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Bruton's Tyrosine Kinase Inhibitors: Approaches to Potent and Selective Inhibition, Preclinical and Clinical Evaluation for Inflammatory Diseases and B Cell Malignancies

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INTRODUCTION

Bruton's tyrosine kinase (BTK) is a member of the Tec tyrosine kinase family.[1] BTK is expressed in most hematopoietic cells such as B cells, mast cells, and macrophages but not in T cells, natural killer cells, and plasma cells.[2] BTK plays key roles in multiple cell signaling pathways including BCR and FcR signaling cascades. Mutations in the human BTK gene cause the inherited disease X-linked agammaglobulinemia (XLA), with lack of peripheral B cells and low levels of serum Ig.[3] In XLA, the primary immune deficit is B cell specific. In fact Rituxan, a CD20 antibody, has impacted B cells on the pathogenesis of many autoimmune diseases, such as RA, SLE, and MS.[4] This has fueled interest by multiple pharmaceutical companies in pursuing small molecule BTK inhibitors in the treatment of autoimmune diseases. Likewise, there is also interest in the development of BTK inhibitors for the treatment of hematological malignancies, as aberrant activating BTK has been implicated in the pathogenesis of B cell lymphoma.[5] Detailed reviews and articles on BTK biology and its therapeutic potentials have been reported.[5−9]

Kinase selectivity is a central issue in discovering efficacious and safe small molecule inhibitors for kinase targets, especially for non-life-threatening diseases such as RA.[10] To prevent adverse toxicological events caused by immunological responses, the pharmaceutical industry has largely focused its small molecule drug discovery efforts on agents that interact noncovalently with their target proteins. This strategy has been followed despite numerous examples of marketed drugs with target-specific covalent mode-of-action.[11,12] In the case of protein kinase targets for which selectivity and efficacy pose major challenges for noncovalent inhibitors, targeted covalent inhibition has provided an attractive alternative.[13] Both approaches, noncovalent and covalent inhibition of protein kinases, benefit tremendously from high resolution structural information from protein crystal structures. This article will review BTK structural biology with a focus on design features for selective BTK inhibitors. We first summarize the publically available structural information on the BTK kinase domain. Then we will provide a brief summary and analysis with key SAR information for the most potent inhibitors reported for the chemical classes that have been disclosed in patents and publications. When available, preclinical and clinical data for advanced compounds will be summarized.

STRUCTURAL BIOLOGY

Protein crystal structures of inhibitor complexes are routinely accessible and successfully used in kinase drug discovery efforts. Structural information on inhibitors binding to BTK, however, has been only recently published. The first X-ray crystal structure of the BTK kinase domain has been determined in its apo form.[14] More recently, crystal structures of complexes with nine distinct inhibitors have been reported (Table 1).[15−17] All crystal structures have been determined with unphosphorylated BTK protein. A Y551E phosphomimetic BTK variant was used for the complex with compound **1** (dasatinib).[15] The spectrum of BTK protein conformations observed in these crystal structures is remarkable, exhibiting a broad range of shapes and properties for the ATP binding region to be targeted by small molecules using structure-based drug design.

Compounds **1**,[15] **5**, and **6**[16] bind to the active state conformation of BTK where helix C (amino acids 439−452) and the DFG sequence (amino acids 539−541) are in their "in" positions and the conserved salt bridge between K430 and the helix C residue E445 is formed. Compounds **2** (B43),[15] **3**, **4** (R406), **7**, **8**,[16] and **9** (CGI1746)[17] stabilize six different conformations that represent inactive states of BTK (Table 1). The pharmacologically highly relevant DFG-out conformation, often associated with slow binding kinetics, is stabilized by compound **8**. An atypical DFG conformation is observed in the presence of **4**.[16] Unique and distinct activation loop (amino acids 542−559) conformations that reshape the ATP binding pocket are induced by **2**, **3**, **4**, and **9**. Helix C is stabilized in varying "out" positions by compounds **2**, **3**, **4**, **7**, and **9**, preventing the formation of the K430-E445 salt bridge.[16]

APPROACHES TO DESIGN KINOME SELECTIVE BTK INHIBITORS

Two general approaches to design selective BTK inhibitors have been used and will be summarized here. One is to target amino acid residues found in BTK but uncommon in the kinome, and the other is to target binding pockets of inactive BTK conformations not commonly observed in other kinases. Inactive conformations of kinases are considered promising starting points for the design of highly kinome-selective inhibitors because they are often specific to particular kinases or kinase families.[18]

Analysis of 491 kinase sequences for positional similarity within the ATP binding pocket revealed that C481 of BTK is the amino acid most unique to BTK. The most common amino acid at this position is D (161 occurrences), followed by S (116), E (91), N (44), T (28), and A (15). The residue

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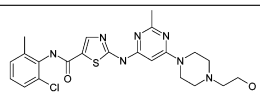
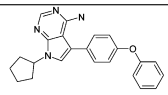
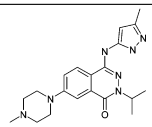
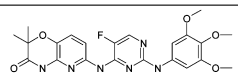
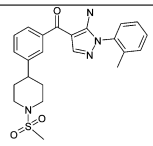
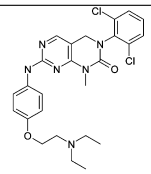
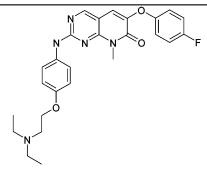
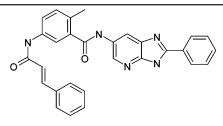
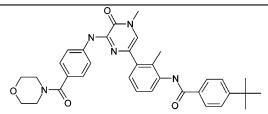
Table 1. Crystal Structures of BTK Kinase Domain in the PDB

BTK Inhibitor	Structure	Helix C	DFG	Activation loop	PDB accession number
apo		Out	In	Resolved	1K2P
1 (Dasatinib)		In	In	Not resolved	3K54
2 (B43)		Out	In	Resolved, forms selectivity pocket	3GEN
3		Out	In	Resolved, reshapes ATP pocket	3PIX
4 (R406)		Out	Atypical	Resolved, reshapes ATP pocket	3PIY
5		In	In	Not resolved	3PIZ
6		In	In	Not resolved	3PJ1
7		Out	In	Not resolved	3PJ2
8		In	Out	Not resolved	3PJ3
9 (CGI1746)		Out	In	Resolved, forms selectivity pocket	3OCS

distribution is given in Figure 1. The presence of the threonine gatekeeper and resulting smaller back pocket is critical to the good selectivity profile of 4-[(4-methylpiperazin-1-yl)methyl]-*N*-(4-methyl-3-{[4-(pyridin-3-yl)pyrimidin-2-yl]amino}-phenyl)benzamide (imatinib, Glivec, STI571), a rationally developed, targeted anticancer drug.[19] About 20% of all human protein kinases share threonine as gatekeeper residue, but when combined with C481, the total number of kinases decreases to just eight.

Gatekeeper Residue T474. The back pocket of protein kinases is a lipophilic region separated from the ATP binding site by the so-called "gatekeeper" residue. The relatively small gatekeeper residue T474 in BTK allows ATP-competitive inhibitors to occupy the back pocket. Rotation of the gatekeeper threonine side chain induced in the mitogen

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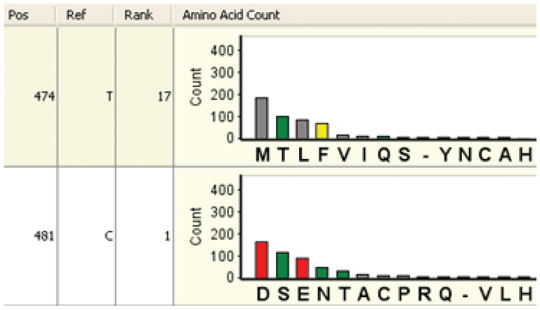

Figure 1. Analysis of corresponding amino acid residues in kinome at the positions aligned with T474 (gatekeeper) and C481 of BTK. *X*-axis refers to the identity of the amino acid, and *Y*-axis is the corresponding total count in the human kinome. For BTK, C481 ranks as the most uncommon residue whereas T474 ranks as the 17th.

activated protein kinase p38α by the phenoxy side chain of compounds like 7[20] has not been observed for BTK.[16] About 20% of all human protein kinases share threonine as the gatekeeper residue, providing a starting point for the rational design of kinome selective BTK inhibitors. To our knowledge, the first attempt to design BTK-specific inhibitors by interacting with T474 dates back to 1999 when researchers at the Parker Hughes Cancer Center used homology modeling and docking to rationally design the leflunomide metabolite **10** (LFM-A13) (IC_{50} = 2.5 μM) (Figure 2).[21] On the basis of the

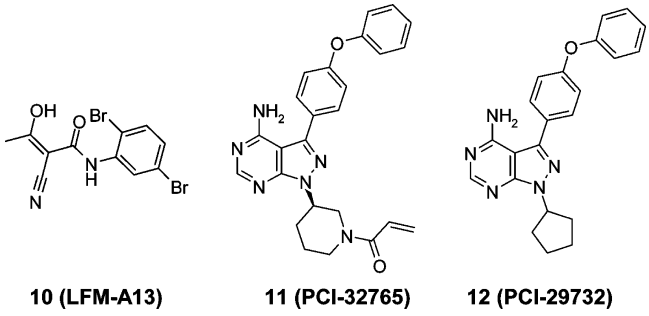

Figure 2. BTK inhibitors **10**, **11**, and **12**.

proposed BTK binding mode of **10** and promising yet limited kinase profiling data, it was suggested that interactions with T474 could yield to selectivity against other kinases.

The hypothesis of gaining BTK specificity for small molecule inhibitors by interacting with the gatekeeper residue T474 (Figure 3) was further strengthened by selectivity data obtained for the irreversible inhibitor **11** (PCI-32765)[22] and its non-covalent precursor **12** (PCI-29732).[22] Screening of **12** against more than 100 kinases by ligand replacement showed that the tightest binding was observed for 13 kinases that all contain threonine as gatekeeper residue.[22] Kinome profiling shows that **11** has the greatest affinity for 10 kinases, which all share the threonine gatekeeper residue.[23] These data strongly indicate that interaction with the gatekeeper residue T474 can significantly contribute to the kinome selectivity of BTK inhibitors.

C481. The second approach reported to yield inhibitors specific for BTK is to interact with the side chain of C481. Sequence alignment of 491 human kinases shows that cysteine occurs at this position in only nine other kinases: BLK, BMX, EGFR, ERBB2, ERBB4, ITK, JAK3, TEC, and TXK.[22] All 10 kinases have as gatekeeper a threonine residue at position 474

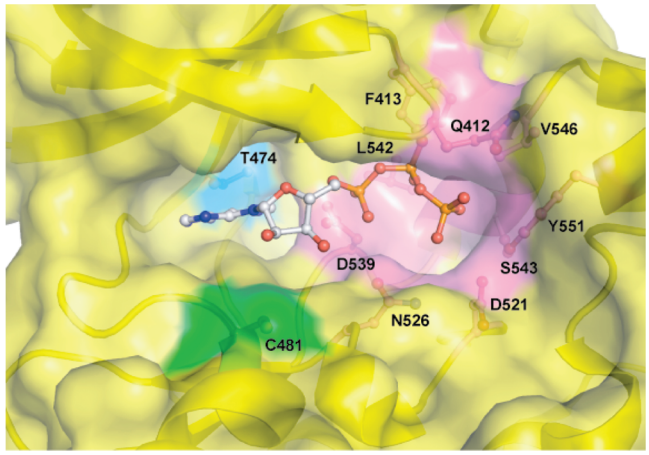

Figure 3. Amino acids in the ATP binding pocket of BTK targeted for the design of selective inhibitors. Shown in yellow is a ribbon representation of the protein component of the BTK crystal structure bound with **9**.[17] Side chains of amino acids targeted for selectivity design are shown in ball-and-stick representation: the gatekeeper residue T474 (cyan), C481 (green), and the specificity pocket (pink). For orientation, an ATP molecule (carbon atoms in gray) has been modeled into the binding site.

except JAK3 and ITK, which have methionine and phenylalanine as the gatekeeper residues, respectively. Compound **12**, a close analogue of **2** with presumably the same BTK binding mode (Figure 4A), shows that this approach can indeed lead to kinome selectivity.

Screening of **12** against more than 100 kinases showed that the tightest binding was observed for nine kinases that contain either cysteine or serine at the position equivalent to the BTK residue C481.[22] The crystal structure of BTK in complex with the close analogue **2** shows that the cyclopentyl ring of the ligand is within 4 Å of the C481 side chain.[15] Presumably, amino acids larger than cysteine or serine at this position, and present in about 70% of all human kinases, are likely to clash with the ligand, which results in significant loss of inhibitory potency.

To further improve the kinome selectivity, BTK inhibitors that bind covalently to C481 have been synthesized.[13] The irreversible inhibitor **11** blocks three protein kinases with subnanomolar potency: BTK (IC_{50} = 0.5 nM), BLK (IC_{50} = 0.5 nM), and BMX (IC_{50} = 0.8 nM), all sharing the rare cysteine.[22] Compound **11** blocks another 12 human protein kinases with IC_{50} < 50 nM.[23] Four of these kinases possess a cysteine residue as the amino acid at the positions equivalent to the BTK residue C481, and eight kinases have serine residues. These kinases also contain a threonine gatekeeper with the exception of RET, which contains a valine gatekeeper. This may explain its relatively modest activity against RET, with an IC_{50} of 37 nM. Consequently, **11** achieves a >100-fold selectivity window in vitro over kinases with amino acids other than cysteine or serine at this position, and it achieves maximum potency against BTK and two other kinases that have the C481 residue. These findings demonstrate that interaction with C481 is a valid approach for the design of kinome selective BTK inhibitors.

Specificity Pocket. A third and very successful approach reported to design BTK-specific inhibitors is to fill a "specificity pocket", also referred to as the "H3 pocket", about 15 Å away from the hinge region (Figure 4B).[17] Compound **9** is a potent (IC_{50} = 1.9 nM), ATP-competitive inhibitor of BTK with

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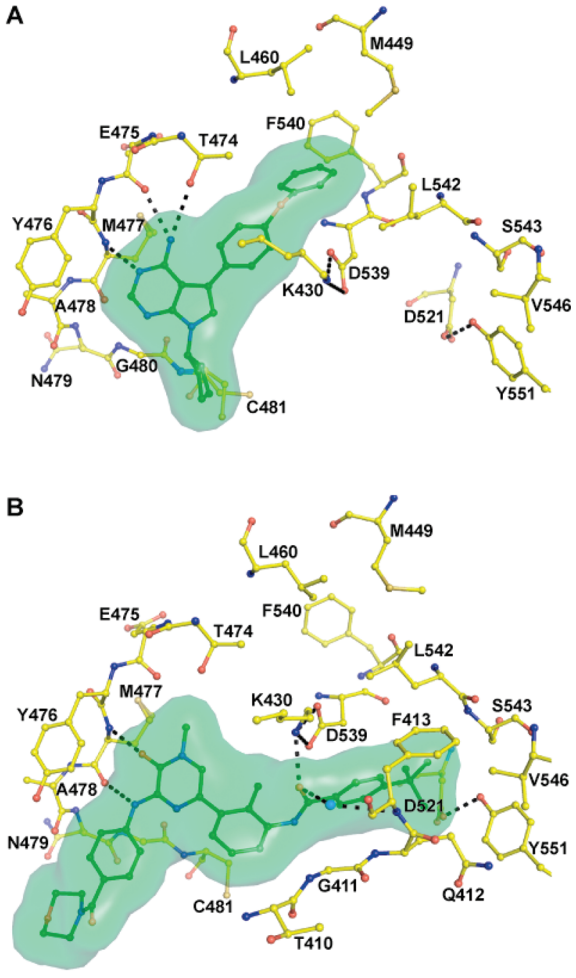

Figure 4. X-ray crystal structures of BTK-inhibitor complexes: (A) compound **2** at 1.94 Å resolution; (B) compound **9** at 1.8 Å resolution. Inhibitors are displayed in green and selected amino acids in yellow. Black dashes indicate hydrogen bonds. A structurally important water molecule is drawn in cyan.

~1000-fold selectivity over Tec and Src family kinases. The high kinome selectivity of the BTK inhibitor **9** and related analogues (vide infra) stems from its *tert*-butylphenyl moiety that occupies the specificity pocket with great complementarity (Figure 4B). This pocket is formed to a large extent by residues L542, S543, V546, and Y551 of the fully ordered activation loop. The side chains of the Gly-rich loop residues Q412 and F413, the catalytic loop residues D521 and N526, and the DFG residue D539 also contribute to the specificity pocket.[17] Interestingly, the specificity pocket is also present in the crystal structure of BTK complexed with **2**, even though this inhibitor does not occupy the pocket. Equivalent pockets created by the same activation loop conformation have been observed in crystal structures of SRC[24] and HCK.[25] A crystal structure of ITK in complex with an inhibitor bound also shows this activation loop conformation, yet the side chain of the phosphorylatable Y512 does not point into the protein, contributing to the specificity pocket, but occupies a solvent-exposed position.[26] It remains to be seen whether more selective kinase inhibitors will emerge from exploiting this specificity pocket.

The side chains of residues S543, V546, and Y551 are presumably responsible for the high selectivity to be gained from filling the BTK specificity pocket. Sequence analysis of 491 human kinases shows that the V546/Y551 pair occurs in only three other kinases, BMX, ITK, and TXK. The triple S543/V546/Y551 is completely unique to BTK. Notably, BMX, ITK, and TXK all have a threonine residue at the BTK position S543. For ITK, it has been proposed that this threonine prevents the activation loop tyrosine rotamer required for formation of the BTK selectivity pocket.[26]

The side chains of two amino acids contributing to the formation of the BTK specificity pocket, unphosphorylated Y551 and D521, form an intramolecular hydrogen bond (Figure 4B). After phosphorylation of Y551, electrostatic repulsion between these two side chains prevents formation of the BTK specificity pocket. Consequently, by binding to and stabilizing the BTK specificity pocket, **9** blocks the activation loop phosphorylation of BTK and to a much lesser extent inhibits the activity of phosphorylated BTK.[17] Surface plasmon resonance data show that **9** binds with 32-fold greater affinity to the nonphosphorylated form than to the phosphorylated form (K_d of 2.9 nM vs 94.1 nM, respectively) of the enzyme. Competition binding of **9** in the Ambit panel of 385 kinases at 1 μM reveals only five kinases with more than 50% displacement of control ligand (BTK 100%, CASK 69%, MINK 77%, NEK11 59%, PDGFRB 59%). Compound **9** binds MINK1, the second most tightly bound kinase, with a K_d of 40 μM vs 1.5 nM for BTK. A biochemical screen of 47 purified

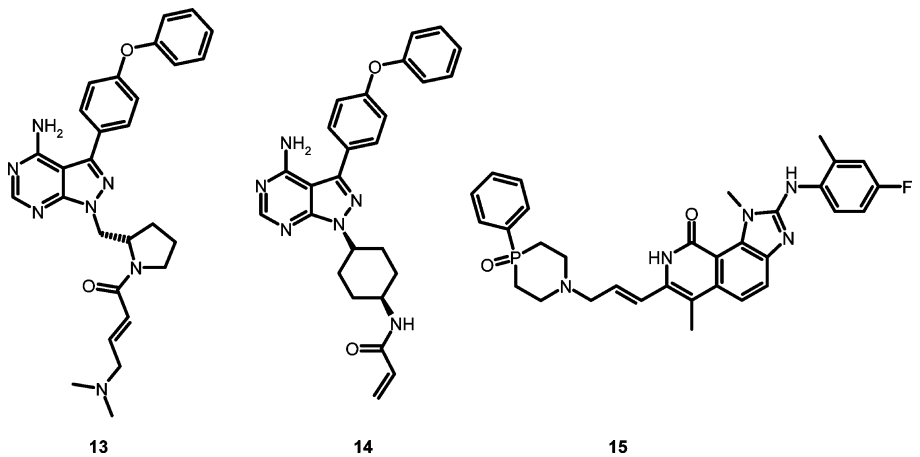

Figure 5. Representative BTK inhibitors from Pharmacyclics.

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kinases at K_m for ATP showed that BMX, with IC_{50} = 1.87 μM, was the next most potently inhibited kinase after BTK, with a nearly 1000-fold reduction in potency relative to BTK.[17]

IRREVERSIBLE INHIBITORS

Pharmacyclics. Pharmacyclics screened a variety of scaffolds for inhibitory activity of BTK and found that compound **12** was a potent (IC_{50} = 8.2 nM) inhibitor. Compound **12** and others from this compound class (i.e., **2**) had previously been found to be potent inhibitors of Itk as well as other kinases from the Tec and Src family. On the basis of the proposed binding mode of **12** in a homology model, a series of inhibitors targeting the C481 with electrophilic Michael acceptors were designed. Of these early published examples, **11** was the most potent in enzymatic assays (IC_{50} < 0.5 nM) and showed cellular selectivity, with >10 times greater inhibition of Ca^{2+} flux in B cell related (Ramos) cells vs T cells (Jurkat).[22] Examples of other BTK inhibitors that have been claimed in Pharmacyclics patents are shown (Figure 5).[27−32]

Pharmacyclics has reported on the development of the selective irreversible Btk inhibitor, **11**, that is currently in phase II clinical trials in patients with relapsed or refractory mantle cell lymphoma[33] and phase Ib/II trials for multiple B-cell related malignancies.[34−36]

As described above, **11** is a selective BTK inhibitor with potent enzyme activity (IC_{50} = 0.5 nM). In cells, **11** blocks BCR-stimulated activation of NF-kB and ERK, inhibiting growth and inducing apoptosis in B cell lymphoma cell lines. It was shown to be efficacious in multiple preclinical oncology models, including a transgenic murine model of BCR-driven lymphoma. In dogs, **11** induced objective clinical responses with spontaneous B-cell non-Hodgkin's lymphoma (NHL). Additionally, orally administered **11** showed activity in a mouse collagen-induced arthritis model, reducing the level of circulating autoantibodies and completely suppressing disease. Finally, **11** also was effective in the MRL-Fas(lpr) lupus model of kidney disease.[23]

A phase I dose-escalating, multicenter, open-label study of **11** in patients with relapsed aggressive NHL was conducted to evaluate its safety and pharmacokinetic profile. Compound **11** was well tolerated with minimal toxicities at doses up to 12.5 mg kg^{-1} day^{-1}, and MTD was not reached. A second primary end point of the study was to obtain a PK/PD assessment, and as determined by ex vivo competition experiments with an irreversible fluorescent probe, full occupancy of BTK was achieved at doses of ≥2.5 mg/kg per day.[37]

Phase I results with multiple ascending doses of **11** have been reported for a variety of B cell malignancies. Generally the compound was well tolerated, with diarrhea and fatigue being the most common side effects and with no hepatic or renal toxicity reported even after more than 6 months of dosing. An objective response rate of 45% was achieved across all patient cohorts with the highest efficacy seen for chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) and mantle cell lymphoma (MCL) with rates of 56% and 78% respectively achieving a complete or partial response. Additionally, data in CLL or SLL patients showed an initial treatment-related lymphocytosis that could be due to a BTK mechanism-driven inhibition of integrin-mediated B cell homing. This shift into the peripheral circulation may contribute to overall CLL regression by loss of malignant B cell survival signals provided by the lymph node and bone marrow microenvironment.[38]

Subsequent phase II clinical trials for **11** are either planned or currently ongoing. A safety and efficacy study using a single agent (560 mg daily) for treatment of relapsed/refractory MCL[33] with or without prior treatment with bortezomib has been initiated as well for diffuse large B cell lymphoma (DLBCL).[39] Phase II trials are also ongoing at a slightly lower dose (420 mg daily) for CLL/SCL[40] in combination with existing therapies. Efficacy data for these larger trials are expected in the first half of 2012. It is interesting to note here that the doses (560 and 420 mg daily) chosen for efficacy studies are significantly higher than the minimal dose determined to give full target occupancy coverage in blood (2.5 mg/kg, ∼150 mg daily).[37] Though no rationales have been given by the investigators, it is possible that higher doses are needed to account for PK variability in a larger patient population. It is also possible that higher doses can provide full target coverage for malignant B cells in bone marrow or in lymph nodes than in blood to maximize the clinical efficacy.

Recently, **16** (PCI-45292, structure not disclosed), another irreversible inhibitor of BTK, was highlighted as a potential treatment for autoimmune disorders. While its structure has not yet been disclosed, a comprehensive patent analysis suggests that the compounds highlighted in Figure 5 may represent this lead series of inhibitors.[41] This compound was described as having a reduced potential for off-target protein binding and improved metabolic stability compared to **11**. Compound **16** dose-dependently inhibited inflammatory synovitis, pannus formation, synovial fluid cytokines, cartilage damage, and bone erosion in both preventive and established murine collagen-induced arthritis (CIA) models.[42] Recently, further studies were halted because of an undesirable organ effect in all dose groups in rats. However, it was noted that the effect was not seen in a second tox species or with **11** and that it did not appear to be related to BTK inhibition. A backup compound for autoimmune applications is expected to be disclosed soon.[43]

Avila Therapeutics. Avila is developing orally available, covalent BTK inhibitors as a potential treatment of B-cell-related hematological cancers such as non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL), as well as autoimmune diseases such as rheumatoid arthritis. Avila's lead compound **17** (AVL-292, structure not disclosed) is likely based on the diaminopyrimidine scaffold decorated with acrylamide warheads which have been described in patents.[44−47] From analysis[41] of the BTK patents from Avila Therapeutics, the structures that may represent their lead series of inhibitors are as represented in Figure 6.

Compound **22** (AVL-101, structure not disclosed) was the first lead compound disclosed by Avila. It was designed to form a covalent bond with C481 of BTK.[48] Studies from mass spectrometry and prolonged inhibition (over 8 h) of cell-based assays after washout confirmed the irreversible inhibition. Compound **22** was shown to inhibit BTK substrate phosphorylation (P-PLCγ2) and B cell proliferation with EC_{50} between 1 and 10 nM. When **22** was tested at 1 μM against 62 kinases, it was found to inhibit eight other kinases at >60% (AURORA-A, BMX, FGFR1, FLT4, ITK, JAK2, RET, and TRKA). No further development of **22** was described.

Avila's most advanced BTK inhibitor **17** has potent activity with enzyme IC_{50} < 0.5 nM and EC_{50} < 10 nM in preventing BTK autophosphorylation and BTK substrate PLCγ2.[49] It was reported to have good selectivity when measured in a panel of 62 kinases. However, without complete profiling of kinome

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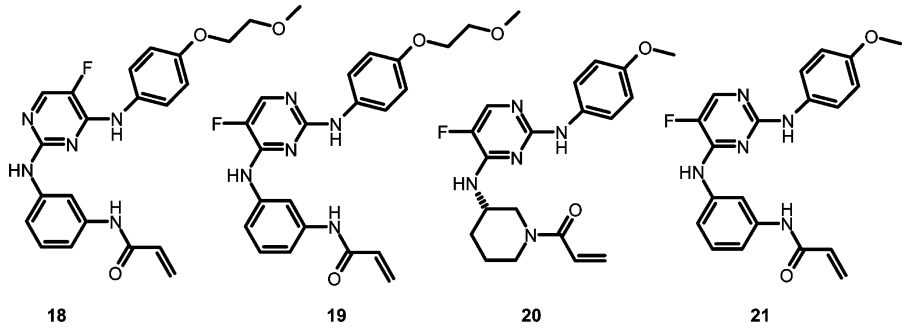

Figure 6. Representative BTK inhibitors from Avila.

selectivity, it is so far not possible to compare Avila's covalent BTK inhibitors with Pharmacyclics' covalent BTK inhibitors. Because **17** and **22** do not seem to bind into the back pocket, thus not taking advantage of the T474 gatekeeper residue, one would expect to see some differences in off-target kinase activities for these two classes of covalent inhibitors. Compound **17** was demonstrated to be efficacious in a collagen induced arthritis model, with significant efficacy at a dose of 3 mg/kg, and at doses of 10 mg/kg and higher, it completely suppressed signs and symptoms of collagen-induced arthritis in both prophylactic and therapeutic settings. A phase Ia single dose trial in healthy volunteers demonstrated that **17** was well tolerated up to a 7 mg/kg dose and a BTK occupancy with patient samples was assessed to determine optimum dosing for phase Ib studies. Preliminary data in patients with relapsed and/or refractory B cell NHL, CLL, and Waldenstrom's macroglobulinemia have recently been reported.[50] Five of six patients receiving once daily dosing of **17** at either the 125 or 250 mg dose level had stable disease and remained on the study for more than 28 days with all three patients at the 125 mg level remaining on treatment for more than 100 days. Consistent with the proposed mechanism and clinical results for **11**,[38] within these cohorts, for all four CLL patients, absolute lymphocyte counts increased after 4 weeks of treatment.

Locus Pharmaceuticals. Scientists at Locus Pharmaceuticals have identified novel pyrrolotriazines acting as inhibitors of BTK that are reported to be useful for the treatment of allergic conditions, autoimmune disorders, thromboembolism, B cell lymphoma, and inflammatory diseases such as rheumatoid arthritis.[51] A representative compound, **23** (Figure 7) inhibited

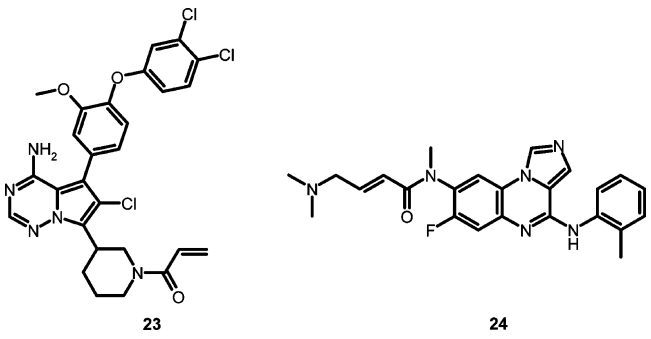

Figure 7. Representative BTK inhibitors reported by Locus Pharmaceuticals and Pfizer.

the activity of BTK (IC_{50} = 2 nM) with selectivity of 1750-, 10000-, and >10000-fold over LCK, LYN, and FYN, respectively. The agent significantly inhibited the activation of murine B-cell splenocytes (IC_{50} = 130 nM). In pharmacokinetic studies in Sprague–Dawley rats, a close analogue (at 2 mg/kg po) displayed the following values: oral bioavailability of 24%, C_{max} = 0.227 μg/mL, AUC = 652 ng·h/mL, and clearance of 0.74 mL h^{-1} kg^{-1}.[51] These inhibitors are structurally related to **11**, with the main difference in the kinase hinge-binding core motif.

Pfizer. Recently scientists at Pfizer described a series of imidazo[1,5-1]quinoxalines as potent and selective irreversible BTK inhibitors.[52] An optimized compound **24** has BTK IC_{50} of 1.93 nM as compared to LYN IC_{50} of 624 nM. It is also active in cells with IC_{50} of 3.41 nM for the inhibition of B cell proliferation. Compound **24** was also evaluated in semi-therapeutic mouse CIA model, where it significantly inhibited the progression of the disease compared to vehicle control at 3 and 10 mg/kg after oral dosing.[52]

REVERSIBLE INHIBITORS

CGI Pharmaceuticals/Genentech. CGI/Genentech pioneered the discovery and development of selective reversible inhibitors of BTK. In 2006, CGI first described a series of imidazo[1,2-*a*]pyrazinylamines with activity IC_{50} < 10 nM in an in vitro biochemical assay and IC_{50} < 500 nM for inhibition of B-cell proliferation.[53−55] Subsequent patent filings focused on replacing imidazo[1,2-*a*]pyrazinylamino with other kinase hinge binding motifs.[56] The discovery of **25** (CGI560, Figure 8),[57] with IC_{50} = 400 nM for BTK, from site-directed

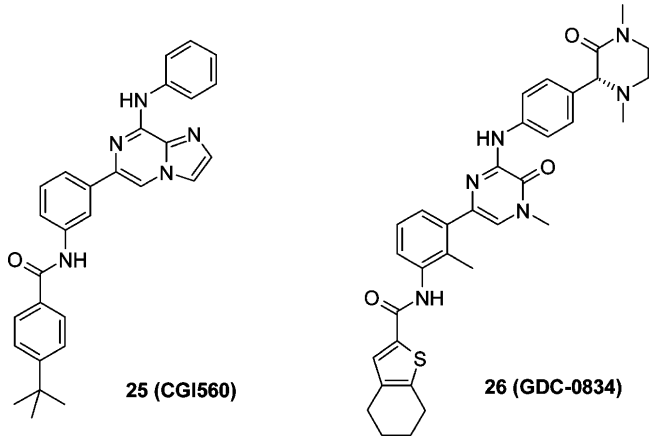

Figure 8. CGI/Genentech's BTK inhibitors **25** and **26**.

libraries and the optimization efforts that led to **9** have been described. Solution of the crystal structure of this inhibitor bound to the human BTK kinase domain (vide supra)

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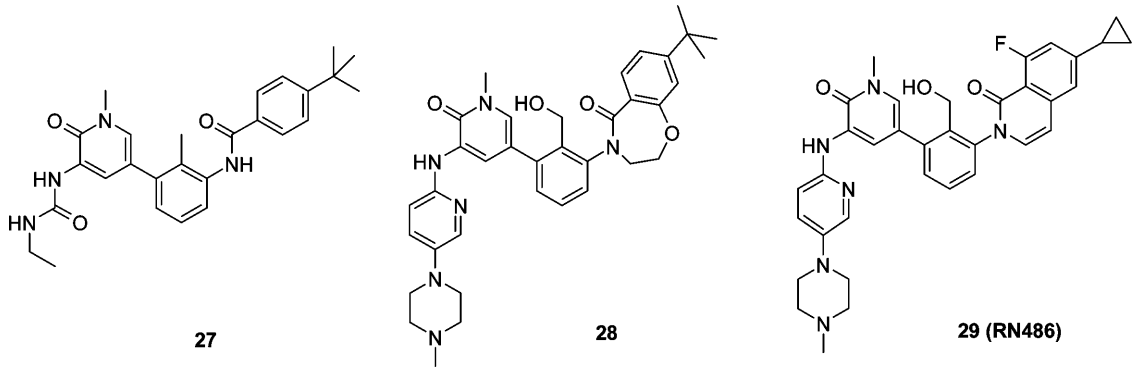

Figure 9. Representative BTK inhibitors reported by Roche.

explained the unique selectivity of this class of compounds and allowed evaluation of this class of inhibitors in a variety of in vivo studies.

Further optimization of **9** focused on improving potency and achieving oral bioavailability.[57] Scaffold hopping and replacement of the key lipophilic *tert*-butyl group with a tetrahydrobenzothiophene moiety reduced the in vivo clearance in rats, giving much improved oral exposure. Solubility was addressed by the addition of an amine group in the solvent-exposed region, ultimately leading to the development candidate **26** (GDC-0834).[57]

The effect of targeting the inactive, nonphosphorylated form of BTK with **9** on cellular signaling and in animal efficacy models was studied. Compound **9** was shown to inhibit the anti-IgM-induced phosphorylation of BTK Y551 and Y223 as well as PLCγ2 Y1217 in both human and murine B cells. Functionally, **9** inhibits anti-IgM-induced human B cell proliferation with an IC_{50} of 42 nM while having no effect on anti-CD3 and anti-CD28-induced T cell proliferation. A variety of animal models of arthritis were also investigated with this new tool compound and showed striking efficacy. Prophylactic, subcutaneous administration of **9** (100 mg/kg b.i.d.) provided IC_{90} coverage for 50% of the dosing period and significantly (>97%) inhibited clinical arthritis scores in a mouse CIA model, superior to dexamethasone. Compound **9** also inhibits FcR signaling, inhibiting TNFα production by stimulation of macrophages with immobilized immune complexes in a dose-dependent manner with an IC_{50} of 25 nM. Identical dosing of **9** (100 mg/kg b.i.d.) was also effective in the passive anti-collagen II antibody-induced arthritis model (CAIA), effectively preventing arthritis development when compared to untreated animals. Analysis of cytokine production in joints showed reduced levels of TNFα, IL-1β, IL-6, MCP1, and MIP1α. Btk inhibition was also found to affect established arthritis, where treatment was initiated 3 days subsequent to secondary collagen immunization. Compound **9** showed efficacy comparable to that of TNFα blockade and significantly reduced bone loss, proliferative synovitis, subsynovial pannus formation, and cartilage destruction.

In summary, across multiple cellular pathways and using various animal models, selective inhibition of Btk through reversible binding to the unactivated protein shows great promise as an effective treatment for rheumatoid arthritis and perhaps other autoimmune and inflammatory diseases. While **9** served as an excellent tool compound, the need for subcutaneous dosing becomes clear when noting that it has rapid clearance (86 mL min^{-1} kg^{-1}) and very low bioavailabilility (<1%) when dosed orally in rats. Additionally, potency in whole blood assay was poor (CD63 inhibition EC_{50} = 1.2 μM).[57]

Compound **26**[58] represents CGI/Genentech's first reported clinical candidate and possesses potent enzyme activity (IC_{50} = 2.3 nM) as well as excellent activity in a human whole blood CD63 assay (IC_{50} = 397 nM). Cellular mode of action studies indicate that efficacy is driven through BCR and FcR signaling as described above for **9**. Additionally, this molecule was reported to have a good PK profile in rat and dog and a good safety profile both in vitro and in vivo. Compound **26** was shown to suppress arthritis in the rat CIA model with dosing at 100 mg/kg b.i.d., giving results comparable to those from methotrexate treatment. Additionally, a PK/PD model was established that linked the level of pBTK inhibition and antiarthritis effect, with >60% inhibition of pBTK required for reduction of ankle diameter and 73% inhibition for half-maximal activity.[58] While this holds promise for future trial design, clinical development of **26** has been suspended because of poor pharmacokinetic properties in humans. Specifically, in a single dose human clinical trial the rapid hydrolysis of the amide bond resulted in below quantitative levels of **26** and the presence of the primary aniline and derivatives as the major metabolites.[59]

Roche. Roche has disclosed various novel classes of specific BTK inhibitors. A class of potent urea compounds was claimed,[60a,b] as exemplified in structure **27** (Figure 9), which has an enzyme IC_{50} of 13 nM. Multiple types of bicyclic compounds with isoquinolone, quinolone, and quinazolinone ring systems were exemplified,[60b−e,g] which have replaced the secondary amide moiety typically conserved in other patent publications. More recent analogues with unusual [7, 6] bicyclic ring systems were claimed to have very good HWB potency, with compound **28** having an HWB IC_{50} of 4 nM for CD69 inhibition.[60f]

Roche reported preclinical studies with a reversible, potent, and highly selective BTK inhibitor, **29** (RN486).[61] It binds the enzyme in a competitive manner in a TR-FRET-based competitive assay with an IC_{50} of 0.3 nM. Compound **29** exhibited a high degree of selectivity over a large panel of 396 kinases, including SYK and JAK, two validated RA targets. The enzyme that was inhibited the most by **29** next to BTK was SLK, an enzyme not implicated in autoimmune responses, with 139-fold selectivity.[61] Together, these data indicate that **29** is a selective and potent inhibitor for Btk. It blocks both BCR and FcR signaling, inhibiting IgM-stimulated CD69 expression in B cells in human whole blood, IgG-FcγR mediated TNFα release in monocytes, and IgE-Fce cross-linking induced histamine release in mast cells with IC_{50} values of 17, 4, and 29.2 nM,

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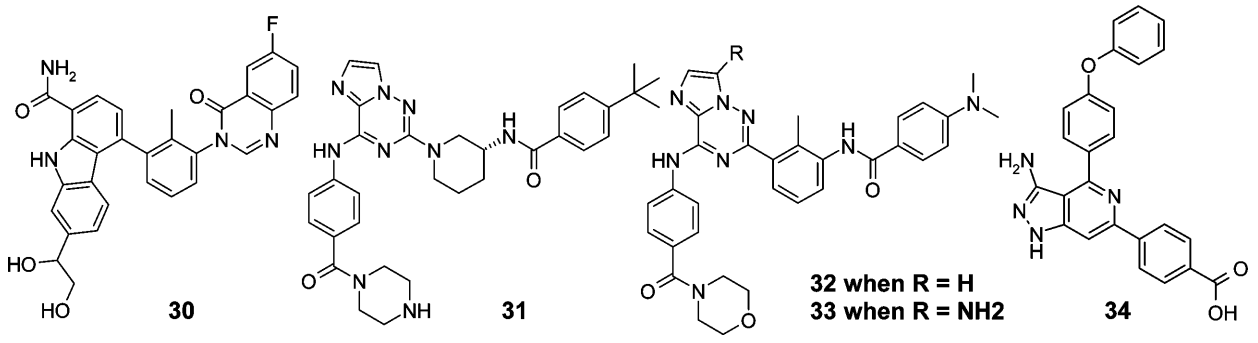

Figure 10. Representative BTK inhibitors reported by BMS.

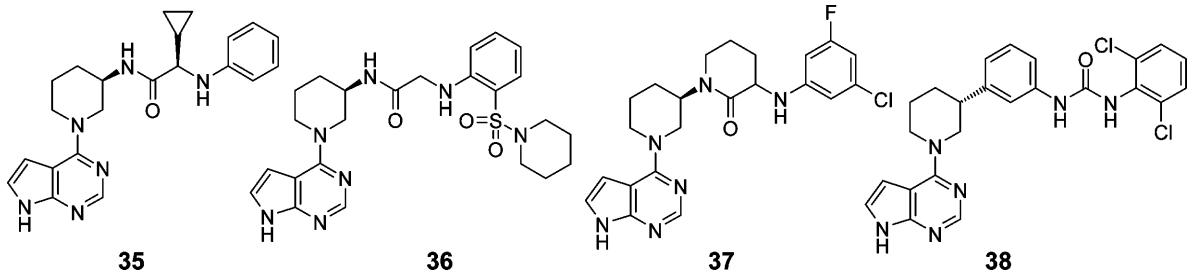

Figure 11. Representative BTK inhibitors reported by Biogen Idec.

respectively. In contrast, as expected, **29** is inactive in the Jurkat T cell calcium flux assay. Compound **29** dose-dependently inhibits disease progression in both mCIA and mCAIA models, as well as demonstrates an additive effect of inhibiting inflammation and bone erosion in adjuvant-induced arthritis. When **29** was administered orally in a preventive mode at doses of 3, 30, and 100 mg/kg in a mouse CIA model, it completely inhibited ex vivo anti-IgD stimulated CD69 expression at 3 and 6 h postdose at all doses and by approximately 50−80% 24 h postdose. Compound **29** showed complete inhibition of arthritis as measured by clinical scores at 100 mg/kg, similar to dexamethasone. When **29** was studied in a therapeutic mode in mCIA and mCAIA models, animals treated with **29** did not show progression of disease at 30 mg/kg. In addition, **29** demonstrated a greater effect (91%, 30 mg/kg) than cyproheptadine, a nonselective antihistamine agent (69% maximum inhibition) in blocking the hypersensitive immune responses in a rat passive cutaneous anaphylaxis (rPCA) model. Both BCR and FcR pathways are known to play an essential role in the development of RA and other autoimmune diseases. Therefore, the blockade of both signaling pathways may have contributed to the anti-inflammatory effects observed with **29** in the various preclinical disease models. BTK inhibition is suggested to protect bone erosion not only indirectly by inhibiting ongoing inflammation but also directly by affecting osteoclast differentiation. In addition, observed additive effects between **29** and low dose methotrexate in a rat AIA model demonstrated the potential of combination therapies for BTK inhibitors.

BMS. The small molecule kinase inhibitor **1** is approved for the treatment of imatinib-resistant chronic myelogenous leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia. The primary targets of **1** are the tyrosine kinases Bcr-Abl and Src family members, and numerous other kinases have been reported to be inhibited at nanomolar concentrations.[62] Chemical proteomics experiments using CML cells have demonstrated that **1** also binds to BTK. **1** inhibits the kinase activity of BTK in vitro with IC_{50} = 5 nM, and it abolishes autophosphorylation on Y223 in cells at 100 nM.[63] Compound **1** has also been shown to bind to BTK in B cells, basophils, and mast cells.[64] Inhibition of BTK with **1** has been indicated to block the secretion of proinflammatory cytokines. At 1 μM, the multitargeted kinase inhibitor almost completely blocks basal and LPS-induced TNFα secretion of vitamin D3-differentiated U937 cells, and it completely inhibits antigen-induced secretion of IL6 in murine mast cells.[63] Inhibition of BTK with **1** has also been indicated to block histamine release. At 1 μM, **1** completely abolishes anti-IgE antibody-induced histamine release from primary human basophils.[63,65] It also significantly reduces antigen-induced histamine release from bone marrow-derived murine mast cells. The level of reduction is similar to that in cells derived from BTK deficient animals.[63] Consequently, **1** has been suggested to be of potential benefit for the treatment of inflammatory and immunological diseases involving BTK-dependent signaling pathways.[63,65] However, its off-target activity against other tyrosine kinases may limit its potential applications for chronic inflammatory diseases.

BMS has also disclosed diverse chemical classes of kinase inhibitors specific for BTK. Substituted carbazoles were claimed to be effective inhibitors of BTK and other Tec family kinases such as ITK with a representative example compound **30** (Figure 10) with BTK IC_{50} of 0.7 nM and Ramos FLIPR IC_{50} of 11 nM.[66] Substituted imidazotriazines[67,68] have some pharmacophore features that are similar to known selective BTK inhibitors (CGI bicyclic core patents). Compound **31**, with a piperidine group instead of a phenyl group, retains potent BTK activity, having IC_{50} values of 13 and 56 nM in human enzyme assays and mouse splenic B cell proliferation assays, respectively.[67] This modification avoids the potential of generating primary aniline metabolites and may provide improved physical properties compared to aromatic amides. An amino group on the imidazole ring was shown to increase potency. For example, compound **33** is about 5-fold more

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active than compound **32**.[68] In the human enzyme assay and the human Ramos cell FLIPR assay, **33** has IC_{50} values of 30 and 32 nM, versus 144 and 142 nM for **32**. The amino group is presumably in proximity to the gatekeeper Thr residue, possibly making favorable H-bond interaction with the Thr group. A more recent patent[69] described a series of 3-aminoazaindazole compounds as BTK inhibitors. A representative compound, **34**, is reported to have a BTK IC_{50} of 1 nM.

Biogen Idec. Multiple compounds with pyrrolopyrimidine cores were recently claimed by Biogen Idec as selective BTK inhibitors.[70] Compounds **35**–**38** (Figure 11) are some representative examples with BTK enzyme IC_{50} less than 100 nM. The vector off the piperidine ring seems to project into the selectivity pocket, albeit with very different functionalities from the *tert*-butyl benzyl group. None of the claimed inhibitors have obvious solvent-exposed groups, unlike the other types of reversible BTK inhibitors. However, lack of more detailed information such as binding affinity and cellular activity precludes meaningful comparison.

■ CONCLUSIONS

BTK inhibition has emerged as a target of growing interest in both oncology and inflammation settings in the past 10 years. Careful analysis of the unique amino acid sequences and structural characterization by X-ray crystallography of ligands bound to BTK have enabled the strategic development of potent and exquisitely selective BTK inhibitors. Targeting the relatively unique C481 of BTK has led to selective *covalent* inhibitors that have provided validation of the target in animal models of inflammation and cancer. Most recently, a compound from this class has advanced to phase II clinical trials in an oncology setting, showing acceptable toxicities at doses that provide complete BTK occupancy and significant clinical efficacy. Concerted screening, crystallography, modeling, and medicinal chemistry efforts have led to highly selective, *noncovalent* BTK inhibitors that bind to a specificity pocket present in the nonphosphorylated form of BTK. Recent disclosures have shown that these molecules exhibit similar in vivo effects in animal models of arthritis, mediated through inhibition of BCR and FcR signaling. Recent patent activity demonstrates that there is continued interest in further exploring both covalent and noncovalent inhibitors of BTK with improved potency and druglike physicochemical properties and safety profiles. Ultimately, more data from ongoing and planned clinical trials in both oncology and inflammation settings will determine whether BTK inhibition with small molecules will play a role in improving human health.

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The authors declare no competing financial interest.

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Timothy D. Owens received his B.S. degree from University of California—San Diego and his Ph.D. from University of California—Berkeley under the guidance of Prof. Jonathan Ellman, designing chiral sulfinamide-based catalysts for asymmetric synthesis. He pursued postdoctoral studies in natural product synthesis at Memorial Sloan-Kettering in New York with Prof. Samuel Danishefsky before joining Roche Palo Alto in 2004. Working at Roche Nutley since 2009, his research included CNS, inflammation, and oncology programs as well as chemical biology approaches to finding novel therapeutic targets. In December 2011, Tim joined Principia Biopharma in South San Francisco, CA, as an Associate Director of Medicinal Chemistry.

Andreas Kuglstatter received an M.S. degree from the University of Frankfurt, Germany, in 1999 for the characterization of photosynthetic reaction centers, and a Ph.D. from the University of Cambridge, U.K., in 2003 for structural analysis of RNA–protein complexes. After 2 years as a Postdoctoral Fellow at F. Hoffmann-La Roche in Basel, Switzerland, he joined Roche Palo Alto, CA, where he was Head of Protein Crystallography. In 2009, Andreas joined the Discovery Technology Department at Hoffmann-La Roche in Nutley, NJ, and in 2011 he returned to F. Hoffmann-La Roche in Basel, Switzerland. His main areas of expertise are kinase inhibitors, fragment-based drug discovery, protein engineering, and external collaborations.

Rama K. Kondru received his B.S. and M.S. from Indian Institute of Technology in Mumbai, India. He received his Ph.D. from the University of Pittsburgh, PA, in 1999. His research interests are in structure-based drug design and computational chemistry. He is currently the Head of the Computational Chemistry and Data Mining Group at Hoffmann-La Roche, Nutley, NJ.

David M. Goldstein received his Ph.D. in Organic Chemistry from the Department of Chemistry at the University of Virginia in 1992. He went on to complete postdoctoral studies at the University of Pittsburgh, PA, in 1994 and subsequently joined Syntex in Palo Alto, CA. David continued on with Roche in 1995 where his research focused on the discovery of small molecule inhibitors in the areas of inflammatory, respiratory, autoimmune, and virology diseases. In 2009, David became a Senior Director and the Head of Inflammation Chemistry at Hoffmann-La Roche in Nutley, NJ. In 2011, David joined Principia Biopharma in South San Francisco, CA, as the Vice President of Drug Discovery.

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■ ABBREVIATIONS USED

AURORA-A, Aurora kinase A; BCR, B cell receptor; BLK, B lymphosite kinase; BMX, kinase encoded by *BMX* gene; BTK, Bruton's tyrosine kinase; CASK, calcium/calmodulin-dependent serine protein kinase; CD63, antigen encoded by the *CD63*

gene; CD69, lectin protein encoded by the *CD69* gene; CIA, collagen induced arthritis; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; EGFR, epidermal growth factor receptor; ERBB2, kinase of epidermal growth factor receptor family (also known as HER2, Neu, and CD340); ERBB4, kinase of epidermal growth factor receptor family encoded by *ERBB4* gene; FcR, Fc receptor; FGFR1, fibroblast growth factor receptor 1; FLIPR, fluorescence imaging plate reader; FLT4, FMS-related tyrosine kinase 4; FYN, tyrosine kinase encoded by the *FYN* gene; HCK, hemopoietic cell kinase; HWB, human whole blood; ITK, IL2-inducible T-cell kinase; JAK2, Janus kinase 2; JAK3, Janus kinase 3; LCK, lymphocyte specific protein tyrosine kinase; LYN, V-yes-1 Yamaguchi sarcoma viral relate oncogene homologue; MCL, mantle cell lymphoma; MINK, misshapen-like kinase 1; MS, multiple sclerosis; NEK11, NIMA (never in mitosis gene a)-related kinase 11; NHL, non-Hodgkin's lymphoma; PDGFRB, platelet-derived growth factor receptor, β polypeptide; RA, rheumatoid arthritis; RET, tyrosine kinase encoded by the *RET* proto-oncogene; rPCA, rat passive cutaneous anaphylaxis; SLE, systemic lupus erythematosus; SLL, small lymphocytic lymphoma; SLK, Ste20-like kinase; SRC, sarcoma kinase; SYK, spleen tyrosine kinase; TEC, tyrosine kinase encoded by the *TEC* gene; TR-FRET, time-resolved fluorescence resonance energy transfer; TRKA, neurotrophic tyrosine kinase receptor type 1; TXK, tyrosine kinase encoded by the *TXK* gene; XLA, X-linked agammaglobulinemia

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