

Discovery of a Potent, Covalent BTK Inhibitor for B-Cell Lymphoma

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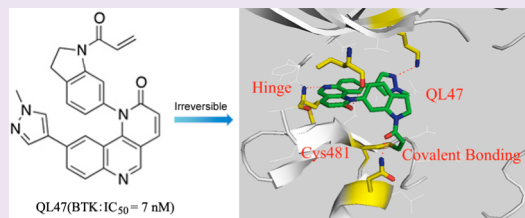
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Supporting Information

ABSTRACT: BTK is a member of the TEC family of non-receptor tyrosine kinases whose deregulation has been implicated in a variety of B-cell-related diseases. We have used structure-based drug design in conjunction with kinome profiling and cellular assays to develop a potent, selective, and irreversible BTK kinase inhibitor, QL47, which covalently modifies Cys481. QL47 inhibits BTK kinase activity with an IC_{50} of 7 nM, inhibits autophosphorylation of BTK on Tyr223 in cells with an EC_{50} of 475 nM, and inhibits phosphorylation of a downstream effector PLC γ 2 (Tyr759) with an EC_{50} of 318 nM. In Ramos cells QL47 induces a G1 cell cycle arrest that is associated with pronounced degradation of BTK protein. QL47 inhibits the proliferation of B-cell lymphoma cancer cell lines at submicromolar concentrations.



B-lymphoma's tyrosine kinase (BTK) is a member of TEC family non-receptor tyrosine kinases that plays a critical role in B-lineage lymphoid cells.^{1,2} BTK signals downstream of the B-cell receptor (BCR) where it regulates survival, activation, proliferation, differentiation, and maturation of B-cells.^{3,4} BTK is predominately expressed in hematopoietic lineage cells with the exception of T, NK, and plasma cells. BTK was originally linked to diseases following the discovery of mutations in BTK that result in X-linked agammaglobulinemia (XLA), an immune disorder where patients fail to produce mature B-cells.^{5–7} Full activation of BTK following engagement of the B-cell receptor is a multistep process that requires generation of PIP3 on the inner leaflet of the membrane by PI3K, which serves as a docking site for the pleckstrin homology domain of BTK followed by phosphorylation of Tyr551 by kinases such as Lyn and Syk as well as autophosphorylation of tyrosine 223.^{8,9} BTK activation results in induction of multiple

signaling pathways including Stat5, PI3K/Akt/mTOR, and NF- κ B.^{10–13} The most well-defined effector molecule of BTK signaling is PLC γ , whose activation results in calcium mobilization and activation of NF- κ B and MAP kinase signaling pathways.¹⁴ Recently the deregulation of BTK has been observed in numerous B-cell-derived malignancies such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), non-Hodgkin's lymphoma (NHL), mantle cell lymphoma (MCL), Waldenström's macroglobulinemia (WM), and multiple myeloma (MM).^{15–20} These findings have spurred an intense effort to develop both covalent and non-covalent BTK inhibitors that have exhibited efficacy in clinical trials against various human tumors.²¹ Ibrutinib (PCI-

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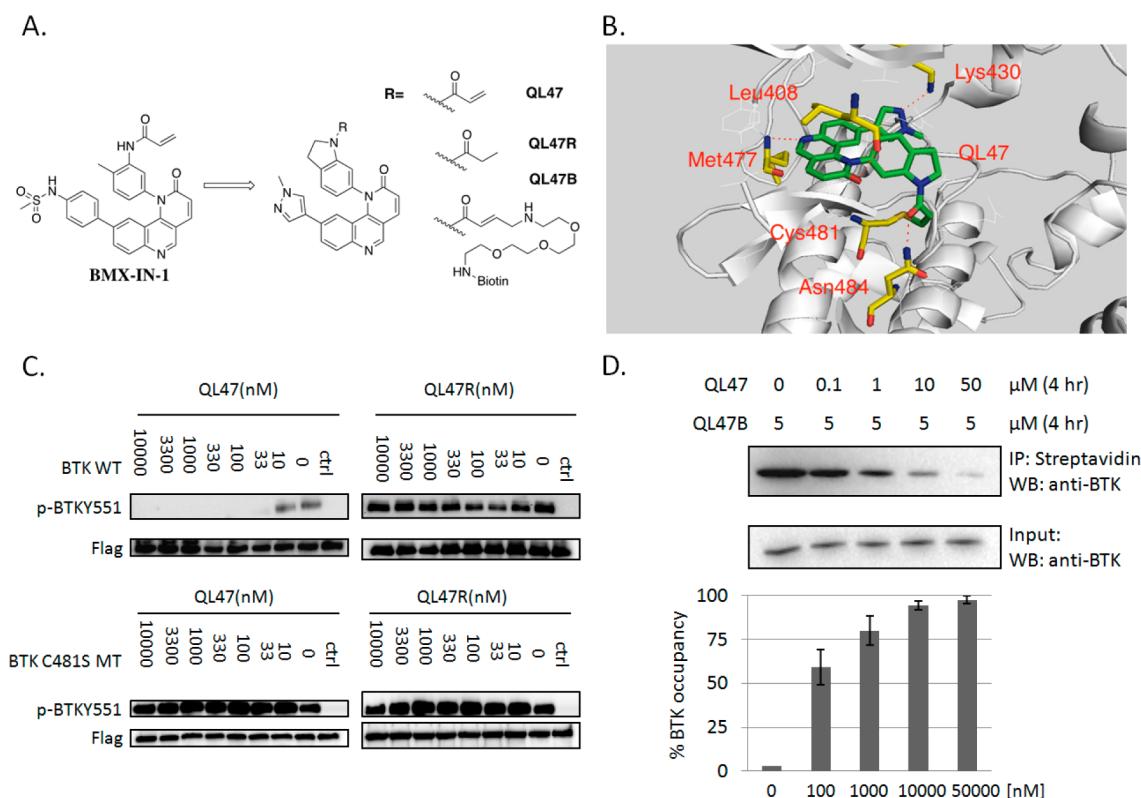


Figure 1. Characterization of QL47 as an irreversible BTK inhibitor. (A) Chemical structures of QL47, QL47R and QL47B. (B) Predicted mode of binding of QL47 to BTK based upon molecular modeling (PDB id: 3GEN). (C) *In vitro* kinase assay using FLAG-tagged BTK of either wild-type or C481S immunopurified from HEK293T cells showed that QL47 inhibits only wild-type BTK with an IC_{50} of less than 30 nM, whereas QL47R fails to inhibit both wild-type and C481S BTK. (D) Target engagement studies using QL47B and QL47 show that QL47 binds BTK in cells.

32765), the most clinically advanced irreversible BTK kinase inhibitor, has demonstrated efficacy in patients with CLL, MCL, and WM and recently has been approved for clinical use for MCL.^{22,23} A number of additional covalent and noncovalent BTK inhibitors including AVL-292(CC-292), Dasatinib, LFM-A13, ONO-WG-307, and GDC-0834 are in various stages of preclinical or early clinical development.²¹ In addition, a highly selective, reversible BTK inhibitor, CGI-1746, has demonstrated efficacy in mouse models of inflammation.²⁴ Here we report the identification and biological characterization of QL47, a compound with potent BTK inhibitory activity and excellent kinome selectivity that inhibits growth and survival of B-cell lymphomas more potently than several existing agents.

All currently reported covalent BTK inhibitors target Cys481 located in the kinase hinge segment, which is conserved among all five Tec-family kinases, JAK3, EGFR, HER2, HER4, and BLK.^{25,26} In order to identify ATP-site directed scaffolds that possess reasonable selectivity and potency for inhibiting BTK, we queried our historical database of kinase selectivity data generated using a combination of enzymatic binding (KinomeScan) and chemical proteomic (KiNativ) approaches. This analysis revealed that a subset of our collection of tricyclic quinoline-based compounds demonstrated potent binding to BTK. Previously we have elaborated this class of tricyclic quinolones to generate potent noncovalent inhibitors of mTOR such as Torin1 and Torin2 and covalent inhibitors of BMX such as BMX-IN-1.^{27–29} We next screened this collection of compounds for antiproliferative activity in Ramos and other cancer cell lines to identify compounds with cellular efficacy. BMX-IN-1 is a potent covalent inhibitor of both BMX and

BTK in biochemical assays but interestingly does not possess potent antiproliferative activity against B-cell lines that are reported to be sensitive to potent covalent BTK inhibitors such as Ibrutinib.²⁹ In order to determine whether the acrylamide-containing tricyclic quinoline chemotype exemplified by BMX-IN-1 could be further elaborated to achieve potent inhibition of B-cell proliferation we generated focused libraries informed by docking of BMX-IN-1 to BTK (PDB id: 3GEN).³ (Supplemental Figure 1) Members of the focused library were evaluated for ability to inhibit BTK biochemically, broader kinase selectivity profiles and their ability to inhibit the proliferation of B-cell lymphoma lines. As described further below, this effort culminated in the discovery of QL47 (Figure 1A). Molecular modeling of QL47 using the reported crystal structure of BTK kinase (PDB id: 3GEN) suggests that the electrophilic acrylamide is poised in a suitable position to allow for covalent bond formation with Cys481 (Figure 1B). QL47 inhibits BTK kinase activity with an IC_{50} of 6.6 nM as determined using a Z'lyte assay at a fixed time point (1 h).³⁰ Broad-based kinase selectivity of QL47 was performed against a panel of 34 kinases using enzymatic assays. This analysis revealed that QL47 also potently inhibits BMX with an IC_{50} of 6.7 nM but impressively displays more than 100-fold selectivity against EGFR, HER2, JAK3, BLK, TEC, and ITK that possess an equivalently placed cysteine (Table 1). In particular, QL47 displays a significantly improved selectivity profile compared to Ibrutinib (PCI32765) especially across the TEC and SRC family of tyrosine kinases.^{31,32} In addition, more extensive kinome wide selectivity profiling was performed by DiscoverX's KINOMEscan technology against 456 kinases, and it revealed

Table 1. Biochemical Comparison of QL47 and Ibrutinib

kinase	QL47 IC ₅₀ (nM)	Ibrutinib IC ₅₀ (nM)	kinase	QL47 IC ₅₀ (nM)	Ibrutinib IC ₅₀ (nM)
BTK	6.59	0.5	ABL	>10000	86
BMX	6.73	0.8	FYN	>10000	96
BLK	366	0.5	RIPK2	5690	152
EGFR	4310	5.6	c-SRC	>10000	171
Her2	8530	9.4	LYN	>10000	200
ITK	2830	10.7	PDGFRa	>10000	718
JAK3	5180	16.1	FMS	1720	5545
TEC	195	78	FER	>10000	8070
CSK	>10000	2.3	JAK1	>10000	>10000
FGR	6180	2.3	JAK2	>10000	>10000
BRK	>10000	3.3	NEK2	>10000	>10000
HCK	>10000	3.7	P38	>10000	>10000
YES	>10000	6.5	PI3K	696	>10000
FRK	>10000	29.2	PLK1	>10000	>10000
LCK	4460	33.2	RSK1	>10000	>10000
RET	4210	36.5	SYK	>10000	>10000
FLT3	3990	73	mTOR	1200	ND

that only BTK was potently inhibited at 1 μ M ($S(35) = 0.01$), which indicates that QL47 has exquisite selectivity among the kinome (Supplemental Table 1).

We next sought to establish if covalent bond formation is required for QL47 to achieve potent biochemical and cellular inhibition of BTK. First, we synthesized an approximately isosteric analogue, QL47R, in which the electrophilic acrylamide is replaced with a nonreactive propyl amide (Figure 1A). QL47R did not inhibit the biochemical kinase activity of BTK at concentrations below 10 μ M, demonstrating a stringent requirement for the acrylamide functional group (Figure 1C). Second, we evaluated the ability of QL47 to inhibit the activity of a mutant (Cys496Ser) BTK in which the reactive cysteine was mutated to a serine, which is incapable of undergoing a Michael addition reaction. The kinase activity of this BTK mutant is at least 300-fold less potently inhibited by QL47 relative to wild-type BTK (Figure 1C). To establish that QL47 can label native BTK, we designed a 'target-engagement' assay. For this we synthesized a biotinylated analogue of QL47, QL47B, where we installed the biotin moiety in an analogous position to where BODIPY has previously been appended to Ibrutinib³¹ (Figure 1A). QL47B maintains the ability to inhibit BTK kinase activity albeit with a less potent IC₅₀ of 1.3 μ M (Supplemental Figures 2 and 3). Subsequently we performed a competition assay where cell lysates were treated with increasing concentrations of QL47 for 4 h followed by incubation with QL47B to 'pull-down' BTK not bound by QL47. This analysis revealed that after 4 h, 100 nM QL47 was sufficient to label approximately 50% of the available BTK (Figure 1D). Taken together, the biochemical experiments with QL47R and BTK C481S and cellular target engagement studies provide strong evidence that QL47 covalently labels BTK in the cellular environment.

We next investigated the effect of QL47 on the BTK signaling pathway in Ramos cells in comparison to Ibrutinib and CGI-1746 as reference covalent and noncovalent inhibitors, respectively. Upon IgM activation, kinases including Syk located upstream of BTK activate BTK, which in turn activates the downstream mediator PLC γ . As shown in Figure 2A we observed robust phosphorylation of Y223 and Y551 of BTK and of the pathway components PLC γ (Y759) and Syk

(Y525/526) in Ramos cells. All three BTK inhibitors were capable of inhibiting the BTK autophosphorylation site Y223 at a concentration of 1 μ M with QL47 in particular showing dose-dependent inhibition with complete suppression at a concentration of 3.3 μ M (Figure 2A). Furthermore, QL47 blocked downstream phosphorylation of Y759 of PLC γ 2 to a greater extent than Ibrutinib, CGI-1746, and QL47R at 1 μ M with a measured EC₅₀ of 318 nM. In addition, at 1 μ M Ibrutinib has greater efficacy than QL47 and CGI-1746 in blocking PLC γ 1 Y783 phosphorylation and similar effect on the PLC γ 2 Y1217 phosphorylation (Supplemental Figures 4 and 5). None of the inhibitors impacted the phosphorylation of Syk at Y525/526. In addition, QL47 did not affect other growth-dependent signaling pathways including the PI3K/mTOR or Raf/Mek/Erk pathway as assessed at the levels of S6K and Erk phosphorylation (Supplemental Figure 6).

We next investigated the antiproliferative effects of QL47 in two well-defined B-cell lymphoma cells lines: Ramos and U2932. QL47 inhibited the proliferation of Ramos and U2932 cells with GI₅₀'s of 370 nM and 200 nM, respectively, while the reversible QL47R did not display antiproliferative activity at concentrations of less than 10 μ M (Table 2). Interestingly, Ibrutinib, AVL-292, and CGI-1746 were all considerably less potent against these cell lines. BMX-IN-1, which is equipotent to QL47 as a biochemical BTK inhibitor, was also approximately 10-fold less potent as an inhibitor of cell proliferation. We noted that Ramos cells do not express BMX, suggesting that alternative pharmacology on BTK or additional unidentified targets of QL47 may account for the differential effects of these compounds (Supplemental Figure 7).

We extended investigation of the antiproliferative effects of QL47 to a panel of cell lines derived from a variety of B-cell malignancies where deregulation of BTK has been implicated. QL47 displayed two to three digit nanomolar antiproliferative potency against a variety of cell lines derived from multiple myeloma and lymphomas (Table 3); Ibrutinib was less potent against all cell lines evaluated.

Subsequently we investigated whether the observed antiproliferative activity of QL47 is a consequence of growth inhibition or cell death. In Ramos cell line QL47 induced apoptosis in a dose dependent manner with effects manifesting at 5 μ M and more significantly at 10 μ M after 8 h as assessed by PARP cleavage and caspase-3 activation. In contrast, Ibrutinib did not induce apparent apoptosis at concentrations below 10 μ M. (Figure 2B) Cell cycle analysis using flow cytometry indicates that QL47 can arrest the cell cycle at the G1 phase. (Figure 2C)

Recently several covalent and noncovalent kinase inhibitors have been observed to result in degradation of their respective kinase targets.³³ To investigate whether treatment of cells with QL47 results in the degradation of BTK we treated HEK293T cells expressing FLAG-tagged wild-type or C481S BTK with the inhibitor. This analysis revealed that QL47 at a concentration of 1 μ M does lead to approximately 50% degradation of BTK in the HEK293T system; this result was confirmed for endogenous BTK in the Ramos cell line (Figure 2D, 2E). The degradation seems to be a consequence of covalent bond formation to BTK as it was not observed for QL47R or in the C481S BTK expressing cells.²⁹ Interestingly, the degradation of BTK was only observed when blocking nascent protein synthesis by cycloheximide. Degradation of BTK in the Ramos cell line appears to be proteasome-mediated as inclusion of the well-characterized proteasome inhibitor

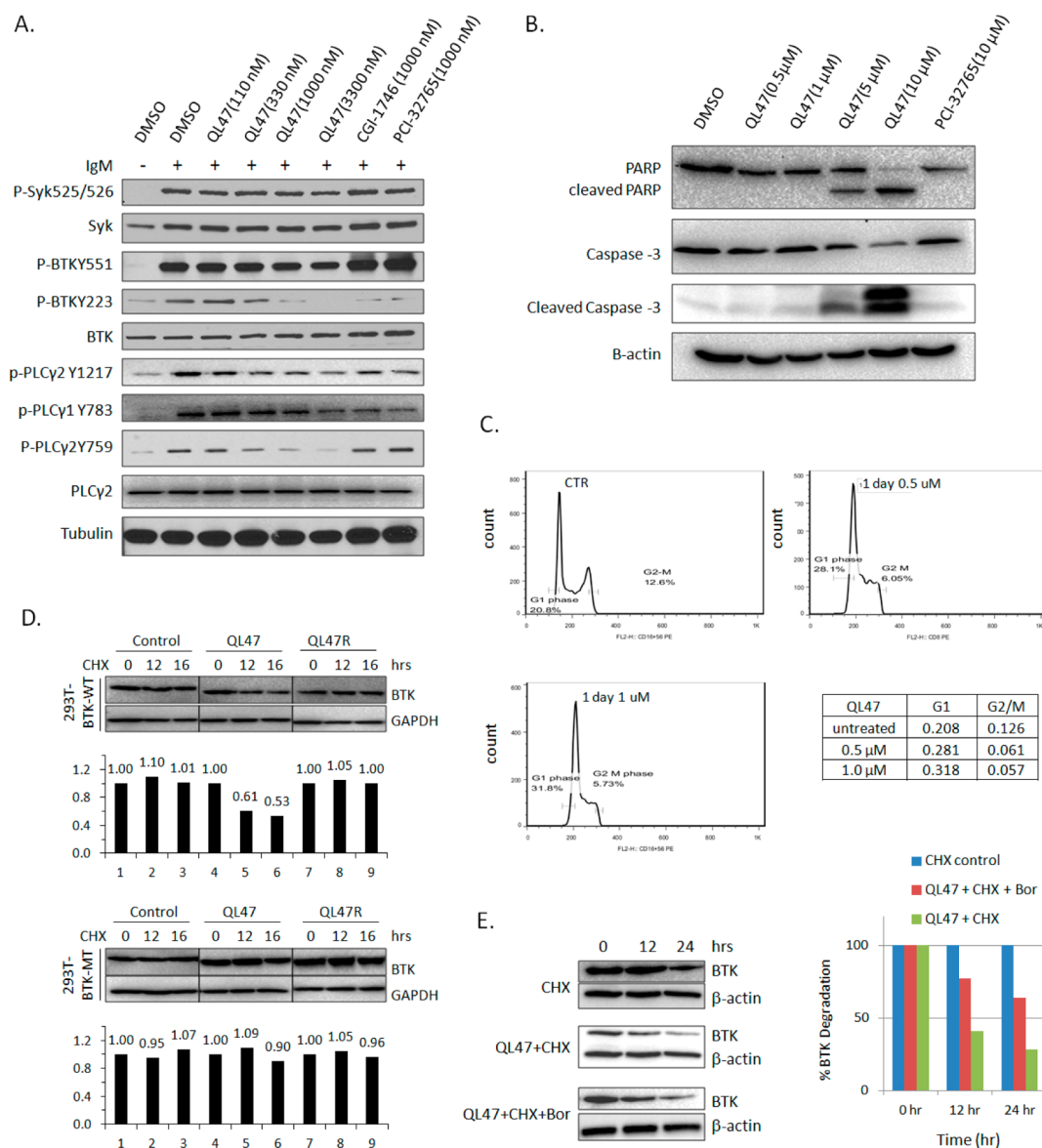


Figure 2. Effect of QL47 on B-cell cancer cells. (A) Effects of QL47 on BTK mediated signaling pathway in the Ramos cell line. (B) QL47 induced apoptosis of Ramos cell line. (C) Effect of QL47 on cell cycle in Ramos cells. (D) QL47 (1 μM) induces degradation of wild-type but not C481S BTK in stably transfected HEK293T cells. (E) QL47 induces BTK protein degradation in Ramos cells, and the proteasome inhibitor Bortezomib can block this process.

Table 2. QL47's Anti-proliferation Effect on B-Cell Lymphoma Cell Lines

cell line	GI ₅₀ (μM)					
	QL47	QL47R	BMX-IN-1	AVL-292	PCI-32765	CGI-1746
Ramos	0.37	>10	1.8	9.2	>10	>10
U2932	0.2	>10	2.15	4.3	5.0	>10

Bortezomib results in a partial rescue of the observed degradation (Figure 2E). In addition, to exclude the possibility that QL47 affects Hsp90 protein and that activity accounts for the protein degradation, we looked at the effect of QL47 on Hsp70 and CRAF protein levels in HCT116 cells and did not observe an effect consistent with Hsp90 inhibition (Supplemental Figure 8). To determine if the observed degradation of BTK is commonly observed when covalent BTK inhibitors are employed, we performed a similar analysis with Ibrutinib, AVL-292, CGI-1746, and QL47 at a concentration of 1 μM. All three covalent inhibitors induced BTK degradation, while the

reversible inhibitor (CGI-1746) did not (Supplemental Figure 9).

An accumulating body of evidence suggests that BTK is a potential therapeutic target in B-cell-related lymphocytic diseases. The positive clinical results obtained with Ibrutinib and AVL-292 on relapsed CLL and MCL have generated preliminary evidence that BTK may be a critical molecular target in these diseases. However, Ibrutinib is a highly multitargeted kinase inhibitor, displaying almost equipotent activity against all five TEC family kinases and many SRC family kinases.³¹ Therefore it is important to generate

Table 3. Anti-Proliferative Activity of QL47 against a Panel of B-Cell Cancer Cell Lines

cell line	disease	GI ₅₀ (μM)	
		QL47	PCI-32765
MM1S	myeloma	0.26	7.56
RPMI8226	myeloma	0.12	>10
KMS12BM	myeloma	0.49	>10
BCWM.1	lymphoma	0.12	0.57
MWCL-1	lymphoma	0.19	0.54
KU812	leukemia	0.45	3.08

alternative chemical series that possess alternative kinase selectivity profiles that can be used as pharmacological probes. Here, we used kinome-wide screening and structure-based design to prepare QL47, a potent covalent inhibitor of BTK that displays considerably improved kinase selectivity relative to Ibrutinib. QL47 covalently targets cysteine 481 using its acrylamide moiety, and this modification is required to achieve potent inhibition of BTK-dependent cellular inhibition. Interestingly, we also discovered that QL47 not only inhibits BTK kinase activity but also induces BTK degradation at low concentrations (2–3 times of GI₅₀), providing a potential mechanism for antagonizing non-kinase-dependent BTK functions. In addition, we determined that the ability to induce BTK degradation is a common feature of all three available irreversible BTK kinase inhibitors. We also noted that the tricyclic quinoline analogue of QL47, BMX-IN-1, which displays a highly similar biochemical kinase selectivity profile, also possesses approximately 10-fold less potent antiproliferative activity. This suggests that QL47 possesses unique pharmacology and that additional targets may be relevant to its antiproliferative activity. Pharmacological profiling of QL47 demonstrates that the compound possesses very poor microsomal stability and is not likely suitable as an *in vivo* probe in murine studies. QL47 will contribute to our armory of pharmacological tools for investigating BTK and may serve as a lead compound for development of the next-generation targeted therapeutics for B-cell tumors.

METHODS

Chemical synthesis, antibodies, mutagenesis, IP kinase assay, proliferation assay, signaling transduction immunoblotting and molecule modeling are described in details in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Supplemental experimental procedures and figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Schmidt, U., Boucheron, N., Unger, B., and Ellmeier, W. (2004) The role of Tec family kinases in myeloid cells. *Int. Arch. Allergy Immunol.* 134, 65–78.
- (2) Berg, L. J., Finkelstein, L. D., Lucas, J. A., and Schwartzberg, P. L. (2005) Tec family kinases in T lymphocyte development and function. *Annu. Rev. Immunol.* 23, 549–600.
- (3) Kersseboom, R., Ta, V. B., Zijlstra, A. J., Middendorp, S., Juma, H., van Loo, P. F., and Hendriks, R. W. (2006) Bruton's tyrosine kinase and SLP-65 regulate pre-B cell differentiation and the induction of Ig light chain gene rearrangement. *J. Immunol.* 176, 4543–4552.
- (4) Uckun, F. M., Tibbles, H. E., and Vassilev, A. O. (2007) Bruton's tyrosine kinase as a new therapeutic target. *Anticancer Agents Med. Chem.* 7, 624–632.
- (5) Smith, C. I., Baskin, B., Humire-Greif, P., Zhou, J. N., Olsson, P. G., Maniar, H. S., Kjellen, P., Lambris, J. D., Christensson, B., Hammarstrom, L., et al. (1994) Expression of Bruton's agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. *J. Immunol.* 152, 557–565.
- (6) Tsukada, S., Rawlings, D. J., and Witte, O. N. (1994) Role of Bruton's tyrosine kinase in immunodeficiency. *Curr. Opin. Immunol.* 6, 623–630.
- (7) Rankin, A. L., Seth, N., Keegan, S., Andreyeva, T., Cook, T. A., Edmonds, J., Mathialagan, N., Benson, M. J., Syed, J., Zhan, Y., Benoit, S. E., Miyashiro, J. S., Wood, N., Mohan, S., Peeva, E., Ramaiah, S. K., Messing, D., Homer, B. L., Dunussi-Joannopoulos, K., Nickerson-Nutter, C. L., Schnute, M. E., and Douhan, J., 3rd (2013) Selective inhibition of BTK prevents murine lupus and antibody-mediated glomerulonephritis. *J. Immunol.* 191, 4540–4550.
- (8) Mohamed, A. J., Yu, L., Backesjo, C. M., Vargas, L., Faryal, R., Aints, A., Christensson, B., Berglof, A., Vihinen, M., Nore, B. F., and Smith, C. I. (2009) Bruton's tyrosine kinase (Btk): regulation, and transformation with special emphasis on the PH domain. *Immunol. Rev.* 228, 58–73.
- (9) Park, H., Wahl, M. I., Afar, D. E., Turck, C. W., Rawlings, D. J., Tam, C., Scharenberg, A. M., Kinet, J. P., and Witte, O. N. (1996) Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity* 4, 515–525.
- (10) Mahajan, S., Vassilev, A., Sun, N., Ozer, Z., Mao, C., and Uckun, F. M. (2001) Transcription factor STAT5A is a substrate of Bruton's tyrosine kinase in B cells. *J. Biol. Chem.* 276, 31216–31228.
- (11) Glassford, J., Soeiro, I., Skarell, S. M., Banerji, L., Holman, M., Klaus, G. G., Kadowaki, T., Koyasu, S., and Lam, E. W. (2003) BCR targets cyclin D2 via Btk and the p85α subunit of PI3-K to induce cell cycle progression in primary mouse B cells. *Oncogene* 22, 2248–2259.
- (12) Bajpai, U. D., Zhang, K., Teutsch, M., Sen, R., and Wortis, H. H. (2000) Bruton's tyrosine kinase links the B cell receptor to nuclear factor kappaB activation. *J. Exp. Med.* 191, 1735–1744.
- (13) Petro, J. B., and Khan, W. N. (2001) Phospholipase C-gamma 2 couples Bruton's tyrosine kinase to the NF-kappaB signaling pathway in B lymphocytes. *J. Biol. Chem.* 276, 1715–1719.
- (14) Hashimoto, S., Iwamatsu, A., Ishiai, M., Okawa, K., Yamadori, T., Matsushita, M., Baba, Y., Kishimoto, T., Kurosaki, T., and Tsukada, S. (1999) Identification of the SH2 domain binding protein of Bruton's tyrosine kinase as BLNK—functional significance of Btk-SH2 domain in B-cell antigen receptor-coupled calcium signaling. *Blood* 94, 2357–2364.
- (15) de Weers, M., Verschuren, M. C., Kraakman, M. E., Mensink, R. G., Schuurman, R. K., van Dongen, J. J., and Hendriks, R. W. (1993) The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precursor B cell stages preceding

immunoglobulin gene rearrangement up to mature B cell stages. *Eur. J. Immunol.* 23, 3109–3114.

(16) Rodriguez, A., Martinez, N., Camacho, F. I., Ruiz-Ballesteros, E., Algora, P., Garcia, J. F., Menarguez, J., Alvaro, T., Fresno, M. F., Solano, F., Mollejo, M., Martin, C., and Piris, M. A. (2004) Variability in the degree of expression of phosphorylated I κ B α in chronic lymphocytic leukemia cases with nodal involvement. *Clin. Cancer Res.* 10, 6796–6806.

(17) Ortolano, S., Hwang, I. Y., Han, S. B., and Kehrl, J. H. (2006) Roles for phosphoinositide 3-kinases, Bruton's tyrosine kinase, and Jun kinases in B lymphocyte chemotaxis and homing. *Eur. J. Immunol.* 36, 1285–1295.

(18) Uckun, F., Dibirdik, I., Sarkissian, A., and Qazi, S. (2011) In vitro and in vivo chemosensitizing activity of LFM-A13, a dual-function inhibitor of Bruton's tyrosine kinase and polo-like kinases, against human leukemic B-cell precursors. *Arzneimittelforschung* 61, 252–259.

(19) Cinar, M., Hamedani, F., Mo, Z., Cinar, B., Amin, H. M., and Alkan, S. (2013) Bruton tyrosine kinase is commonly overexpressed in mantle cell lymphoma and its attenuation by Ibrutinib induces apoptosis. *Leuk. Res.* 37, 1271–1277.

(20) Liu, Y., Dong, Y., Jiang, Q. L., Zhang, B., and Hu, A. M. (2014) Bruton's tyrosine kinase: potential target in human multiple myeloma. *Leuk. Lymphoma* 55, 177–181.

(21) D'Cruz, O. J., and Uckun, F. M. (2013) Novel Bruton's tyrosine kinase inhibitors currently in development. *OncoTargets Ther.* 6, 161–176.

(22) Byrd, J. C., Furman, R. R., Coutre, S. E., Flinn, I. W., Burger, J. A., Blum, K. A., Grant, B., Sharman, J. P., Coleman, M., Wierda, W. G., Jones, J. A., Zhao, W., Heerema, N. A., Johnson, A. J., Sukbuntherng, J., Chang, B. Y., Clow, F., Hedrick, E., Buggy, J. J., James, D. F., and O'Brien, S. (2013) Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N. Engl. J. Med.* 369, 32–42.

(23) Wang, M. L., Rule, S., Martin, P., Goy, A., Auer, R., Kahl, B. S., Jurczak, W., Advani, R. H., Romaguera, J. E., Williams, M. E., Barrientos, J. C., Chmielowska, E., Radford, J., Stilgenbauer, S., Dreyling, M., Jdrzejczak, W. W., Johnson, P., Spurgeon, S. E., Li, L., Zhang, L., Newberry, K., Ou, Z., Cheng, N., Fang, B., McGreiv, J., Clow, F., Buggy, J. J., Chang, B. Y., Beaupre, D. M., Kunkel, L. A., and Blum, K. A. (2013) Targeting BTK with ibrutinib in relapsed or refractory mantle-cell lymphoma. *N. Engl. J. Med.* 369, 507–516.

(24) Di Paolo, J. A., Huang, T., Balazs, M., Barbosa, J., Barck, K. H., Bravo, B. J., Carano, R. A., Darrow, J., Davies, D. R., DeForge, L. E., Diehl, L., Ferrando, R., Gallion, S. L., Giannetti, A. M., Gribbling, P., Hurez, V., Hymowitz, S. G., Jones, R., Kropf, J. E., Lee, W. P., Maciejewski, P. M., Mitchell, S. A., Rong, H., Staker, B. L., Whitney, J. A., Yeh, S., Young, W. B., Yu, C., Zhang, J., Reif, K., and Currie, K. S. (2011) Specific Btk inhibition suppresses B cell- and myeloid cell-mediated arthritis. *Nat. Chem. Biol.* 7, 41–50.

(25) Pan, Z., Scheerens, H., Li, S. J., Schultz, B. E., Sprengeler, P. A., Burrill, L. C., Mendonca, R. V., Sweeney, M. D., Scott, K. C., Grothaus, P. G., Jeffery, D. A., Spoerke, J. M., Honigberg, L. A., Young, P. R., Dalrymple, S. A., and Palmer, J. T. (2007) Discovery of selective irreversible inhibitors for Bruton's tyrosine kinase. *ChemMedChem* 2, 58–61.

(26) Evans, E. K., Tester, R., Aslanian, S., Karp, R., Sheets, M., Labenski, M. T., Witowski, S. R., Lounsbury, H., Chaturvedi, P., Mazdiyasni, H., Zhu, Z., Nacht, M., Freed, M. I., Petter, R. C., Dubrovskiy, A., Singh, J., and Westlin, W. F. (2013) Inhibition of Btk with CC-292 provides early pharmacodynamic assessment of activity in mice and humans. *J. Pharmacol. Exp. Ther.* 346, 219–228.

(27) Liu, Q., Chang, J. W., Wang, J., Kang, S. A., Thoreen, C. C., Markhard, A., Hur, W., Zhang, J., Sim, T., Sabatini, D. M., and Gray, N. S. (2010) Discovery of 1-(4-(4-propionylpiperazin-1-yl)-3-(trifluoromethyl)phenyl)-9-(quinolin-3-yl)benzo[h][1,6]-naphthyridin-2(1H)-one as a highly potent, selective mammalian target of rapamycin (mTOR) inhibitor for the treatment of cancer. *J. Med. Chem.* 53, 7146–7155.

(28) Liu, Q., Wang, J., Kang, S. A., Thoreen, C. C., Hur, W., Ahmed, T., Sabatini, D. M., and Gray, N. S. (2011) Discovery of 9-(6-aminopyridin-3-yl)-1-(3-(trifluoromethyl)phenyl)benzo[h][1,6]-naphthyridin-2(1H)-one (Torin2) as a potent, selective, and orally available mammalian target of rapamycin (mTOR) inhibitor for treatment of cancer. *J. Med. Chem.* 54, 1473–1480.

(29) Liu, F., Zhang, X., Weisberg, E., Chen, S., Hur, W., Wu, H., Zhao, Z., Wang, W., Mao, M., Cai, C., Simon, N. I., Sanda, T., Wang, J., Look, A. T., Griffin, J. D., Balk, S. P., Liu, Q., and Gray, N. S. (2013) Discovery of a selective irreversible BMX inhibitor for prostate cancer. *ACS Chem. Biol.* 8, 1423–1428.

(30) Carlson, C. B., Mashock, M. J., and Bi, K. (2010) BacMam-enabled LanthaScreen cellular assays for PI3K/Akt pathway compound profiling in disease-relevant cell backgrounds. *J. Biomol. Screening* 15, 327–334.

(31) Honigberg, L. A., Smith, A. M., Sirisawad, M., Verner, E., Loury, D., Chang, B., Li, S., Pan, Z., Thamm, D. H., Miller, R. A., and Buggy, J. J. (2010) The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13075–13080.

(32) Liu, Q., Sabnis, Y., Zhao, Z., Zhang, T., Buhrlage, S. J., Jones, L. H., and Gray, N. S. (2013) Developing irreversible inhibitors of the protein kinase cysteinome. *Chem. Biol.* 20, 146–159.

(33) Polier, S., Samant, R. S., Clarke, P. A., Workman, P., Prodromou, C., and Pearl, L. H. (2013) ATP-competitive inhibitors block protein kinase recruitment to the Hsp90-Cdc37 system. *Nat. Chem. Biol.* 9, 307–312.