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

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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 IC50 of 7 nM, inhibits autophosphorylation of BTK on Tyr223 in cells with an EC50 of 475 nM, and inhibits phosphorylation of a downstream effector PLCγ2 (Tyr759) with an EC50 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<br>ruton’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 plekstrin homology domain of BTK followed by phosphorylation of Tyr551 by kinases such as Lyn and Syk as well as autophosphorylation of tyrosine 223.5,9 BTK activation results in induction of multiple signaling pathways including Stat5, PI3K/Akt/mTOR, and NF-κB.10–13 The most well-defined effector molecule of BTK signaling is PLCγ, whose activation results in calcium mobilization and activation of NF-κB 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), Waldenstrom 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-
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 IC50 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 IC50 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...
that only BTK was potently inhibited at 1 μM (S(3S) = 0.01), which indicates that QL47 has exquisite selectivity among the kinase (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 μM, 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 Ibrutinib.31 (Figure 1A). QL47B maintains the ability to inhibit BTK kinase activity albeit with a less potent IC50 of 1.3 μM (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.

Next we 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 PLCγ. As shown in Figure 2A we observed robust phosphorylation of Y223 and Y551 of BTK and of the pathway components PLCγ (Y759) and Syk (Y525/Y526) in Ramos cells. All three BTK inhibitors were capable of inhibiting the BTK autophosphorylation site Y223 at a concentration of 1 μM with QL47 in particular showing dose-dependent inhibition with complete suppression at a concentration of 3.3 μM (Figure 2A). Furthermore, QL47 blocked downstream phosphorylation of Y759 of PLCγ2 to a greater extent than Ibrutinib, CGI-1746, and QL47R at 1 μM with a measured EC50 of 318 nM. In addition, at 1 μM Ibrutinib has greater efficacy than QL47 and CGI-1746 in blocking PLCγ1 Y783 phosphorylation and similar effect on the PLCγ2 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 μM (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 μM and more significantly at 10 μM after 8 h as assessed by PARP cleavage and caspase-3 activation. In contrast, Ibrutinib did not induce apparent apoptosis at concentrations below 10 μM. (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.32 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 μM 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 cyclohexamide. Degradation of BTK in the Ramos cell line appears to be proteasome-mediated as inclusion of the well-characterized proteasome inhibitor

| kinase | QL47 IC50 (nM) | Ibrutinib IC50 (nM) | kinase | QL47 IC50 (nM) | Ibrutinib IC50 (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   | 8350           | 9.4               | Lyn    | >10000         | 200               |
| ITK    | 2830           | 10.7              | PDGFRα | >10000         | 718               |
| JAK3   | 5180           | 16.1              | FMS    | 1730           | 5545              |
| TIC    | 195            | 78                | FER    | >10000         | 8070              |
| CSK    | >10000         | 2.3               | JAK1   | >10000         | >10000            |
| FGFR   | 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                |
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. Therefore it is important to generate...
pharmacological tools for investigating BTK and may serve as a murine studies. QL47 will contribute to our armorium of somal stability and is not likely suitable as an demonstrates that the compound possesses very poor micropharmacology and that additional targets may be relevant to alternative activity. This suggests that QL47 possesses unique displays a highly similar biochemical kinase selectivity pro tricyclic quinoline analogue of QL47, BMX-IN-1, which BTK degradation is a common feature of all three available material is available free of charge via the Internet at http://

| cell line | disease | 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      |
| MWC1-L    | 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 armorium 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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