Ability of Bruton’s Tyrosine Kinase Inhibitors to Sequester Y551 and Prevent Phosphorylation Determines Potency for Inhibition of Fc Receptor but not B-Cell Receptor Signaling

Andrew T. Bender, Anna Gardberg, Albertina Pereira, Theresa Johnson, Yin Wu, Roland Grenningloh, Jared Head, Federica Morandi, Philipp Haselmayer, and Lesley Liu-Bujalski

TIP Immunology (A.T.B., A.P., Y.W., R.G.) and Discovery Technologies (A.G., T.J., J.H., F.M., L.L.-B.), EMD Serono Research and Development Institute, Billerica, Massachusetts; and TIP Immunology, Merck, Darmstadt, Germany (P.H.)

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ABSTRACT

Bruton’s tyrosine kinase (Btk) is expressed in a variety of hematopoietic cells. Btk has been demonstrated to regulate signaling downstream of the B-cell receptor (BCR), Fc receptors (FcRs), and toll-like receptors. It has become an attractive drug target because its inhibition may provide significant efficacy by simultaneously blocking multiple disease mechanisms. Consequently, a large number of Btk inhibitors have been developed. These compounds have diverse binding modes, and both reversible and irreversible inhibitors have been developed. Reported herein, we have tested nine Btk inhibitors and characterized on a molecular level how their interactions with Btk define their ability to block different signaling pathways. By solving the crystal structures of Btk inhibitors bound to the enzyme, we discovered that the compounds can be classified by their ability to trigger sequestration of Btk residue Y551. In cells, we found that sequestration of Y551 renders it inaccessible for phosphorylation. The ability to sequester Y551 was an important determinant of potency against FcR signaling as Y551 sequestering compounds were more potent for inhibiting basophils and mast cells. This result was true for the inhibition of FcγR signaling as well. In contrast, Y551 sequestration was less a factor in determining potency against BCR signaling. We also found that Btk activity is regulated differentially in basophils and B cells. These results elucidate important determinants for Btk inhibitor potency against different signaling pathways and provide insight for designing new compounds with a broader inhibitory profile that will likely result in greater efficacy.

Introduction

Bruton’s tyrosine kinase (Btk) is expressed in a wide variety of immune cell types and plays a role in multiple signaling pathways (López-Herrera et al., 2014). The most studied and best characterized is the role of Btk in regulating B-cell receptor (BCR) signaling. Btk has also been demonstrated to play a role in Fc receptor signaling, including regulation of both Fcγ (Xu et al., 2012; Hartkamp et al., 2015) and Fcε receptors (Ellmeier et al., 2011). Other reports have implicated Btk in regulating toll-like receptors (López-Herrera et al., 2014), although there is less conclusive evidence for the importance of Btk in toll-like receptor signaling. Given that Btk plays such a central role in immunity, it is an attractive target for treating autoimmunity, inflammation, and allergy. This idea is supported by animal disease model studies testing Btk-deficient mice and Btk inhibitors.

To maximize the clinical utility of a Btk inhibitor, it would be desirable to develop a compound capable of blocking signaling downstream of multiple receptors. A compound that has multiple mechanisms of action can provide better efficacy by preventing several disease pathologic processes. For instance, in autoantibody-driven diseases, a Btk inhibitor may be particularly efficacious because it can block activation of B cells, reduce autoantibodies, and also decrease end-organ damage by preventing activation of myeloid cell Fc receptors (FcRs) by immune complexes. However, Btk may be regulated differently in BCR and FcR signaling pathways in regard to which kinases phosphorylate the enzyme, which scaffolding proteins it binds to, and how it traffics in the cell. Btk is regulated by phosphorylation at Y223 and Y551 (Wahl et al., 1997; Dinh et al., 2007) and is also regulated by association with phosphatidylinositol 3,4,5 trisphosphate (PI(3,4,5)P3) at the plasma membrane (Mohamed et al., 2009). Because of the potential for differential regulation, it is important to characterize how Btk inhibitors can affect Btk activity and signaling in different cell types. Fundamentally, how a kinase inhibitor binds to the enzyme and affects its conformational structure...
could have an impact beyond simply blocking catalysis. Understanding inhibitor binding and effects on protein structure at the molecular level could translate into knowledge about how a compound has effects at the cellular level.

Kinases are attractive drug targets for treating a variety of diseases, and a large number of small molecules have been developed to inhibit them. All kinases transfer phosphate groups from ATP to a substrate, and therefore the enzymes have a high level of secondary as well as tertiary structure conservation. Most kinase inhibitors are ATP competitive, and the ATP binding pocket of kinases has an impressive ability to accommodate a multitude of diverse small-molecule inhibitors. Inhibitors may make contacts with the hinge region, a gatekeeper residue, or one of two hydrophobic pockets in this site (termed hydrophobic pocket I and hydrophobic pocket II) (Zhang et al., 2009). Extensive work has been done in the Akinleye et al., 2013; Gilbert, 2014). Reversible inhibitors have been produced, as have irreversible inhibitors, which use an electrophilic warhead to covalently react with Cys481 (Liu et al., 2013), a relatively nonconserved amino acid at that location. Irreversible inhibitors have the benefit of longer-lasting inhibitory activity, but their binding mode is restricted by the need to reach the C481 residue. Reversible inhibitors are freer to target different areas within the active site. As a general rule, the reversible inhibitors tend to bind deeper in the enzyme (contacting the “H3” pocket and hydrophobic pocket I or II) (Di Paolo et al., 2011) relative to the irreversible inhibitors, which achieve potency and selectivity without more extensive binding.

To date, little is understood about how the binding mode of Btk inhibitors affects their activity against different signaling pathways. This knowledge may allow the design of compounds that can block both BCR and FcR signaling, leading to maximum efficacy in autoimmune indications. Alternatively, it may be possible to design a compound that is selective for FcR signaling but less active against BCR signaling. To this end, we have endeavored to characterize the binding of a series of Btk inhibitors to elucidate how their binding modes translate into inhibition of different signaling pathways in cells. Furthermore, we have performed studies to understand how Btk may be regulated differently in different cell types, particularly through phosphorylation. The results presented herein provide information that should help guide the development of Btk inhibitors with more targeted cellular effects and greater disease-specific activity.

### Materials and Methods

**Compounds**

The synthesis of all compounds used in this study has been reported previously, and all are commercially available. Commercial sources and catalog numbers are GDC-0834, Medchemexpress, HY-15427A; CGI1746, Medchemexpress (Hercules, CA), HY-15999; RN486, Pharmablock, PBR018, RN983, PharmaBloc (Sunnyvale, CA), PB93313; Ibrutinib-Rev, ChemShuttle (Hayward, CA), 132938; ibrutinib, mablock PBR018, RN983, PharmaBloc (Sunnyvale, CA), PB93313; CGI1746, Medchemexpress (Hercules, CA), HY-11999; RN486, PharmaBloc, PBR018, RN983, Synnovator; Q E-3531; CNX 774, Synnovator (Durham, NC), SYN00305; CC-292, Combi-Blocks, QJ-1395, ONO-4059, PharmaBlock, PBSQ134.

**Btk Crystallography**

### Crystallography

Crystallization. Crystals of BTK kinase domain (residues 382–659) with a tool compound were obtained via co-crystallization with microseeding in hanging or sitting drop vapor diffusion, incubated at room temperature. Protein solution at 11 mg/ml in 20 mM Tris pH 8, 50 mM NaCl, and 3 mM DTT was mixed with a reservoir solution consisting of 0.1 M BisTrisPropane pH 7–7.5, 200 mM sodium acetate, 15%–25% w/v PEG 3350, and 3 mM TCEP pH 7.0. These tool compound co-crystals were soaked overnight with ligand molecules at 1–8 mM in a solubilizing solution consisting of 0.1 M BisTrisPropane pH 7, 200 mM sodium acetate, 28% w/v PEG 3350, 3 mM TCEP, pH 7.0, ±0.05% Tween detergent.

Data Collection and Processing. The diffraction data were collected at 100 K at X-ray wavelength of 1.00 Å at beamline X06SA/ Swiss Light Source using a Pilatus 6 M detector and integrated using the software XDS and XSCALE (Kabsch, 2010). The parameters used in the data collection have been deposited in the Protein Data Bank (PDB). The crystal system was determined to be orthorhombic with space group P212121. The unit cell dimensions were consistent overall at ~71 Å × 104 Å × 38 Å.

Structure Solution, Refinement, and Validation. The structures were solved via molecular replacement using the software PHASER (McCoy et al., 2007) (CCP4, Oxon, UK) with Btk reference structures used as search models. Subsequent model rebuilding was done in multiple rounds using COOT (Emsley et al., 2010). Refinement was performed using the REFMACS (Murshudov et al., 1997) and BUSTER (Smart et al., 2012) programs with bulk solvent correction and TLS parameterization. The water model was built with the “Find Waters” algorithm of COOT by putting water molecules in peaks of the 2Fo–Fc map contoured at 1.8 σ, followed by manual verification of all waters. Side chains without electron density were clipped to their Cβ atom. The final refinement residual factors, Rwork and Rfree, are deposited in the PDB. The Ramachandran plot of the final models shows 98% of all residues in the favored region and 0.05% in the outliers region. In all cases, MolProbity (Chen et al., 2010) (Desert Scientific Software, Sydney, AU) was used for validation, with percentile ranks ≥99%.

### B-Factor Normalization

In the B-factor factor (also called the temperature factor or Debye-Waller factor) describes the uncertainty or mobility of an atom in a protein structure, quantifying the displacement or atomic disorder in the protein crystal. Lower B factors are associated with relatively well ordered atoms or domains, and larger B factors suggest higher mobility or disorder. B factors were normalized on a structure-by-structure basis to enable comparisons of structures refined with different programs at different resolutions according to the formula:

\[
B' = (B - <B>) / \Sigma(B)
\]

(Parthasarathy and Murthy, 1997). In this equation \(<B> = \Sigma B_i / N_i\) where \(B_i\) is the B value for the Ca of the ith residue and \(N_i\) is the total number of residues in the protein.

Structure Deposition. The atomic coordinates and structure factors have been deposited into the Protein Data Bank (http://www. rcsb.org) with the following deposition records: D_1001400384 / 5P9F, D_1001400385 / 5P9G, D_1001400386 / 5P9H, D_1001400387 / 5P9I, D_1001400388 / 5P9J, D_1001400389 / 5P9K, D_1001400390 / 5P9L, and D_1001400391 / 5P9M.

### Btk Inhibition Assays

rBtk. The potency of compounds against recombinant wild-type Btk and Y551E Btk was determined using a previously described method (Bender et al., 2016). The assay uses purified protein, ATP, and a peptide substrate, and ATP phosphorylation is measured by the Caliper LabChip 3000 (Caliper Life Sciences, Waltham, MA).
### TABLE 1
Btk inhibitors tested

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Designation</th>
<th>Company</th>
<th>Binding</th>
</tr>
</thead>
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<td>1</td>
<td><img src="image1.png" alt="Structure Image 1" /></td>
<td>GDC-0834</td>
<td>CGI Pharmaceuticals</td>
<td>Noncovalent</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Structure Image 2" /></td>
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<td>CGI Pharmaceuticals</td>
<td>Noncovalent</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure Image 3" /></td>
<td>RN486</td>
<td>Roche Palo Alto</td>
<td>Noncovalent</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Structure Image 4" /></td>
<td>RN983</td>
<td>Hoffmann-LaRoche</td>
<td>Noncovalent</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Structure Image 5" /></td>
<td>Ibrutinib-Rev</td>
<td>Chinese Academy of Sciences</td>
<td>Noncovalent</td>
</tr>
</tbody>
</table>

(continued)
**FcεR.** The ability of Btk inhibitors to block FcεR signaling in basophils was determined using a previously reported method (Bender et al., 2016). Briefly, the method uses activation of the FcεR by anti-IgE for 15 minutes and then measures CD63 upregulation by flow cytometry. Human cord blood isolated CD34+ stem cells (AllCells, Alameda, CA) were differentiated to mast cells by culture for 8 weeks. Cells were maintained in StemPro-34 media (Gibco, Waltham, MA) supplemented with interleukin 6 (IL-6) at 50 ng/ml, and stem cell factor at 100 ng/ml all from R&D Systems (Minneapolis, MN). To the media were also added L-glutamine (Gibco) and penicillin/streptomycin (Gibco). After 7 weeks, IL-4 (R&D Systems) was added to a concentration of 10 ng/ml and IL-3 to 10 ng/ml. The differentiated mast cells were collected and plated in 96-well plates with 100 μl per well at 1 × 10⁶ cell/ml. The cells were incubated overnight in 1 μg/ml of IgE (BioRad, Hercules, CA). Cells were then washed and pretreated with compounds for 1 hour at 37°C. Cells were activated by cross-linking of the FcεR by addition of 0.1 μg/ml anti-IgE (BioRad). After 1 hour of incubation, the media were collected and the production of histamine was measured by enzyme-linked immunosorbent assay (Oxford Biomedical Research, Rochester Hills, MI).

**BCR.** The ability of compounds to block BCR signaling in human whole blood was determined as previously described (Bender et al., 2016). The assay used anti-human IgM (Fab')₂ (Dianova, Hamburg, Germany) to stimulate the cells overnight and CD69 upregulation was subsequently measured by flow cytometry the following day. The ability of compounds to inhibit BCR activation was also measured using Ficoll-Paque purified human peripheral blood mononuclear cells (PBMCs) using the same method.

**FcγR.** The compounds were tested for their ability to block CD64 activation in U937 cells transfected with an NF-κB-luciferase reporter gene. U937 cells were stably transfected with the signal lent NF-κB-luc reporter gene (Qiagen, Hilden Germany) and maintained in RPMI 1640 with 1 μg/ml puromycin. To activate CD64, 96-well plates...
were coated overnight with 20 μg/ml anti-CD64 (clone 10.1, BioLegend, San Diego, CA), and the following day cells were added to the well at a final concentration of 4 × 10^6 cells/ml. In experiments where compounds were tested, the cells were pretreated for 30 minutes before adding them to the anti-CD64–coated plates. The cells were incubated with anti-CD64 for 4 hours, and then the luciferin substrate was added to a final concentration of 100 μg/ml. The luminescence was read on an Envision instrument (PerkinElmer, Waltham, MA).

**Phosphorylation Studies**

Studies on phosphorylation of Btk and other proteins were conducted in the Ramos Burkitt’s lymphoma cell line (CRL-1596; American Type Culture Collection, Manassas, VA) and the RBL-2H3 rat basophilic leukemia cell line (ATCC CRL-2256). Ramos cells were maintained as a nonadherent culture at 37°C in a CO₂-regulated tissue culture incubator in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. On the day of the experiment, cells were collected and counted. Cells were diluted to a concentration of 8 × 10^7 cells/ml in serum-free RPMI 1640 and plated in wells of a round-bottom 96-well tissue culture plate. Cells were incubated with compounds for 30 minutes at 37°C at concentrations of 0, 0.001, 0.01, 0.1, and 1 μM. After compound treatment, the cells were stimulated with an anti-IgM F(ab’)2 antibody (Southern Biotech, Birmingham, AL) at a concentration of 5 μg/ml to activate the BCR. The cells were incubated with the anti-IgM for 5 minutes. After treatment, the cells were collected by centrifugation at 500 g for 5 minutes. The media were aspirated, and 150 μl of ice-cold mammalian protein extraction reagent lysis buffer (Thermo Fisher, Waltham, MA) containing HALT protease/phosphatase inhibitor (Thermo Fisher) was added to the cells. The cells were resuspended in the buffer and homogenized by sonication. The homogenate was centrifuged at 100,000g for 5 minutes, and the supernatant was used for the analysis of phosphorylation. The phosphorylation level of Btk was determined by Western blot analysis using specific antibodies against phosphorylated residues.

**TABLE 2**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Y551 Sequestration</th>
<th>Averaged B’ for Y551</th>
<th>rBtk WT/ Y551E</th>
<th>Whole-Blood FccR/BCR</th>
<th>Ramos pY551/ pY223</th>
<th>RBL-2H3 pY551/ pY223</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDC-0834</td>
<td>Yes</td>
<td>−0.35</td>
<td>0.03</td>
<td>1.11</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>CGI1746</td>
<td>Yes</td>
<td>−0.63</td>
<td>0.01</td>
<td>1.60</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>RN486</td>
<td>Yes</td>
<td>0.26</td>
<td>0.15</td>
<td>1.46</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>RN983</td>
<td>Yes</td>
<td>−0.27</td>
<td>0.02</td>
<td>1.32</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Ibrutinib-Rev</td>
<td>No</td>
<td>2.22</td>
<td>0.29</td>
<td>2.55</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ibrutinib</td>
<td>No</td>
<td>3.08</td>
<td>0.40</td>
<td>22.86</td>
<td>10,000</td>
<td>100</td>
</tr>
<tr>
<td>CNX 774</td>
<td>No</td>
<td>2.51</td>
<td>0.29</td>
<td>3.63</td>
<td>100</td>
<td>1000</td>
</tr>
<tr>
<td>CC 292</td>
<td>No</td>
<td>Absent</td>
<td>0.80</td>
<td>6.83</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>ONO-4059</td>
<td>No</td>
<td>2.64</td>
<td>0.57</td>
<td>27.08</td>
<td>10,000</td>
<td>10</td>
</tr>
</tbody>
</table>

*The averaged B factor for the residues in the Y551 residue was determined from the crystal structure of the compound in complex with Y551. The term absent is used when the mobility of the residues was too great for it to be accurately localized in the determined structure.*

*The ratio of the IC₅₀ for inhibition of Btk pY551 to Btk pY223 was calculated by dividing the values determined for pY551 by pY223 (as described in Fig. 3).*

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**Fig. 1.** Btk crystal structures. The kinase domain of Btk was crystallized in the absence of any ligand (A) or in the presence of RN486 (B) or ibrutinib (C). The thickness of the backbone represents its mobility, with regions of higher mobility represented as thicker areas. The backbone and side-chain atoms of the Y551 residue are colored according to their normalized B factor; atoms with a higher B factor are colored red. An overlay of RN486 (gold) and ibrutinib (pink) bound to Btk is shown (D). The differing position of Y551 when it is sequestered and not sequestered is marked by an arrow.
Following day, the media were removed and the cells were washed with buffer for 10 minutes with gentle agitation every 2 minutes. The inhibitor was added to the cells. The cells were incubated with lysis extraction reagent lysis buffer containing HALT protease/phosphatase inhibitor for 30 minutes at 37°C at concentrations of 0, 0.001, 0.01, 0.1, and 1 μM. Cells were then scraped into 10-cm dishes in complete minimum Eagle’s medium at a concentration of 1 × 10^6 cell/ml. The cells were activated for 5 minutes, and the lysates were then loaded on a Wes assay plate along with the primary antibody, the secondary antibody, streptavidin-HRP, luminol peroxide mix, and wash buffer. The assay plate was then transferred to the Wes instrument and run according to the manufacturer’s protocol. At the conclusion of the run, the intensities of the bands of interest were quantitated using the Compass software program (Compass Software, Inc., Atlanta, GA). The primary antibodies used were anti-Btk pY551 (BD Biosciences, San Jose, CA), anti-Btk pY223 (Cell Signaling, Danvers, MA), and anti-PLCγ2 pY1217 (Cell Signaling).

**Results**

Crystallographic Characterization of Compound Binding. A large number of Btk inhibitors have been developed, including ibrutinib (IMBRUVICA), which is currently used for oncologic indications. Other Btk inhibitors are now in clinical trials (CC-292, ACP-196, etc.), but still many more are in preclinical development stages. The compounds all bind in the ATP-binding pocket, but they vary in their binding modes and in the residues they contact within the active site. Furthermore, both irreversible and reversible compounds have been described. To determine how best to block the multiple signaling pathways that Btk regulates, we have characterized a panel of Btk inhibitors that vary in the way they interact with the enzyme. A table of the nine compounds with their structures, designations, and binding modes is shown in Table 1. A group of compounds was chosen that represents a broad spectrum of binding activities and provides structural diversity.

The cocystal structure of all the compounds with the Btk kinase domain was determined to characterize how the compounds bind and affect the orientation of the protein (Fig. 1). In native Btk, the residue Y551, which is part of the activation loop, is solvent accessible and oriented outward from the protein (Fig. 1A); however, we found that a subset of the Btk inhibitors bind and induce Y551 to fold back into the protein, rendering it solvent inaccessible and thus "sequestering Y551." We have used the term sequestration to describe this effect of triggering Y551 to fold into the protein as this was previously described for CGI1746 in the literature (Di Paolo et al., 2011). We also considered how inhibitor binding may affect other aspects of the Btk protein structure, particularly the DFG motif position and the αC helix position. We noted that all of the inhibitors’ binding can be classified as DFG-in. Also, the αC helix adopts an almost identical conformation in the presence of all inhibitors and appears to be αC helix-out. Thus, the compounds are most readily differentiated on the position of the Y551 residue, and we investigated this observation in depth.

Of the nine inhibitors studied, it was found that four are capable of triggering Y551 sequestration, whereas five do not trigger sequestration (Table 2). Representative structures illustrating Y551 sequestration are shown in Fig. 1. Ibrutinib is a prototypical irreversible binding compound that does not trigger sequestration as evidenced by the Y551 residue...
existing in solvent accessible space (Fig. 1C). In contrast, RN486 is a prototypical Y551 sequestering compound and in the structure of RN486 with Btk, the Y551 residue is folded into the protein making it solvent inaccessible (Fig. 1B). The same effect is noted for the other compounds listed as Y551 sequestering (Table 1 compounds 1–4). These compounds do not make direct contacts with the Y551 residue, but they seem to trigger sequestration by causing an undefined rearrangement of the local protein environment. The compounds can further be evaluated by their ability to affect the conformational flexibility and mobility of the Y551 residue. The metric to illustrate atomic mobility in crystallography is the B factor. Residues that are less ordered and have higher flexibility and motion have a higher B factor and are shown in Fig. 1 as thicker areas of the main chain and by color with “hotter” colors, such as red, indicating a higher B factor. In the ibrutinib structure (Fig. 1C), the activation loop is relatively thick and Y551 is colored red, indicating a greater freedom of motion. Almost as a rule, the compounds that triggered Y551 sequestration had a lower B factor for that residue (Fig. 2A and Table 2). Although the preponderance of compounds that trigger Y551 sequestration are reversible binders, we did not find that reversibility of binding was a determinant for Y551 sequestration or inhibitory activity. Evidence for this is seen for the highly similar properties of ibrutinib and an exact copy of ibrutinib that has the acrylamide warhead modified (Ibrutinib-Rev, compound 5). Instead, it seems that a compound’s ability to bind the H3 pocket of the enzyme is likely the determinant for Y551 sequestration. Prior crystallographic work has shown that Y551 forms the bottom of the
H3 pocket. Interestingly, since the H3 pocket is unique to Btk and a few other kinases, targeting the pocket can result in exquisite kinase selectivity (Johnson et al., 2016).

Compound Potency against Wild-Type and Y551E Mutated Btk. To characterize the compounds of interest, their potency for inhibition of purified recombinant Btk was determined. To investigate what impact the position of Y551 has on compound binding and potency, we measured the inhibition of wild-type Btk and Btk with Y551 mutated to glutamic acid (E) (Fig. 2B). Glutamic acid carries a negative charge and is a rough mimic of a polar phosphate group. Thus, this mutation could induce a conformational change in the structure of the protein because of interactions at that position with other hydrophobic and hydrophilic residues, that is, similar to the change triggered by phosphorylation of that residue. We found that compounds that do not trigger Y551 sequestration have nearly equal potencies for wild-type Btk and Y551E Btk (Fig. 2B). Data points plotted are means for at least two tests of each compound. Each symbol represents one compound, and the shaded bar represents the median for each group. **Significantly different $P < 0.01$ (Mann Whitney $t$ test).

Inhibitory Activity against Btk Y223 and Y551 Phosphorylation. To explore the functional consequence of Y551 sequestration, we determined the effects of the compounds on Y551 and Y223 phosphorylation. Y223 has been recognized as a site of autophosphorylation, whereas Y551 is reportedly phosphorylated by the upstream kinases Lyn and Syk, although it has also been reported to be a site of autophosphorylation as well (Dinh et al., 2007; Mohamed et al., 2009). Phosphorylation at both sites has been reported to regulate Btk catalytic activity as well as localization within the cell. Phosphorylation of Y223 and Y551 was measured in the Ramos cell line after BCR activation by IgM cross-linking and in the RBL-2H3 rat basophil cell line after FceR activation induced by IgE anti-DNP/BSA cross-linking. In Ramos cells, BCR activation led to strong phosphorylation at both Y223 and Y551 (Fig. 3A). In contrast, in RBL-2H3 cells, there was a basal level of Y223 phosphorylation that was slightly increased by FceR activation (Fig. 3B). In the RBL-2H3 cells, however, Y551 was also strongly phosphorylated after activation. In both cell types, phosphorylation was a rapid event occurring within 5 minutes after activation. The ability of Btk inhibitors to affect Btk pY223 binding or may create steric clashes that reduce compound binding. The differential relative potency of compounds for wild-type Btk and Y551E Btk confirms the disparate binding of the two classes of compounds and their regulation of Y551.
and pY551 was determined in both cell types. Cells were pretreated with compounds for 30 minutes before activation. Representative data are shown in Fig. 3, A and B, for CGI1746 and a reversible version of ibrutinib (Ibrutinib-Rev), which are examples of Y551 sequestering and non-Y551 sequestering compounds, respectively. Y551 sequestering compounds such as CGI1746 are able to block pY551 as well as pY223, whereas nonsequestering compounds show activity selectively against pY223 and are less able to prevent pY551.

Phosphorylation was measured using the ProteinSimple Wes, an automated Western blotting instrument, which allowed determination of an approximate IC50 value for each compound for inhibition of pY223 and pY551. When the ratio of the IC50 values for pY551 and pY223 was calculated, we found that the compounds that sequester Y551 nearly equally inhibited pY551 and pY223 in Ramos cells as the ratio of the IC50 values for those compounds was a median of 1, whereas the pY551 IC50/pY223 IC50 ratios for the nonsequestering compounds had a median of 1000 (Fig. 3C). Likewise, in the RBL-2H3 cells, the Y551 sequestering compounds were better at blocking Y551 phosphorylation relative to Y233 phosphorylation. In fact, the Y551 sequestering compounds had lower IC50 values for pY551 compared with pY223 as their pY551 IC50/pY223 IC50 ratios had a median of 0.1.

**Potency against BCR, FcεR, and FcγR Signaling.** To determine the inhibitory activity of the compounds in cells, we evaluated their IC50 values for inhibition of BCR and FcεR signaling. Compounds were first tested for their ability to inhibit BCR activation by anti-IgM cross-linking on B cells in human whole blood. Second, we measured the potency for inhibition of FcεR cross-linking and activation by IgE/anti-IgE on basophils in whole blood. When the IC50 values were compared for the two cell types, it was discovered that compounds that sequester Y551 are equally potent for inhibiting signaling in both cell types, whereas compounds that do not sequester Y551 have a reduced activity against FcεR signaling relative to their ability to inhibit BCR signaling (Fig. 4A). When the compounds were grouped by their Y551 sequestering ability (Fig. 4B), a significant difference in the FcεR IC50/BCR IC50 ratio was seen, with the Y551 sequestering compounds demonstrating nearly equal activity against the two signaling pathways. There was a similar effect when we tested the compounds for activity against FcεR in human mast cells differentiated from CD34+ stem cells and BCR activation in B cells in PBMC preparations (Supplemental Fig. 1). With mast cells, the nonsequestering compounds were less effective against FcεR relative to the BCR, although the effect was less dramatic compared with that in whole blood. This result may have been a consequence of using purified mast cells for determining FcεR potency and a mixed cell population (PBMC) for determining BCR potency. The mast cell IC50 values may be generally lower because it could be easier to see an effect in a more purified population or because the mast cell assay takes much longer to run compared with the basophil assay. In conclusion, the inability to sequester Y551 results in a loss of potency against FcεR signaling, and phosphorylation at Y551 is likely more...
important for regulation of Btk activity in mast cells and basophils than in B cells.

To determine whether Y551 sequestration also affects the potency of the compounds for inhibition of FcγR signaling, we tested the compounds for blockade of CD64 activation in U937 cells, a monocyte model cell line (Fig. 5). When U937 cells were treated with the Btk inhibitor compounds before activation of CD64 by anti-CD64 cross-linking, there was a reduction in nuclear factor (NF-κB) activation, which serves as a downstream readout of receptor activation. The IC₅₀ for inhibition of FcγR activation in U937 cells was compared with the IC₅₀ for inhibition of BCR activation in PBMCs (Fig. 5A), and again nonsequestering compounds were in general less potent against FcγR signaling compared with BCR signaling. A statistically significant difference was seen between the Y551 sequestering and nonsequestering compounds (Fig. 5B), as again an inability to sequester Y551 tended to decrease the ability to block FcγR signaling. Thus, it appears that Btk phosphorylation at Y551 plays an important role in regulating activity of the enzyme in the FcγR signaling pathway as well as the FcεR pathway.

**Cis and Trans Regulation of Btk Phosphorylation and Activation.** To provide a greater understanding of why Y551-sequestering compounds have better activity against FcγR signaling, we performed experiments to determine how upstream kinases and phosphorylation regulate Btk activity. In initial studies, Btk appeared to be constitutively phosphorylated on Y223 in RBL-2H3 cells but not in Ramos cells (Fig. 3, A and B). Reports in the literature indicate that Y223 is a site of autophosphorylation (Mohamed et al., 2009). To determine whether Y223 is constitutively phosphorylated in RBL-2H3 cells owing to constant Btk activation, we tested the ability of Btk inhibitors to reduce pY223 in the absence of FcγR signaling. RBL-2H3 cells were treated with three different Btk inhibitors (GDC-0834, RN486, and RN983) and at different time points after the compound addition the cells were collected, and Btk pY223 was detected by Western blotting (Fig. 6). After the addition of Btk inhibitor, the Btk pY223 signal was reduced, starting at 10 minutes, and a substantial reduction was observed at 60 minutes. This observation suggests that under basal conditions in Ramos cells, Btk exists in a conformation that is catalytically inactive, whereas in RBL-2H3 cells, it is constitutively active and hence a high basal level of Btk pY223 is observed. Since phosphorylation at Y223 is not a mechanism for regulation of Btk in RBL-2H3 cells, phosphorylation at Y551 is likely more important for controlling Btk and regulating receptor activation in those cells compared with in Ramos cells. This finding seems consistent with the finding that inhibitors that are more effective at blocking Btk pY551 also have better potency for inhibition of FcεR signaling.

To gain greater insight into how Btk Y551 is phosphorylated in the two cell types, we tested Lyn and Syk kinase inhibitors for their ability to prevent Btk phosphorylation at Y551. Previous reports have indicated that both Lyn and Syk can phosphorylate Btk and are known to be upstream of Btk. It is unclear for BCR and FcεR signaling, however, whether Lyn and Syk act in sequence or independently in parallel pathways to regulate Btk. To address this question, Ramos and RBL-2H3 cells were treated with INNO-46 (bafetinib), which is a Bcr-Abl/Lyn inhibitor, and the Syk inhibitor R406 and then activated. Phosphorylation of Btk Y223 and Y551, as well as PLC-γ2 Y1217, which is directly downstream of Btk, were measured. R406 inhibits Syk with an IC₅₀ of 41 nM in vitro (McAdoo and Tam, 2011) and has been reported to be relatively selective for Syk (Davis et al., 2011). INNO-406 is a dual Bcr-Abl/Lyn inhibitor that blocks Lyn in vitro with an IC₅₀ of 26 nM (Niwa et al., 2007). In Ramos cells stimulated with anti-IgM, INNO-406 inhibited both pY223 and pY551 at a concentration of around 10 nM (Fig. 7A) and also blocked PLC-γ2 pY1217 with slightly lower potency. Inhibiting Syk with R406 also reduced Btk pY223 and pY551 in Ramos cells but at higher concentrations compared with the Lyn inhibitor. R406 had a slightly greater effect on PLC-γ2 pY1217 compared with INNO-406. In RBL-2H3 cells, when the FcεR was activated with IgE-anti-DNP/DNP-BSA, neither compound strongly blocked Btk pY223 (Fig. 7B). In contrast to the Ramos cells, R406 was more potent at blocking Btk pY551 than was INNO-406, although neither compound strongly blocked Btk
phosphorylation at this site. The two compounds comparably blocked PLC-γ2 phosphorylation in RBL-2H3 cells, and both compounds more potently inhibited in RBL-2H3 cells compared with Ramos cells. These results suggest that both Lyn and Syk can regulate Btk phosphorylation and may do so independently of one another instead of in sequence. The results also illustrate that Btk is differentially regulated in the two cell types as it is clearly cataclysmically inactive in the Ramos cells before BCR activation and phosphorylated at Y551 by Syk or Lyn. Interestingly, in the RBL-2H3 cells, both INNO-406 and R406 had a noticeable effect on PLC-γ2 phosphorylation despite a minimal impact on Btk phosphorylation, which could be interpreted to mean that both Lyn and Syk activity is needed for FcεR signaling and PLC-γ2 phosphorylation, but either one or the other can phosphorylate Btk. The regulation of Btk by upstream kinases appears to be complex, and there are certainly differences as to how Btk phosphorylation is regulated in different cell types and signaling pathways. Furthermore, the two sites may be subjected to differences in how tightly they are regulated by phosphatase activity, adding another mechanism for differential regulation (Hamasy et al., 2016).

Hierarchical Clustering of Btk Inhibitors. To classify the compounds based on their aggregate biochemical and cellular properties in an unbiased manner, hierarchical clustering was performed using the Tibco Spotfire software program (TIBCO Software Inc., Palo Alto, CA) (Fig. 8). The B-cell factor (Fig. 2a), the ratio of IC50 of rBtk and rBtk Y551E (Fig. 2B), the ratio of IC50 of Y223 and Y551 phosphorylation in RBL-2H3 and Ramos cells (Fig. 3C), and the ratio of IC50 of the BCR and FcεR (Fig. 4B) and FcεR (Fig. 5B) were used as the data inputs for clustering of the compounds. The UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method was used to cluster the compounds based on the input data; the compounds that sequester Y551 (compounds 1–4) clustered together, whereas the compounds that did not trigger Y551 sequestration were clustered separately (compounds 5–9). These clustering results illustrate how the compounds’ binding and biochemical properties are linked to their cellular activity. There are differences in the compound’s specificity and cell permeability, so their correlations with pure biophysical and biochemical properties are not perfect, but their activities are still well predicted by their Y551 sequestering ability.

Discussion

In the studies reported here, we have found that Btk inhibitor potency can vary between cell types based on how a compound binds and its ability to sequester Y551. How this will translate into efficacy in vivo is unclear. Although Btk clearly plays a prominent role in BCR signaling, evidence not only from these studies, but also from the literature, suggests a role for Btk in FcεR signaling as previously demonstrated using bone marrow derived mast cells from knockout (KO) mice (Kuehn et al., 2008; Ellmeier et al., 2011). Btk KO has been demonstrated to reduce mast cell degranulation, and Btk is phosphorylated upon activation of FcεR. Prior evidence has shown that a Btk inhibitor can block FcεR activation (MacGlashan et al., 2011), but few studies have tested Btk inhibitors in human mast cells and basophils, and no evidence of their effects on Btk phosphorylation in those cell types has been found. It would be predicted that compounds that trigger sequestration of Y551 and consequently have greater potency for FecεRα, may be useful for treatment of diseases where IgE activation of the FcεR is involved; however, in vivo data on Btk inhibitor effects on FcεR activation and disease modeling is lacking. Nevertheless, the potential for Btk inhibitor compounds blocking basophil and mast cell activation is supported by these studies.

Y551 sequestering compounds may also have an added benefit in autoimmune indications where they are able to block FcγR signaling as well as BCR signaling. Earlier work has shown that Btk is expressed in RA patient synovial tissue and the Btk inhibitor RN486 was able to block inflammatory cytokine production triggered by IgG production (Hartkamp et al., 2015). Preclinical studies have demonstrated the ability of Btk inhibitors to reduce disease in RA models as well as lupus models and at least part of this efficacy has been attributed to inhibition of FcγR signaling (Xu et al., 2012; Bender et al., 2016). Our data on inhibition of CD64 signaling in U937 cells and literature results showing FcγR cross-linking on human monocytes induces pY223 as well as pY551 (Chang et al., 2011; Hartkamp et al., 2015), also indicates a role for Btk in regulating FcγR signaling. Our results further suggest that compounds which sequester Y551 may have a better potency for inhibition of FcγR signaling and an advantage over non-Y551 sequestering compounds in the context of treating end-organ damage in autoantibody-mediated diseases.

The knowledge of how Btk inhibitor binding regulates protein conformation and susceptibility to phosphorylation can help guide the design and selection of the best compounds for treating disease. Given the evidence that compounds which do not sequester Y551 have a loss of potency against FcεR signaling, it seems desirable to pursue compounds which do trigger sequestration, unless there is a reason to try to produce an inhibitor which has selective activity against BCR signaling relative to FcεR signaling.

The findings in this report may have broader utility for kinase inhibitor design as phosphorylation of a tyrosine in the activation loop is a mechanism for activation of other kinases as well. For instance Itk is phosphorylated at Y511, similarly to Btk Y551, which directly increases activity, and Src activity is increased by phosphorylation at Y416 in its activation loop (Bradshaw, 2010). It may be a more general rule that kinase inhibitors which have more than one mechanism of action may be more efficacious. If an inhibitor is not only ATP competitive, but also blocks another upstream activation mechanism such as phosphorylation, it may be more effective than an inhibitor which only has one means by which it achieves inhibition. A comparison of the activation loop and Y551 position with that of other kinases has suggested that it may be possible to design such dual inhibitors for ITK or SRC (data not shown).

Although kinases inhibitors with a dual mechanism of action may have advantages over those with only one, both types of compounds may be clinically successful. A highly potent compound may still achieve significant inhibition in multiple cell types and designing a compound to be an irreversible inhibitor may also help it achieve strong inhibition. This has been demonstrated by prior published results with irreversible and reversible Btk inhibitors in preclinical disease models; however, direct head-to-head comparisons are lacking. Further testing in this regard is warranted and
particularly in allergy or asthma models where FceR inhibition would likely be the primary mechanism of action of Btk inhibition.

In summary, the results of these studies elucidate molecular determinants of how Btk inhibitor binding affects regulation of the enzyme and compound potency in different cell types. We hope the knowledge provided here will aid in the design of new compounds with greater therapeutic activity to reduce disease.

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Authorship Contributions

**Participated in research design:** Bender, Gardberg, Pereira, Johnson, Wu, Gremningloh, Head, Morandi, Haselmayer, Liu-Bujalski.

**Conducted experiments:** Bender, Gardberg, Pereira, Wu, Head, Morandi, Liu-Bujalski.

**Performed data analysis:** Bender, Gardberg, Pereira, Johnson, Wu, Head, Morandi, Haselmayer, Liu-Bujalski.

**Wrote or contributed to the writing of the manuscript:** Bender, Gardberg, Pereira, Johnson, Wu, Head, Liu-Bujalski.

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**Address correspondence to:** Dr. Andrew Bender, EMD Serono Research and Development Institute, 45A Middlesex Turnpike, Billerica, MA 01821. E-mail: andrew.bender@emdserono.com