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Letter

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Aminopyrazole Carboxamide Bruton's Tyrosine Kinase Inhibitors. Irreversible to Reversible Covalent Reactive Group Tuning

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ABSTRACT: Potent covalent inhibitors of Bruton's tyrosine kinase (BTK) based on an aminopyrazole carboxamide scaffold have been identified. Compared to acrylamide-based covalent reactive groups leading to irreversible protein adducts, cyanamide-based reversible-covalent inhibitors provided the highest combined BTK potency and EGFR selectivity. The cyanamide covalent mechanism with BTK was confirmed through enzyme kinetic, NMR, MS and X-ray crystallographic studies. The lead cyanamide-based inhibitors demonstrated excellent kinome selectivity and rat pharmacokinetic properties.

KEYWORDS: BTK, EGFR, irreversible covalent inhibitor, reversible covalent inhibitor

Bruton's tyrosine kinase (BTK) is a non-receptor tyrosine kinase belonging to the Tec family of kinases.[1] BTK is broadly expressed in hematopoietic cells, especially B lymphocytes and myeloid cells, but notably not in T cells. In B cells, BTK is essential for competent signaling through the B cell antigen receptor (BCR) and is required for B cell development and differentiation.[2] The critical role BTK plays in B cell development is evident by the phenotype of patients suffering from X-linked agammaglobulinemia (XLA), a hereditary immunodeficiency disease that results from mutations in the gene encoding BTK.[3-5] XLA patients have very few circulating mature B cells as a result of a developmental arrest at the pre-B cell stage in the bone marrow and the further failure of immature B cells that do reach the peripheral compartment to mature.[6] As a consequence, XLA patients display a compromised humoral immune response leading to low levels of immunoglobulin and a failure to develop antibody responses. BTK is also an essential component to enable signaling through the Ig Fc receptors (FcεR and FcγR) and specific toll-like receptors in myeloid cells.[7] Signaling through BTK in these circumstances regulates inflammatory cytokine and chemokine production by macrophages and can lead to degranulation of mast cells.

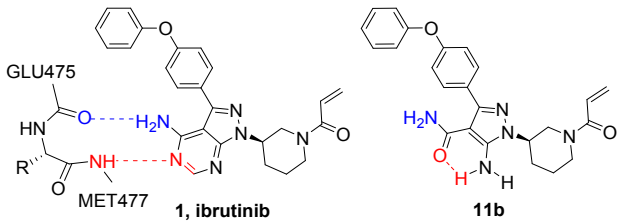
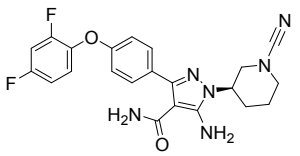


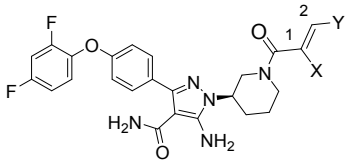
Figure 1. Structure of BTK inhibitor ibrutinib (**1**) and aminopyrazole carboxamide **11b**

The inhibition of BTK activity is an attractive therapeutic approach to treat autoimmune diseases where B cells and myeloid cells play an important role such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).[8-12] BTK is also a promising target in cancer chemotherapy since it is required for the survival and proliferation of leukemic B cells, and myeloid cells are important components of the tumor microenvironment.[13] Ibrutinib (**1,** Figure 1) and acalabrutinib have been approved as second-line therapy for mantle cell lymphoma (MCL). Ibrutinib has also been approved for the treatment of chronic lymphocytic leukemia (CLL) and Waldenström macroglobulinemia

Table 1. Impact of covalent reactive group selection on compound pharmacology[a]



9a



9b-i

Cmpd	X	Y	ω[b]	IC50 (nM)[c]				k_{inact}/K_i (1/M·s)[d]		BTK T½ (h)[e,g]	THLE IC50 (μM)
				BTK WT	BTK C481S	EGFR	SRC	BTK WT[e]	EGFR[f]		
1			0.83	0.1	3.4	18	38	226,000	45,040	>24	11
9a			0.44	1.5	43	16,800	1,630	123,900	56	6	137
9b	H	H	0.83	0.25	17	26	4,030	206,500	25,110	>24	15
9c	CH_3	H	0.48	247	176	>20,000	3,350	<1[h]	ND	ND	ND
9d	H	CH_3	0.60	61	66	>20,000	3,410	4,910	ND	>24	149
9e	F	H	0.69	5.3	170	570	6,480	35,300	435	>24	67
9f	H	CH_2OH	0.61	2.3	33	2,910	4,610	26,650	213	>24	43
9g	H	CH_2F	0.65	2.7	90	2,180	3,140	85,770	332	>24	49
9h	H	CHF_2	0.81	0.41	115	274	5,840	80,500	ND	>24	8
9i	H	CH_2NMe_2	0.59	3.7	65	149	370	19,180	514	>24	15

[a]All values are the mean of two or more independent assays. ND, not determined. [b]Electrophilicity index calculated using DMol3 at PBE/TNP/COSMO level of theory in water with LowModeMD. [c]Lanthascreen assay, [ATP] = K_m. [d]For reversible inhibitors the analogous second-order rate constant is given. [e]TR-FRET binding assay. [f]Caliper enzymatic assay. [g]Apparent dissociation rate. [h]Kinetics consistent with reversible inhibition, time dependence not observed.

(WM). Both of these agents are considered targeted, covalent-irreversible inhibitors of the kinase.[14-16] They react covalently with a non-catalytic cysteine residue (Cys481) in the ATP binding site to form an irreversible protein adduct. Covalent inhibitors have drawn interest for their potential to offer a) improved biochemical efficiency, b) improved selectivity versus targets lacking a reactive amino acid residue, and c) a prolonged pharmacodynamic-driven duration of action compared to reversible, substrate competitive inhibitors.[17] However, the concern over indiscriminant covalency leading to idiosyncratic adverse drug reactions (IADR) has largely limited the application of this drug class to life threatening indications.[18] In response to the latter concern, reversible inhibitors of BTK have also advanced into clinical trials[19,20] as well as a third class, reversible-covalent inhibitors.[21] Inhibitors that form a reversible-covalent bond with the cysteine residue of the kinase offer the advantage of prolonged residence time on the target but theoretically lack the long-lived protein adduct that may be a trigger for drug hypersensitivity liabilities.

Although compound **1** is a potent inhibitor of BTK, its incomplete off-target selectivity does not fully exploit the potential of covalent design. Indeed it potently inhibits several other kinases that also have an equivalent cysteine in their active site such as EGFR and ITK.[15] It is also a potent, reversible inhibitor of SRC family kinases such as SRC, LCK, and LYN. Inhibition of wild-type EGFR leads to skin rash and gastrointestinal side effects while inhibition of ITK and SRC family kinases risk expanded immune suppression beyond the B and myeloid cell compartments. Herein, we describe the discovery of potent BTK inhibitors based upon an aminopyrazole carboxamide kinase hinge binding scaffold. Through fine tuning of the covalent reactive group (CRG) and structural discrimination in non-covalent kinome affinity, highly selective inhibitors of BTK have been identified spanning irreversible to reversible-covalent modalities.

As revealed in the co-crystal structure of **1** with the human BTK kinase domain,[22] the aminopyrimidine ring forms two hydrogen bond contacts with the kinase hinge residues, specifically a) the amino substituent and the backbone carbonyl of Glu475 and b) the N5 ring nitrogen atom with the backbone NH of Met477. We hypothesized that comparable hinge interactions could be achieved by excising the pyrimidine ring and replacing the 4-aminopyrazolopyrimidine scaffold by a 5-aminopyrazole 4-carboxamide as in compound **11b**, Figure 1. A proposed intramolecular hydrogen bond between the carbonyl of the carboxamide and the 5-amino substituent would maintain the rigidity provided by the pyrimidine ring in compound **1** while the amino group may facilitate further hinge contacts.

This approach was indeed successful with compound **11b** demonstrating comparable potency to **1** for BTK inhibition in a Lanthascreen assay (IC_{50} = 0.18 nM compared to 0.13 nM, **1**). Unfortunately, the undesired attributes of EGFR and SRC kinase inhibition (IC_{50} = 15 nM and 72 nM, respectively) were maintained. In order to explore the potential of the aminopyrazole carboxamide scaffold to deliver improved kinome selectivity, we undertook a matrix survey of two regions of the molecule. These included 1) replacement of the acrylamide with alternative CRGs possessing different electronic or steric factors and 2) modification of the terminal phenoxy substituent through substitution or pyridine replacement. The desired aminopyrazole carboxamides were prepared through a convergent route (See Supporting Information).

A series of compounds with various CRGs was profiled in Lanthascreen assays for inhibition of BTK, EGFR and SRC as well as in time dependent kinetics experiments against the former two kinases, Table 1. The electrophilicity index (ω) was calculated for each as an approximation of the intrinsic CRG reactivity. Potency against the BTK C481S mutant, incapable of forming the covalent adduct, was measured to approximate the intrinsic binding (K_i) component. The unsubstituted acrylamide **9b** was found to be the most potent BTK inhibitor of the series; however, it was also a potent inhibitor of EGFR. Consistent with the dominant role of covalency in potency, compound **9b** was 68-fold less potent against the BTK C481S mutant (2 h incubation). Other CRG derivatives showed modestly weaker inhibition toward the mutant suggesting the sterics and/or electronics of the CRG can influence the reversible binding component of the two step inhibition mechanism. The CRG choice did not have a significant influence on SRC kinase inhibition with the exception of dimethylamino-butenamide **9i** owing to the selection of the phenoxy substituents (*vide infra*). Substitution of the acrylamide C1-position by methyl (**9c**) was the most detrimental to BTK potency and led to a compound lacking time dependent inhibition. This can be attributed to a 1,3-strain interaction disfavoring coplanarity of the carbonyl and olefin π-systems. Crotyl analog **9d** also suffered significant erosion of BTK potency presumably due to reduced electrophilicity and steric clash with the cysteine nucleophile. Increasing the electrophilicity of the reactive carbon center by fluorine substitution of the terminal methyl as in **9g** and **9h** recovered BTK potency. The substituted crotyl analogs displayed a significantly reduced time dependent kinetics with respect to EGFR compared to acrylamide **9b** with compounds **9f** and **9g** showing the best overall BTK potency and selectivity. The structurally distinct cyanamide **9a** also showed excellent BTK potency and superior EGFR selectivity to the Michael acceptor-based analogs.

Notably, compared to the acryl- and crotylamide analogs which were effectively irreversible-covalent inhibitors of BTK (dissociation $T_{½}$ > 24 h), cyanamide **9a** displayed a finite residence time on BTK ($T_{½}$ = 6 h), Table 1. Therefore, cyanamide **9a** would be considered a reversible-covalent inhibitor of BTK. This finding is consistent with cyanamide-based covalent inhibitors of the cathepsins previously reported.[23,24] The lack of a long-lived covalent adduct with **9a** was attractive to us for the application to non-life threatening disease indications. Also, compared to the other potent CRGs studied, compound **9a** was less cytotoxic to THLE cells, a potential risk indicator of IADR in addition to hapten generation.[25] Consequently, lead identification was directed towards the cyanamide as the preferred CRG.

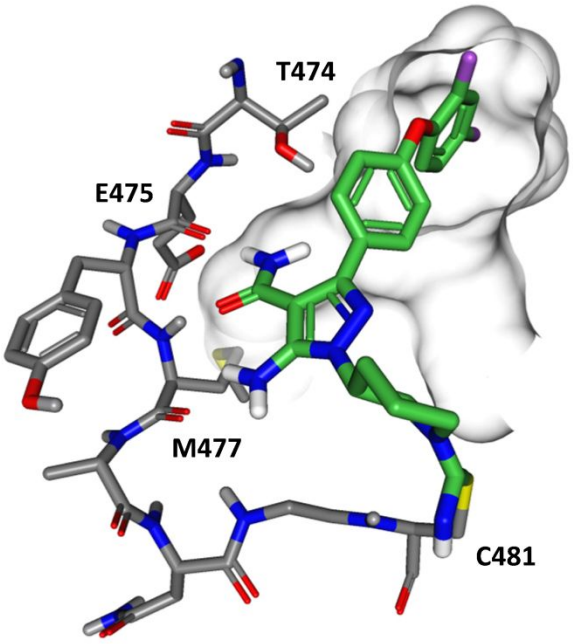
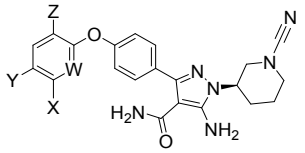


Figure 2. Co-crystal structure of the covalent adduct between compound **9a** and the mouse BTK kinase domain.

In order to confirm that cyanamide **9a** formed a discrete protein adduct, three biophysical approaches were examined. First, compound **9a** was incubated with recombinant human BTK kinase domain and the resulting product was characterized by mass spectroscopy. A shift in the parent protein mass consistent with the mass of **9a** was observed supporting the formation of a 1:1 (BTK:**9a**) adduct, Figure S1 (See Supporting Information). Second, compound **9a** was isotopically labelled with a ^{13}C atom at the cyanamide carbon. Upon incubation with BTK protein, the intensity of the parent ^{13}C signal (119.3 ppm) was diminished resulting in a broad signal at 167.2 ppm (25 Hz linewidth) consistent with formation of the isothiourea adduct, Figure S2 (See Supporting Information).[23] Lastly, compound **9a** was co-crystallized with the kinase domain of mouse BTK which confirmed adduct formation between the cyanamide carbon and the thiol of Cys481, Figure 2. As anticipated, the primary carboxamide of **9a** formed hydrogen bond contacts with hinge residues Glu475 and Met477. The latter also forms a favorable contact with the

Table 2. Impact of aryl ether substituent on pharmacology of reversible-covalent cyanamides[a]



Cmpd	W	X	Y	Z	IC_{50} (nM)[b]				k_{inact}/K_i (1/M·s)[c]		BTK T½ (h)[d,f]
					BTK WT	BTK C481S	EGFR	SRC	BTK WT[d]	EGFR[e]	
9a	CH	H	F	F	1.5	43	16,800	1,630	123,900	56	6
10	CH	H	Cl	H	0.64	48	3,890	1,400	118,300	87	6
11a	CH	H	H	H	0.37	2.8	1,710	4.2	135,400	17	7
20a	N	H	Cl	H	6.2	92	15,400	>20,000	24,370	121	7
20b	N	CF_3	H	H	2.6	36	>20,000	14,400	55,870	7	3
20c	N	H	Cl	F	7.6	230	>20,000	>20,000	60,970	155	5

[a]All values are the mean of two or more independent assays. [b]Lanthascreen assay, [ATP] = K_m. [c]For reversible inhibitors the analogous second-order rate constant is given. [d]TR-FRET binding assay. [e]Caliper enzymatic assay. [f]Apparent dissociation rate.

Table 3. Cellular potency of cyanamides[a]

Cmpd	IC_{50} (nM)			
	Proliferation		HWB histamine	CD69 expression
	B Cell	T Cell		
9a	2.7	>10,000	64	53
10	1.7	8,150	21	46
20a	1.6	>10,000	95	45
20b	14.9	10,100	42	33
20c	19.1	>10,000	50	89

[a]All values are the mean of two or more independent assays.

amino substituent of **9a**.

A representative survey of the terminal phenoxy substituent in the cyanamide series is depicted in Table 2. Irrespective of the terminal substituent, all of the cyanamides demonstrated excellent selectivity against EGFR with very weak time dependent inhibition. As with **9a**, all were reversible-covalent inhibitors of BTK with dissociation rates from the recombinant enzyme of 3-7 h. The unsubstituted phenoxy analog **11a** was the most potent inhibitor of both wild-type BTK and the C481S mutant. However, as with acrylamide-based inhibitors **1** and **11b** also having the phenoxy substituent, **11a** was a very potent inhibitor of SRC kinase. On the other hand, substituted phenyl (**9a** and **10**) or pyridinyl (**20a-c**) ethers showed significantly weaker inhibition of SRC kinase. Substitution also had a detrimental effect on the reversible binding (K_i) component of BTK inhibition as shown by their reduced potency against the BTK C481S mutant. For example, compound **9a** was 15-fold less potent than **11a**. However, SRC kinase was more sensitive to this modification yielding a 388-fold reduction. The size of the phenoxy pocket is largely governed by the conformational state of the C-helix (in vs. out). The observed selectivity may suggest greater accommodation of ligands through the inactive C-helix out conformation for BTK. Indeed, in an analysis of PDB structures by Möbitz,[26] BTK structures were predominantly in a C-helix out conformation while SRC-family kinases predominately were C-helix in. Since SRC kinase lacks the homologous cysteine residue to BTK, this non-covalent intrinsic selectivity is further amplified when the covalent nature of the inhibitors is realized (e.g. 1,290-fold, **9a**).

Selective cyanamide-based BTK inhibitors were further profiled in cellular in vitro assays, Table 3. All compounds potently inhibited B cell proliferation while displaying minimal inhibition of T cell proliferation. Potent inhibition of histamine release from Mast cells was observed in a human whole blood assay. Similar inhibition was observed towards anti-IgD-mediated CD69 upregulation on B cells. The substituted cyanamide analogs also displayed favorable ADME properties with low in vitro human microsomal clearance (HLM CL < 8 μL/min/mg) and good permeability (RRCK cells, $>10\times10^{-6}$ cm/sec). After IV dosing to rats, the compounds showed low to moderate in vivo clearance with the phenyl analogs (**9a** and **10**) having the longest half-lives, Table S1 (See Supporting Information). All compounds demonstrated excellent oral bioavailability following oral dosing in rats (76-93%).

Compound **9a** showed high kinome specificity when profiled against a panel of 51 kinases (1 mM ATP, 1 μM compound), Table S2 (See Supporting Information). Dose response curves were obtained for the 11 kinases that share a homologous cysteine residue to BTK C481 and representative SRC family kinases (IC_{50}, 1 mM ATP), Table S3 (See Supporting Information). Significant inhibition by **9a** was only observed against a subset of the kinases with a

shared cysteine (BMX, TEC, and TXK; 5–29 fold BTK). BTK and all three other kinases share a common gatekeeper residue (Thr) as well as a common Cys+3 residue (Asn). The nature of the Cys+3 residue has been postulated to strongly influence the pKa of the cysteine thiol and consequently its nucleophilicity.[27] The pKa for the reactive cysteine thiol in BTK and other family members sharing an Asn residue has been estimated to be the most acidic (i.e. most nucleophilic at physiological pH) of the 11 kinases.[16] The pKa was calculated to be higher (i.e. less nucleophilic) for other members in the family having an Asp or Glu residue with EGFR being one of the highest. In contrast, the calculated electrophilicity of the cyanamide was significantly lower than the corresponding acrylamide, Table 1. Consequently, the greater differential in CRG electrophilicity and cysteine nucleophilicity is a plausible hypothesis driving the excellent EGFR selectivity seen with the cyanamide analogs such as **9a**. In addition, a weak stabilizing interaction between the isothiourea of the protein adduct and the Cys+3 Asn residue may contribute to the selectivity observed.

The identification of compound **9a** as a potent and selective BTK inhibitor demonstrates the importance of considering reversible ligand affinity and CRG selection in covalent inhibitor design. Aligning the steric environment of the CRG reactive site and its degree of electrophilicity with the nature of the reactive cysteine residue, especially nucleophilicity (pKa), were found to be important factors for achieving selectivity among covalently amenable targets. As shown here, the acceptance of diminished reversible binding affinity towards the covalent target (**9a** vs. **11a**, Table 2) in favor of greater affinity loss against an off-target lacking covalent enablement is a useful tactic to maximize target selectivity of covalent inhibitors. The potential advantage of reversible covalency towards safety must be balanced with the need for more sustained pharmacokinetic exposure since BTK enzyme turnover ($T_{½}$ > 24 h)[28] is no longer the dominant pharmacodynamics determinant. Fortunately, we were able to incorporate good metabolic stability into the cyanamide series as evident by the low clearance and high oral bioavailability in rat especially for **9a**. The success of the covalent-reversible strategy in the case of BTK is further supported by the excellent in vivo efficacy of **9a** we have previously described in the murine NZBxW lupus model[29] and suggests that cyanamides may have broad applicability in covalent drug design.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.0000000.

Synthetic procedures and characterization for final analogs and intermediates, single crystal determination supporting absolute configuration assignment, protein co-crystallography and MS adduct characterization methods, and pharmacology assay procedures (PDF)

Accession Codes

PDB accession code 6MNY (**9a**). Authors will release the atomic coordinates and experimental data upon article publication.

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Notes

The authors declare no competing financial interest.

All procedures performed on animals were in accordance with regulations and established guidelines and were reviewed and approved by the Pfizer Institutional Animal Care and Use Committee.

Compound **9a** (PF-06250112) is commercially available via MilliporeSigma (catalog # PZ0220).

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ABBREVIATIONS

ADME, absorption, distribution, metabolism, and excretion; BTK, Bruton's tyrosine kinase; CRG, covalent reactive group; IARD, idiosyncratic adverse drug reactions; THLE, SV40 T-antigen-immortalized human liver epithelial cells; XLA, X-linked agammaglobulinemia.

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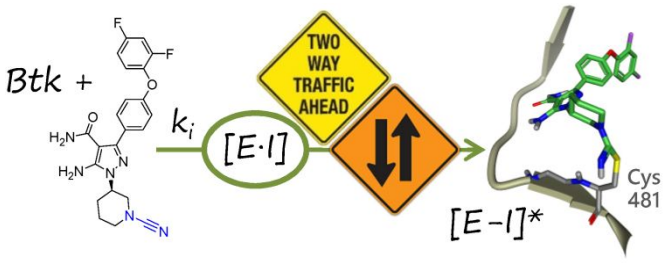
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