacent helix (η7 3_{10}), which is made up of the adjacent four residues, to support the DFG-out conformation of the activation loop (Dibb et al., 2004;Foster et al., 2004; Liang et al., 2016). Upon ATP binding these interactions are disrupted and the activation loop swings outward to the DFG-in orientation (Foster et al., 2004); Aspβ9 pairs with the β6 strand (which paired with the JM domain in the auto-inhibited conformation), to form the β6β9 sheet, which stabilizes the activation loop in its open conformation (Dibb et al., 2004). These critical roles of the Aspβ9 residue are conserved among class III RTKs (Dibb et al., 2004; Liang et al., 2016; Mol et al., 2004).

The tyrosine residue within the activation loop of class III RTKs (KIT Y823, PDGFRA Y849, FLT3 Y842, **Fig. 2B**) serves as a pseudo-substrate, hydrogen-bonding with the catalytic base (KIT D792, PDGFRA D818, FLT3 D811) in the DFG-out conformation to block access to the catalytic site (DiNitto et al., 2010; Foster et al., 2004). In most kinases, phosphorylation of the pseudo-substrate residue within the activation loop drives activation of the kinase (Dibb et al., 2004). However, phosphorylation of this tyrosine of KIT (Y823) has been shown to be one of the last phosphorylation events and can occur after the activation loop has opened, suggesting it does not further increase KIT kinase activity (DiNitto et al., 2010; Foster et al., 2004). Instead, phosphorylation of this tyrosine only further stabilizes the DFG-in conformation of the activation loop. The unphosphorylated tyrosine is capable of making contacts to stabilize the active conformation, but the interactions with the phosphotyrosine are more energetically favored (Foster et al., 2004). Moreover, mutation of this residue has been observed in tumors, suggesting its phosphorylation is not required for activity (Heinrich et al., 2006).

Removal of auto-inhibition by the JM domain allows for binding of ATP in the catalytic pocket, which promotes the dynamic conformational change of the DFG, swinging the phenylalanine of the DFG outward and orienting the catalytic aspartic acid in towards the catalytic site (**Fig. 5**) (Foster et al., 2004; Liang et al., 2016). This causes the activation loop to extend outward, allowing substrates access to the catalytic site (DiNitto et al., 2010; Gajiwala et al., 2009). Trans-phosphorylation of the dimeric partner receptors at multiple tyrosine residues serve as docking sites for phospho-tyrosine binding proteins that transmit signaling down signaling pathways. Activation of wild type receptors by ligand binding is quickly downregulated to prevent extensive signaling. The activated pathways that promote proliferation also signal to negative feedback loops that dephosphorylate and internalize receptors to shut down kinase activation.

2.4. The role of activating KIT, PDGFRA, or FLT3 mutations in cancer

Activating mutations in multiple members of the class III RTK family have been shown to drive human tumors. KIT mutations drive greater than 80% of cases of mastocytosis and mast cell leukemia (Valent et al., 2017; Verstovsek, 2013) and 75% of gastrointestinal stromal tumors (GIST) (Bannon et al., 2017). In addition, KIT mutations are also known to drive oncogenesis in subtypes of seminoma, melanoma, and acute myeloid leukemia (AML) (Beadling et al., 2008; Care et al., 2003; Kemmer et al., 2004). Activating mutations in FLT3 are observed in approximately 30% of patients with AML, both adults and children (Alvarado et al., 2014; Yamamoto et al., 2001). Intragenic, in frame PDGFRA mutations are seen in 10% of GIST cases (Bannon et al., 2017) and can be rarely found in hematologic malignancies. Some cases of hypereosinophlia are associated with PDGFRA fusion translocation events, most notably FIP1L1-PDGFRA (Gotlib et al., 2004). Fusions involving PDGFRB (ETV1-PDGFRB) have also been observed in a minority of hematologic malignancies, typically chronic myelomonocytic leukemia (Wlodarska et al., 1997). Finally, mutations in CSF1R are very rarely seen in cancer, but wild type CSF1R activation by amplification or hyperactivation has been shown to contribute to breast and ovarian cancer progression (Kacinski, 1997; Lin, Nguyen, Russell, & Pollard, 2001).

KIT and CSF1R were originally discovered as homologs of viral oncogenes (*v-kit* and *v-fms*, respectively) (Besmer et al., 1986; Donner, Fedele, Garon, Anderson, & Sherr, 1982). These viral oncoproteins are truncated versions of the cellular genes that have constitutive activity because they lack the cis-inhibitory regions found in the full-length genes and are constitutively dimerized. Similarly, constitutive activity of KIT, PDGFRA, or FLT3 in cancer is most commonly achieved by missense mutation, in-frame deletions/insertion, or gene fusion that interferes with the kinase auto-inhibitory regions (the JM domain or the

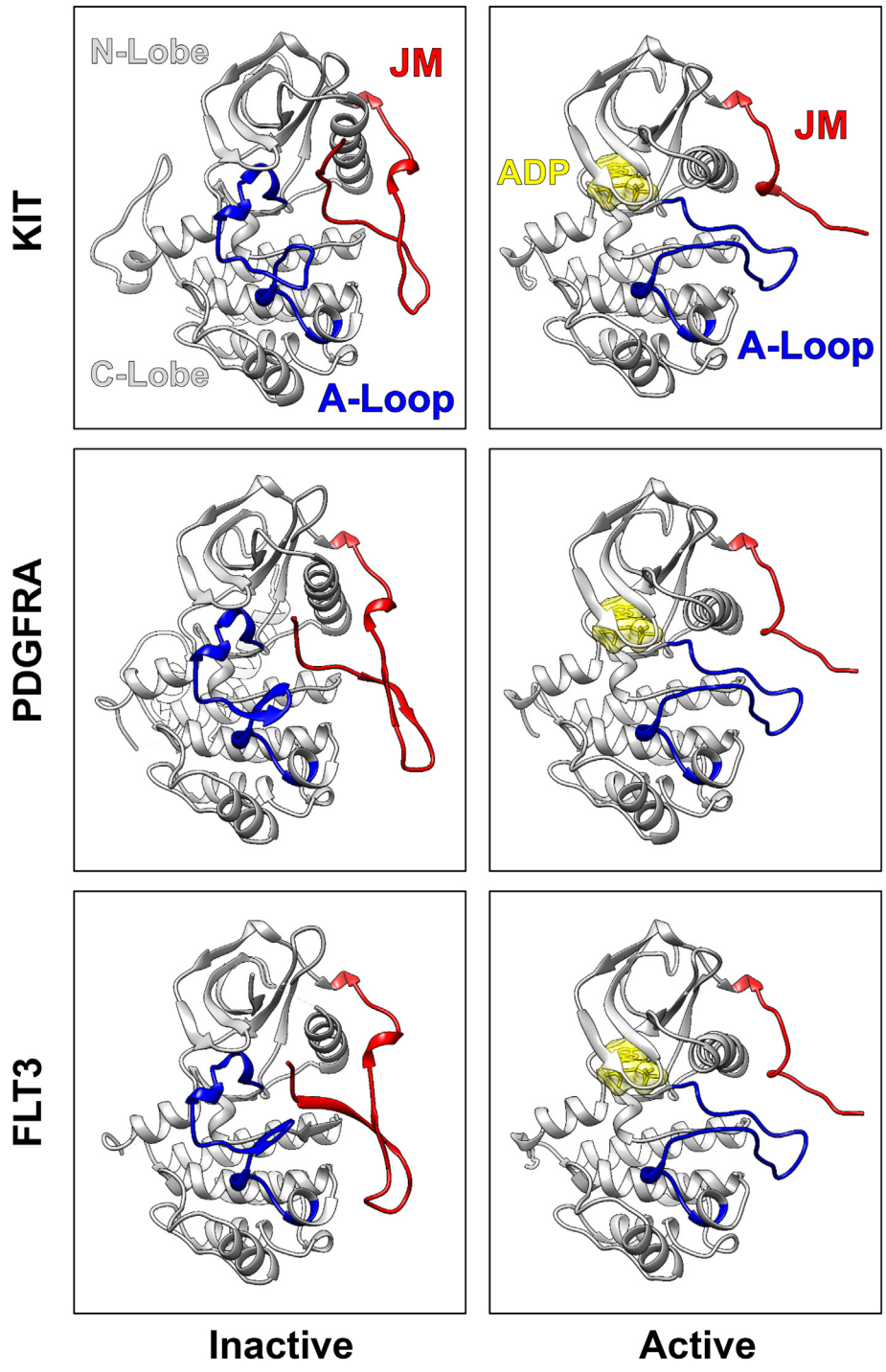

Fig. 3. Intracellular domain crystal structures display dramatic conformational changes during activation. Inactive crystal structures of KIT, PDGFRA, and FLT3 (PDB: 1T45, 5K5X, and 1RJB, respectively) are depicted left. The active confirmation of PDGFRA and FLT3, modeled using the crystal structure of active KIT (PDB: 1PKG) as template with SWISS-MODEL, are depicted right. The N lobe is smaller and mostly made up of beta sheets. The C lobe is larger and mostly helical. Juxtamembrane domain (JM) is shown in red. Activation loop (A-loop) is shown in blue. ADP (yellow) is bound in the active conformation.

activation loop). The mechanisms by which these cis-inhibitory regions are disrupted have different consequences for overall kinase structure and activation state equilibria.

2.5. Juxtamembrane (JM) domain mutations

Mutations affecting the JM domain of class III RTKs are known to cause constitutive kinase activation and thereby drive various human tumors. As discussed above, the JM domain stabilizes the inactive conformation through multiple contacts with both the activation loop and the αC helix near the catalytic site (Dibb et al., 2004). Deletion or point mutation of critical residues within the JM domain disrupt these interactions, destabilizing the auto-inhibited inactive state and shifting the equilibrium towards the non-auto-inhibited states (**Fig. 4B**) (Wodicka et al., 2010). JM mutations essentially eliminate the rate limiting initial step of kinase activation, and, thus, result in constitutive activation.

KIT JM mutations are the most common primary mutations seen in GIST, accounting for ~66% of GIST cases (Bannon et al., 2017). These include KIT point mutations and in-frame deletions that are commonly

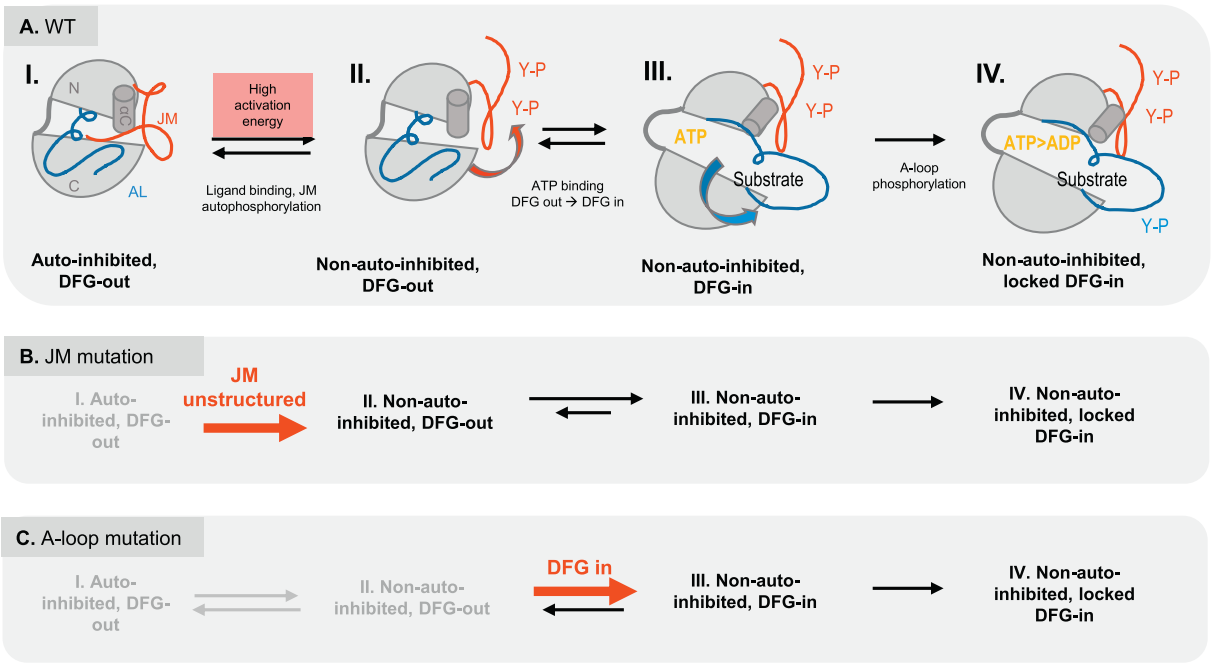

Fig. 4. Kinase domain conformation states for class III receptor tyrosine kinases. **A.** Wild type receptors exist at equilibrium between four states. Autoinhibition is achieved by looping of the JM domain into the catalytic site (I). Contacts that promote this conformation are disrupted by phosphorylation within the JM. At this point, the RTK is still in an overall inactive conformation, but is non-auto-inhibited and ATP can enter the catalytic pocket (II). Rotation of the DFG from an "out" to and "in" conformation induces a dramatic conformational change of the activation loop (AL) so it no longer occludes the catalytic site and substrates can gain access for phosphorylation. Kinase is active (III). The final step of activation loop tyrosyl-phosphorylation further stabilizes the activation loop in the DFG in conformation to promote activity (IV). **B.** Point mutations or deletions/insertions affecting the JM domain disrupt contacts between the catalytic pocket and the JM, which achieves a non-auto-inhibited conformation without phosphorylation. JM mutations skew the conformation equilibrium away from auto-inhibited to non-auto-inhibited, but still inactive. **C.** Mutations affecting the activation loop, particularly Aspβ9, destabilize the activation loop in the DFG-out conformation. Receptors with activation loop mutations greatly favor the active DFG-in conformation.

recurrent (V559D, V560D, W557-K558del, among others, **Fig. 2A**). JM point mutations or deletions are rarer in PDGFRA, but are seen in 2% of GIST patients (Bannon et al., 2017). PDGFRA JM point mutations most frequently affect codon V561, homologous to KIT V559, a critical residue that maintains the inactive conformation (**Fig. 2A**). FLT3 mutations drive about one third of AML cases and most bear in-frame duplications in the JM domain, termed FLT3-internal tandem duplication (FLT3-ITD, 15% of pediatric and 25% of adult AML) (S. H. Lim et al., 2017; Yamamoto et al., 2001)(Meshinchi et al., 2008; Schittenhelm et al., 2006). Internal tandem duplications of *FLT3* can vary in their location and length, but range from 3 to hundreds of nucleotides and most commonly result in the duplication of residues between Y591 and Y597 (**Fig. 2A**) (Meshinchi et al., 2008; Stirewalt et al., 2006).

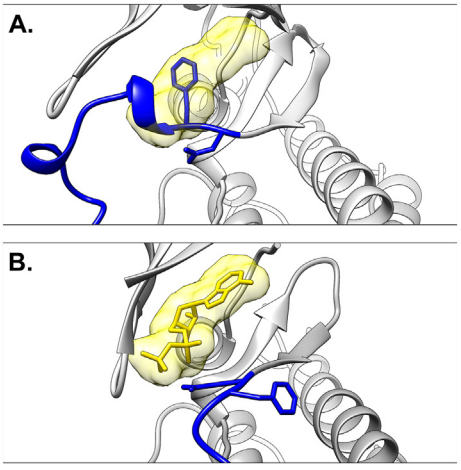

Fig. 5. Structural changes of the activation loop at the DFG. Zoomed in view of the crystal structure of the DFG in (A.) inactive KIT (PBD: 1T45) and (B.) active KIT (PDB: 1PKG). **A.** KIT F811 points into the ATP binding site (DFG out) in the inactive conformation, occluding ATP binding. **B.** KIT F811 rotates out and away from the ATP binding site (DFG-in) in the active conformation allowing binding of ATP to the catalytic site. Blue delineates the activation loop. Side-chains are shown for D810 and F811. ADP and its electron cloud are shown in yellow for the inactive conformation (B) and the ATP binding site is represented in the inactive conformation (B.) by showing the electron cloud from the active.

Class III RTK JM-mutant kinases become non-auto-inhibited, but are not found exclusively in the active form; the activation loop remains at equilibrium between the inactive DFG-out and the active DFG-in activation state (**Fig. 4B**), making these mutant proteins susceptible to inhibition by type II TKIs, which exclusively bind to the non-auto-inhibited, inactive conformation. Imatinib, a type II TKI, binds the non-auto-inhibited conformation of KIT and PDGFRA, and achieves great target specificity by utilizing a hydrophobic pocket adjacent to the ATP binding site that is only present in the inactive form (Liu & Gray, 2006; Zhang, Yang, & Gray, 2009). Other type II TKIs, such as sunitinib and quizartinib utilize this same strategy. One common mechanism of resistance to type II inhibitors is to minimize time spent in the inactive conformation, most often achieved by secondary mutation of the activation loop to promote the active conformation, as described below.

2.6. Activation loop mutations

The activation loop of kinases undergoes a dramatic conformational change to achieve full kinase activity (as discussed above, **Fig. 5**). Mutations within the activation loop, particularly in the N-terminal region of the activation loop, can result in constitutive activity in kinases other than class III RTKs because this structure often contains residues that are critical for stabilizing the inactive conformation (Dibb et al., 2004). As is seen in class III RTKs, missense mutations or insertions/deletions that disrupt key interactions can promote adoption of the active conformation, resulting in constitutive activity of the kinase (Dibb et al., 2004). The skewing of active state equilibrium from the DFG-out inactive state

to the DFG-in active state is a primary mechanism of constitutive activation of class III RTKs (**Fig. 4C**).

The most common missense mutations of the activation loop in KIT, PDGFRA, and FLT3 are observed at homologous residues, highlighting the conservation of structures and functional interactions between the residues in this region (**Fig. 2A**). Activation loop mutations in class III RTKs most often affect the Aspβ9 residue (KIT D816, PDGFRA D842, and FLT3 D835) (Abu-Duhier et al., 2001; Care et al., 2003; Valent et al., 2017; Yamamoto et al., 2001). Based on data deposited in the Catalog of Somatic Mutations in Cancer (COSMIC), 80-95% of activation loop point mutations seen in KIT, PDGFRA, or FLT3 affect Aspβ9 (**Fig. 6A**) (Forbes et al., 2017; Sanger Institute, 2018). Activating mutations affecting the Aspβ9 of CSF1R have also been reported to drive cell proliferation using *in vitro* cell models, but these mutations are rarely seen in cancer (Glover, Baker, Celetti, & Dibb, 1995; Reilly, 2002).

Aspβ9 is known to stabilize the inactive (DFG-out) conformation of the activation loop by stabilizing the dipole moment of the adjacent small helix (η7 3_{10}), which is conserved in class III RTKs (Dibb et al., 2004; Foster et al., 2004; Gajiwala et al., 2009; Liang et al., 2016; Mol et al., 2004) (**Fig. 3**). Mutation of the Aspβ9 position destabilizes this helix, allowing the flexible activation loop to move freely into the active conformation (**Fig. 4C**). In addition, the substitution of a hydrophobic residue (most commonly valine) for the hydrophilic aspartic acid forces this residue towards the interior of the protein, which additionally promotes the active conformation of the activation loop, perhaps explaining why this asp (D) to val (V) substitution at this position is most frequently observed in tumors (Dibb et al., 2004).

Activation mutations of PDGFRA primarily affect Aspβ9 (D842) or the surrounding residues that make up the η7 3_{10} helix. The point mutation D842V is most common, accounting for 7% of GIST. However, various in-frame deletions or compound deletions that involve D842 or the subsequent residues that make up the η7 3_{10} helix (in particular I843-S847) have also been observed (19.5% of activation loop mutations, **Fig. 6B**) (Corless et al., 2005; Forbes et al., 2017; Sanger Institute, 2018). Deletions surrounding Aspβ9 have been seen in FLT3 as well (1% of cases), but generally do not affect Aspβ9 itself. Instead, FLT3 activation loop deletions almost exclusively affect residues within the η7 3_{10} helix (I836-M837). Finally, activation loop deletions are very rare in KIT, where point mutations are more common (D820, N822, Y823, etc.) (**Fig. 6A**) (Forbes et al., 2017; Sanger Institute, 2018).

The similarities and differences in the frequencies of mutations types across this class of RTKs indicate similar, but not entirely identical, kinase structure. Deletions of Aspβ9 and the surrounding residues may be more common in PDGFRA due to the increased electron density seen around these residues compared to in KIT or FLT3, indicating that these residues are more crucial for maintaining the inactive conformation of the activation loop in PDGFRA (Liang et al., 2016). Conversely, in KIT, specific residues that have been shown to make critical contacts within the activation loop to maintain the inactive conformation are

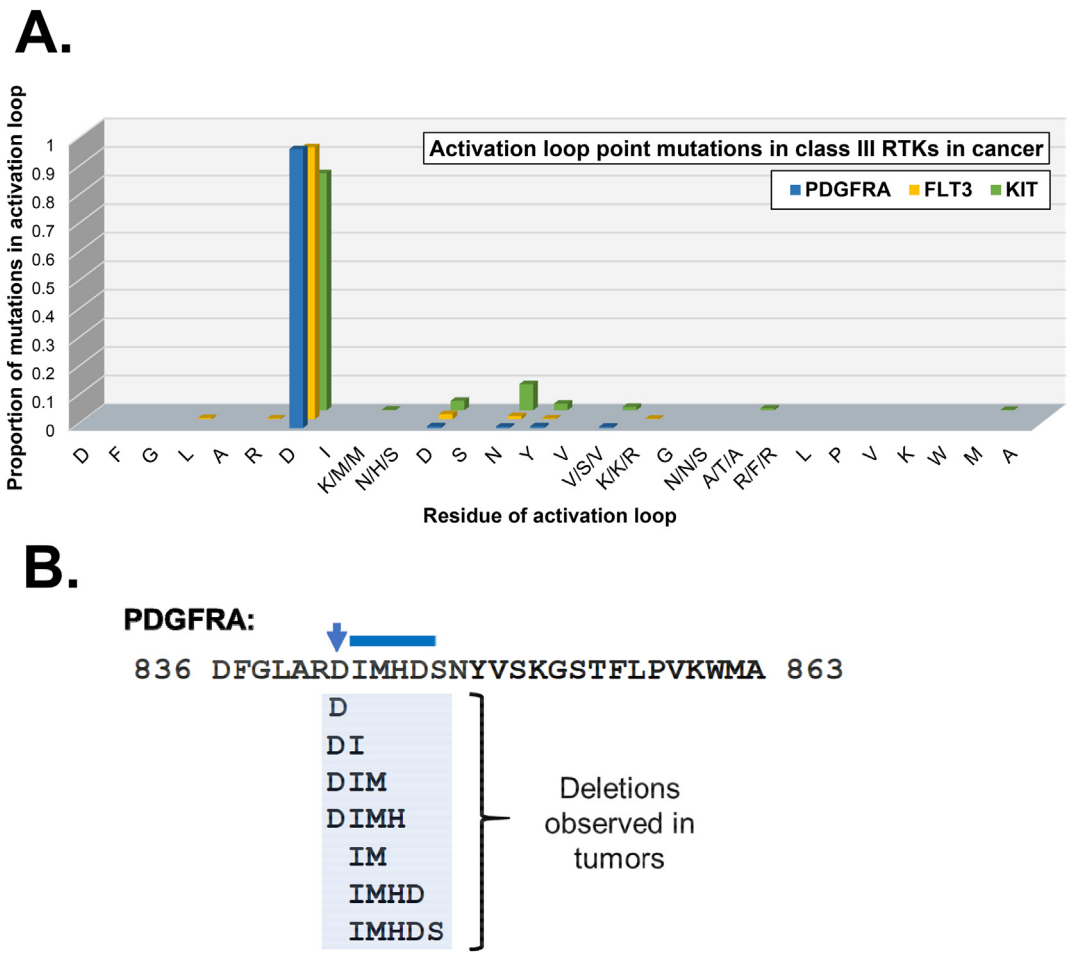

Fig. 6. Proportion of cancer cases reported with point mutations affecting the activation loop of class III RTKs. **A.** Proportions of point mutations in the activation loop of class III RTKs seen in cancer. Activation loop residues are shown in consecutive order (PDGFRA 836-863, FLT3 829-856, and KIT 810-837). Each residue is shown at positions that are not conserved (KIT/PDGFRA/ FLT3, respectively). Data from COSMIC (*Forbes et al., 2017; Sanger Institute, 2018*). Includes point mutations seen in greater than 3 samples per study. **B.** Most common deletions affecting the activation loop of PDGFRA aligned to wild type. Blue arrow indicates Aspβ9 residue (D842). Blue line denotes residues involved in η7 3_{10} helix.

those that we observe in human tumors (Laine, Chauvot de Beauchene, Perahia, Auclair, & Tchertanov, 2011). Mutations of residues in the activation loop of KIT, apart from the Aspβ9, are almost always seen as secondary mutations to primary JM mutations, emerging after first line therapy in GIST patients treated with imatinib or other type II KIT TKI (M. C. Heinrich et al., 2006; Heinrich et al., 2008).

Activation loop mutations, in particular Aspβ9 mutation, in KIT, PDGFRA, and FLT3 confer resistance to type II TKIs (Alvarado et al., 2014; Antonescu et al., 2005; Gajiwala et al., 2009; Gramza, Corless, & Heinrich, 2009; M. C. Heinrich et al., 2006; Heinrich, Maki, et al., 2008; Liegl et al., 2008; Lim et al., 2017; Smith, Lin, Stecula, Sali, & Shah, 2015; Smith et al., 2017). As discussed above, type II inhibitors are only capable of binding the DFG-out conformation of the kinase because of the orientation of the DFG sidechains in the active DFG-in conformation causes steric hindrance (**Fig. 7A**). Activation loop mutants promote the active conformation; Aspβ9 mutants can become activated more than 100 times faster than wild type because the active conformation is so energetically favored (DiNitto et al., 2010). Thus, activation loop mutations are thought to confer resistance because they greatly reduce the time that the kinase spends in the inactive conformation, preventing type II inhibitors from binding (**Fig. 4C**).

Alternatively, type I inhibitors have the ability to bind active DFG-in conformations of kinases. In general, type I inhibitors exclusively occupy the ATP binding pocket and do not protrude in the adjacent hydrophobic pocket occupied by type II inhibitors in the inactive conformation (Liu & Gray, 2006; Zhang et al., 2009). Therefore, the orientation of the DFG sidechains do not impact the binding of most type I inhibitors (**Fig. 7B**). In fact, type I KIT and PDGFRA TKIs have comparable IC_{50} values between JM mutants (inactive conformation) and activation loop mutants (**Fig. 7C**) (Evans et al., 2017; Wodicka et al., 2010). While a general lack of target specificity has limited the clinical use of many type I inhibitors in the past, the use of rational drug design, incorporating structural biology information, is capable of producing potent and specific type I KIT and PDGFRA TKIs. Below we discuss the clinical use and success of these inhibitors.

2.7. Treatment of cancers caused by constitutively activated (activation loop mutant) RTKs

As discussed above, KIT, PDGFRA, or FLT3 activating mutations within the activation loop are seen in cancer patients. Historically, tumors with these mutations have been difficult to treat clinically, as the activation loop mutations most often confer resistance to conventional TKI therapeutic approaches, which are usually type II kinase inhibitors. Here we will review the clinical challenges of treating patients with activation loop mutations of class III RTKs and the recent advances in treatment due to the rational design and development of type I inhibitors. As examples, we will focus on TKI treatment of KIT activation loop mutations in systemic mastocytosis, and KIT and PDGFRA activation loop mutations in GIST. For information on FLT3 activation loop mutations and use of TKIs to treat FLT3-mutant AML, the reader is referred to several recent comprehensive reviews (Larrosa-Garcia & Baer, 2017; Lim et al., 2017; Nguyen et al., 2017).

2.8. Activation loop mutations of KIT in systemic mastocytosis

Mastocytosis comprises a number of clonal mast cell diseases which are characterized by increased activation of mast cells leading to the release of numerous mediators as well as variable infiltration of different tissues. Symptoms are related to mast cell activation (degranulation) and/or organ damage due to the effects of chronic degranulation and mast cell infiltration (Gotlib, 2017; Verstovsek, 2013). The most common type of mastocytosis is cutaneous mastocytosis in which mast cell infiltration is limited to the skin. However, there are a variety of mastocytosis with systemic involvement (systemic mastocytosis, SM), the mildest of which is indolent SM. Survival of patients with indolent SM is similar to age-matched controls. In contrast, patients with more advanced forms of SM such as aggressive SM, SM with an associated hematologic neoplasm (almost always a form of myeloid neoplasm), or mast cell leukemia have median overall survival of 3.5, 2 years, and less than 6 months, respectively (Georgin-Lavialle et al., 2013; Lim et al., 2009). The vast majority of cases of SM are characterized by the

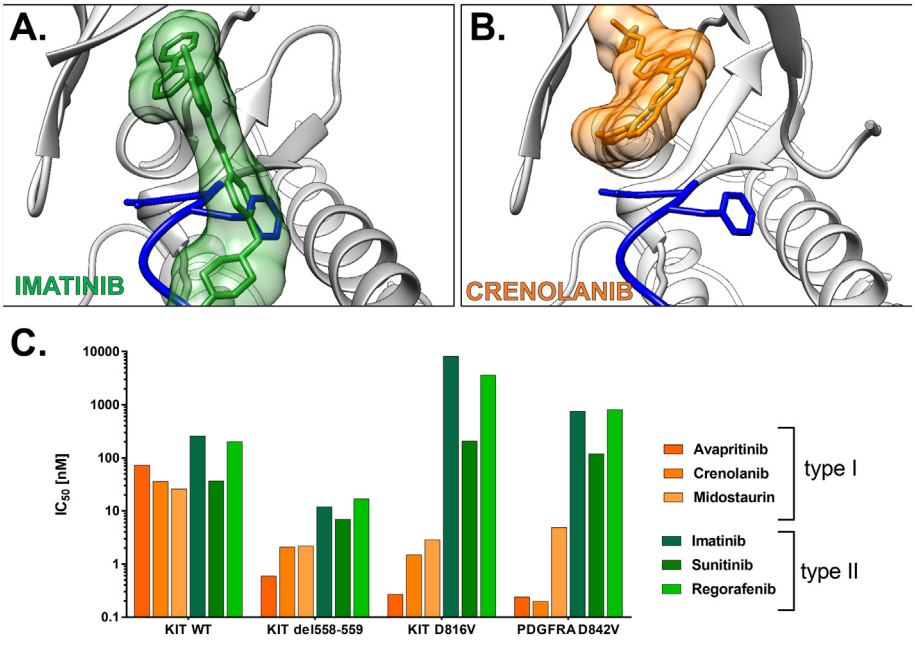

Fig. 7. Type I, but not type II, kinase inhibitors bind and inhibit the active conformation of class III RTKs. **A.** Active DFG-in conformation of KIT (PDB: 1T46) is not capable of binding type II inhibitor, imatinib due to steric clash with the phenylalanine of the DFG (F811). Imatinib and its electron cloud are shown in green. **B.** The type I inhibitor crenolanib can bind to the active DFG-in conformation of PDGFRA. The structurally similar human calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2), co-crystalized with crenolanib (PDB: 6BQP), was superimposed on active PDGFRA using the MatchMaker tool in UCSF Chimera. Crenolanib and its electron cloud are shown in orange. The kinase domain ribbon structure shown in gray with the activation loop residues are denoted in blue. Sidechains of DFG are shown. **C.** Biochemical IC_{50} values (nM) of type II vs. type I inhibitors against KIT WT, KIT JM mutant (del558-559), and activation loop mutant isoforms of KIT (D816V) and PDGFRA (D842V). Adapted from Evans et al. (2017).

prototypic KIT D816V (Aspβ9) mutation described above (Verstovsek, 2013). In addition to this mutation, rare cases of SM have other KIT activation loop mutations (D816F, D816Y, D816T, indels of codons 815-817, or mutation of *KIT* exons 8-11) (Heinrich et al., 2008; Valent et al., 2017).

Due to the presumed pathogenic role of mutant KIT, therapy with KIT inhibitors has long been postulated as an effective treatment for advanced SM. However, the majority of cases of advanced SM have the D816V mutation, which is insensitive to type II inhibitors. Therapeutic trials of imatinib and nilotinib (both type II inhibitors), failed to show any clinical benefit except for patients whose SM lacked the classical D816V mutation (Vega-Ruiz et al., 2009). Imatinib has been FDA-approved for treatment of advanced SM with KIT mutations other than D816V, including rare cases with no detectable KIT mutations.

In vitro studies of the type I inhibitor dasatinib, demonstrated activity against D816V-mutant KIT (Schittenhelm et al., 2006; Shah et al., 2006). However, pre-clinical activity did not translate into clinical benefit when this agent was tested in a phase 2 study of patients with indolent or advanced SM (Verstovsek et al., 2008); the overall response rate to dasatinib was 33% (11/33), but the only two complete responses were in patients whose disease lacked the KIT D816V mutation. The partial responses observed in the other nine patients consisted of improved symptoms only. Notably, there was no objective evidence of a decrease in neoplastic mast cells such as a decrease in serum tryptase or mast cell tissue infiltration (Verstovsek et al., 2008). It is unclear if these results were due to the short plasma half-life of dasatinib and/or the inability to achieve an adequate D816V inhibitory plasma concentration.

More recently, several new therapies for D816V-mutant KIT using type I inhibitors have demonstrated substantial clinical activity for treatment of advanced SM. Midostaurin was evaluated in a phase 2 study which has recently published updated results with a median of 10 years of patient follow up (DeAngelo et al., 2018). Midostaurin, administered at 100 mg twice daily, had an overall response rate of 69% (50% major + 19% partial response) and rare cases of complete response. The median overall survival for all patients was 40 months and 18.5 months for patients with mast cell leukemia. Overall, the treatment was safe and well tolerated. These results were confirmed in an open-label, international, multi-site phase 2 study. In this latter study, the overall response rate was 60%, with 45% of patients having a major response defined as complete resolution of at least one type of SM-related organ damage. In this confirmatory study, the median overall survival was almost 29 months and the median progression-free survival was 14.1 months (Gotlib et al., 2016). Based on these studies, in 2017 the FDA approved midostaurin for treatment of advanced SM.

Avapritinib (previously BLU-285), is another type 1 inhibitor with promising *in vitro* and clinical activity against KIT D816V-mutant mast cells. Avapritinib has been shown to potently inhibit KIT D816V *in vitro* ($IC_{50} < 1$ nM) (Evans et al., 2017). In a preliminary report of results from a phase 1 study of avapritinib in advanced SM, the overall response rate was 72%, with 56% reporting complete or partial responses (NCT02561988) (DeAngelo et al., 2017; Drummond et al., 2016). This study is ongoing and further results should be available later in 2018.

2.9. Primary and secondary activation loop mutations of KIT in GIST

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumor (sarcoma) of the gastrointestinal tract. Each year 5000–7000 patients are diagnosed with GIST in the U.S. These tumors are found throughout the gastrointestinal tract, including the stomach and small intestine and arise from the pacemaker cells of the gut, called the interstitial cells of Cajal (ICC) (Corless, Barnett, & Heinrich, 2011). Distinct driver mutations (including KIT, PDGFRA, and SDH) have been identified in GIST patients. Identification of molecular drivers has immense potential to inform patient prognosis and treatment (Bannon et al., 2017). In particular, treatment of KIT and PDGFRA-mutant GIST has been transformed by the use of KIT/PDGFRA kinase inhibitors.

KIT mutations are common, seen in 77% of all GIST, and the vast majority of primary KIT mutations affect the JM domain encoded by exon 11 (V559D, V560D, or small deletions in this area) (Bannon et al., 2017; Heinrich et al., 2003; Heinrich, Maki, et al., 2008). As noted above, these mutations abrogate the auto-inhibitory effect of the JM domain. Despite shifting the equilibrium to favor activation compared with WT KIT, *KIT* exon 11-mutant kinases spend the majority of the time in the inactive state, allowing them to be potently inhibited by type II inhibitors such as imatinib. A minority of GIST patients have primary KIT mutations involving codons 820, 822, or 823 in the activation loop (~1% of primary GIST). Notably, primary mutations of D816 (Aspβ9) are not found in primary GIST. Thus, the spectrum of KIT mutations in GIST and SM are mirror images: KIT JM mutations predominate in GIST with rare activation loop mutations (but not affecting Aspβ9), whereas KIT Aspβ9 mutations dominate in SM with rare cases of JM mutations. These data suggest that there are quantitative and/or qualitative differences in signaling between KIT JM-mutant and KIT activation loop-mutant kinases. These unknown differences in signaling must dictate the spectrum of KIT mutations associated with a particular disease state.

Imatinib was approved as the first-line therapy for advanced GIST in 2002 based on data showing that imatinib was capable of disease control in over 75% of GIST patients (Demetri et al., 2002; Verweij et al., 2004). The greatest benefit of imatinib is observed when treating KIT JM-mutant GIST. As a type II inhibitor, resistance to imatinib could arise from secondary KIT mutations that further shift the kinase structure equilibrium to favor the activated state. This was clinically validated by the demonstration of compound mutant kinases of the primary KIT JM mutation with a secondary *in cis* mutation of the activation loop, most commonly involving codons 816 (Aspβ9), 820, 822, 823 or 829. Depending upon the specific mutant residue, these secondary mutations also confer partial or total resistance to other clinically available type II (or type 1.5) inhibitors such as sunitinib and regorafenib (Antonescu et al., 2005; Heinrich et al., 2006; Heinrich, Maki, et al., 2008; Heinrich et al., 2012; Liegl et al., 2008; Wardelmann et al., 2006).

More recently, avapritinib (formerly BLU-285), a type I TKI, has been tested *in vitro* and in a phase 1 human study (Evans et al., 2017). As shown in **Fig. 7C**, avapritinib is a potent and specific inhibitor of the prototypic D816V mutation and has shown markedly improved potency compared with imatinib for other activation loop mutations as well. Preliminary results of a phase I study of avapritinib for drug-resistant, KIT-mutant GIST have been reported, showing encouraging safety, tolerability, and efficacy results (NCT02508532) (Heinrich et al., 2017). Based on these data, a randomized phase 3 study of regorafenib vs. avapritinib for patients with imatinib- and sunitinib-resistant GIST was initiated in mid-2018.

2.10. Activation loop mutations of PDGFRA in GIST

Activating mutations of PDGFRA are found in a minority of cases of GIST (Heinrich, Corless, Demetri, et al., 2003; Heinrich et al., 2003). In a population-based series of 492 primary GISTs in France, the frequency of *PDGFRA* mutations was 15%, whereas only 2% of cases in two large clinical series of metastatic GIST were driven by *PDGFRA* mutations (Emile et al., 2012). These observations, which have been confirmed in other series, suggest that *PDGFRA*-mutant GISTs generally have a lower risk of recurrence than *KIT*-mutant GIST. The most common PDGFRA mutations in GIST involve the activation loop, encoded by *PDGFRA* exon 18. Mutations affecting the PDGFRA JM are found in approximately 1% of GIST (Bannon et al., 2017; Corless et al., 2005). As discussed above, these mutations lead to a loss of the auto-inhibitory function of the JM domain.

The prototypic Aspβ9 mutation, D842V, accounts for at least 70% of all *PDGFRA* mutations seen in GIST (Bannon et al., 2017; Corless et al., 2005; Heinrich, Corless, Duensing, et al., 2003). A minority of GIST have alternative mutations involving the Aspβ9 mutations, including amino acid substitutions other than valine (e.g. tyrosine) and deletion/

insertions involving Aspβ9. As discussed above, substitution mutations involving the PDGFRA Aspβ9 codon (D842) strongly favor the activated kinase conformation, whereas many of the deletions/insertions have a lesser shift in kinase conformation equilibrium, with a greater percentage of these mutant kinases found in the inactive conformation. Consistent with these observations, type II inhibitors such as imatinib have no useful clinical activity against PDGFRA D842V-mutant GIST but can have activity against tumors with particular PDGFRA activation loop deletions (Corless et al., 2005). Cassier et al. reported that imatinib treatment of PDGFRA D842V-mutant GIST was associated with a median progression-free survival (PFS) of 2.8 months and an overall survival from the start of imatinib treatment of 14.7 months (Cassier, et al., 2012). In contrast, GIST with PDGFRA mutations other than D842V had a median PFS of 28.5 months with imatinib treatment and the median overall survival from the start of imatinib treatment was not reached in their series. In addition to this data, secondary PDGFRA D842V mutations have been reported in cases of imatinib-resistant GIST with primary PDGFRA JM-mutations (Debiec-Rychter et al., 2005; Heinrich, Maki, et al., 2008).

Recently, several type I PDGFRA inhibitors with activity against D842V mutant kinases have been reported and have entered into clinical studies. Crenolanib was tested in a phase 2 study and preliminary reports indicated some useful clinical activity (Blay et al., 2017). The final report of this study has not yet been published. However, based on the preliminary results, a randomized, placebo-controlled study of crenolanib for treatment of PDGFRA D842V-mutant GIST has been initiated (NCT02847429). In addition, the newly-developed type I PDGFRA/KIT inhibitor avapritinib potently inhibits D842V *in vitro* (Evans et al., 2017). Patients with PDGFRA D842V-mutant GIST have been treated with avapritinib as part of the phase 1 study discussed above (NCT02508532). In the preliminary report, the partial response rate of D842V patients to avapritinib was noted to be a 60%, with a median PFS that had not been reached (PFS 25th percentile 11.2 months) (Michael C. Heinrich et al., 2017).

3. Future

The use of type I kinase inhibitors for the specific targeting of activation loop mutants in the class III RTK family has been successful for the treatment of patients previously unresponsive to many of the clinically approved kinase inhibitors, most of which are type II inhibitors. This has especially been true when rational design strategies based on the knowledge of the structural/ conformational consequences of various activating mutations have been employed, such as with the development of avapritinib. The success of avapritinib demonstrates that treatments can and should be tailored to improve patient outcomes.

In the case of GIST, molecular diagnosis has vastly improved outcomes for patients. Not only determining which gene is mutated (*KIT* vs. *PDGFRA*), but further defining the specific mutation (PDGFRA V561D vs. PDGFRA D842V) and its consequences can inform treatment and result in a patient receiving treatments that are more effective. In the case of advanced systemic mastocytosis, treatment paradigms are likely to undergo a profound evolution with the clinical development of inhibitors such as midostaurin that can effectively target the KIT D816V mutation. Likewise, the care of patients with PDGFRA D842V-mutant GIST is likely to be transformed by the use of avapritinib.

Development and use of specific type I inhibitors for targeting activation loop-mutant RTKs has the potential to overcome type II inhibitor resistance (either primary or secondary resistance) However, from studies performed with selective type I EGFR inhibitors (i.e. gefitinib and erlotinib) in patients with non-small cell lung cancer, resistance to type I inhibitors is possible. Resistance mechanisms vary between different kinases and in regard to specific drugs. For example, most transforming EGFR mutations seen in lung cancer promote the active conformation, making type II inhibitors ineffective, while type I inhibitors, such as gefitinib and erlotinib, have shown clinical efficacy in the majority of EGFR-mutant patients (Douillard et al., 2014; Rosell et al., 2012). Unfortunately, like what is seen with many other kinase inhibitor treatments, over time tumors can develop drug resistance due to acquired intra-allelic mutation of EGFR, most often at the gate keeper position (T790M) in the ATP binding pocket that is critical to drug binding (Ko, Paucar, & Halmos, 2017). It is inevitable that resistance mutations to KIT and PDGFRA type I TKIs will also appear with prolonged treatment, most likely by mutations that affect that conformation of the ATP binding pocket, thereby destabilizing drug binding.

In some cases, switching to an alternative type I inhibitor with a different binding mode will overcome secondary resistance (e.g. osimertinib in the case of T790M EGFR-mutant lung cancer (Goss et al., 2016)), but in other cases such ATP binding pocket mutations may provide cross-resistance to all available agents. Theoretically resistance due to ATP binding pocket mutations could be overcome by chemically modifying the original compound to accommodate the changes within the pocket. The use of prospective *in vitro* resistance mutation identification, such as by random mutagenesis (i.e. using N-ethyl-N-nitrosourea), could allow for mutation-specific drug design to combat resistance (as has been done with imatinib and sunitinib) (Guo et al., 2009).

Unfortunately, TKI drug development becomes an arms race (to use a phrase from microbiology related to the problem of bacterial resistance to antibiotics); resistance will be possible as long as the kinase can mutate in a way that prevents drug binding but does not drastically impair kinase function. Despite this, an iterative drug design strategy has vastly improved patient outcomes (for example with GIST patients) and should not be discounted. Alternative approaches to this problem could include combination therapy using two type I inhibitors, a type I and a type II inhibitor, or a type I inhibitor and an agent that targets a downstream pathway. Future preclinical and clinical testing will be required to determine the relative merits of these approaches.

The identification of targetable oncogenic kinases led to the development and clinical use of kinase inhibitors to treat cancer patients safely and effectively. Class III RTKs KIT, PDGFRA, and FLT3 are classic examples of targetable oncogenes and kinase inhibitors, mostly type II, are approved for the treatment of cancers caused by these oncogenes. While type II inhibitors, such as imatinib have been immensely successful in improving patient outcomes, especially for GIST patients, acquired drug resistance remains a clinical problem. Mutations within the binding pocket (e.g. gatekeeper mutations) can confer resistance to some type II inhibitors by interfering with the binding of the drug in the pocket, but not shifting activation state. In this case, new type II inhibitors can be designed to overcome this particular resistance mechanism (e.g. sunitinib, regorafenib). However, mutations affecting the activation loop confer resistance by skewing the kinase structure towards its active conformation, which can only be bound by type I inhibitors. Using these insights, rational design strategies have resulted in highly specific and potent type I inhibitors for targeting of activation loop mutant class III RTKs, such as avapritinib that have shown clinical success. Further drug development of kinases inhibitors for class III RTKs will continue to be informed by the identification of resistance mechanisms to existing drugs and must be guided by our understanding of the fundamental structural biology of these kinases.

Conflict of Interest statement

L.R.K. and J.D.K. do not declare any conflicts of interest. M.C.H. is a consultant for Novartis, Deciphera Pharmaceuticals, Blueprint Medicines, Ariad Pharmaceuticals, Bayer Pharmaceuticals, and MolecularMD. M.C.H. has provided expert testimony and has a patent licensed to Novartis. M.C.H receives research support from Ariad, Deciphera, Blueprint Medicines and has equity interest in MolecularMD.

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