A Small Molecule Inhibitor of Bruton’s Tyrosine Kinase Involved in B-Cell Signaling

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ABSTRACT: Protein kinases are fundamental within almost all cellular signal transduction networks. Among these, Bruton’s tyrosine kinase (Btk), which belongs to the Tec family of proteins, plays an imperative part in B-cell signaling. Owing to its role, Btk has been established as an important therapeutic target for a vast range of disorders related to B-cell development and function, such as the X-linked agammaglobulinemia, various B-cell malignancies, inflammation, and autoimmune diseases. Herein, using computer-based screening of a library of 20 million small molecules, we identified a small molecule capable of directly binding the Btk kinase domain. On the basis of this hit compound, we conducted a focused structure-similarity search to explore the effect of different chemical modifications on binding toward Btk. This search identified the molecule N2,N6-bis(2,3-dihydrobenzo[1,4]dioxin-6-yl)-9H-purine-2,6-diamine as a potent inhibitor of Btk. The latter small molecule binds Btk with a dissociation constant of 250 nM and inhibits Btk activity both in vitro and in-cell.

INTRODUCTION

Bruton’s tyrosine kinase (Btk) is a member of the Tec family kinases (TFKs), which form one of the largest kinase families in mammals.1,2 TFK expression in different tissues plays an important role in a vast range of cellular signaling.3–5 and TFKs have been recognized as key players in B-cell antigen receptor (BCR) signaling. Within this family, the cytoplasmic Btk is mainly expressed in myeloid- and B-cells acting within a broad range of processes, including cell-cycle progression and apoptosis, and Btk has been directly linked to inflammatory responses and chronic inflammatory diseases.6 Figure 1A shows a schematic representation of Btk’s primary structure. It consists of 659 amino acids arranged into five distinct functional domains, each having a unique structure—function implication within the context of the cellular pathway.7 Following the binding of an antigen to the BCR, activated Btk is phosphorylated by the SRC kinases Lyn or Syk, and is also autophosphorylated at a specific Tyr residue.8–11 These phosphorylation events further trigger the downstream phosphorylation of phospholipase-C-γ-2 (PLCγ2) by Btk.12 Activated PLCγ2 hydrolyzes phosphatidylinositol, resulting in the activation of protein kinase C (PKC).13 It has been shown that the magnitude and duration of Btk-driven PKC signaling are dependent on PLCγ2 activity, which can also affect the activation of important transcription factors, such as NFκB, AP-1, and NFAT.13–16 Considering the crucial part Btk plays in the phosphorylation and activation of PLCγ2, and henceforth in the activation of the NFκB pathway,17,18 Btk has an immense role in B-Cell regulation and is further involved in its development, proliferation, maturation, and apoptosis.19–21 In addition, a large number of cellular abnormalities were found to be associated with nonfunctioning mutant Btk or dysregulation of Btk. For example, although the phenotype of X-linked agammaglobulinemia was discovered more than half a century ago,22 it took about two decades to link it to a mutation in the gene encoding the Btk.23,24 In addition, key roles of Btk were shown in different malignancies such as chronic lymphocytic leukemia, acute myeloid leukemia (AML),25,26 acute myeloid leukemia (AML),27 and multiple myeloma.28 This involvement of Btk in various immune-mediated disorders and cancer contributes to the on-going challenge to develop small molecule inhibitors that can bind and inhibit Btk, and thus act as a therapeutic substance in various Btk-related pathways. Indeed, a variety of different inhibitors have been discovered and developed29–35 but currently only the non-reversible inhibitor ibrutinib has received approval as a drug for targeting Btk.36,37 Herein, we present the discovery of a reversible small molecule inhibitor that can directly bind the Btk kinase domain and inhibit its kinase activity. Using computer-aided drug design tools, we virtually screened a library of 20 million compounds that were filtered according to preset...
criteria. Following the virtual screening, we applied a set of biophysical, biochemical, and cellular characterization tools to validate the activity of the new molecule targeting Btk.

**RESULTS**

**In Silico Screening.** To find new, small molecule inhibitors that can target the Btk kinase domain, we virtually screened a database of about 20 million available small molecules. Figure S1 illustrates the workflow of the virtual screening process designed to search and filter potential Btk inhibitors. This process consisted of the following steps: (i) structural analysis of all available structures of Btk to generate a single averaged model, (ii) analysis of known Btk binders from the publicly available cocrystals and extraction of the required pharmacophore constraints, (iii) crude filtering of the virtual library according to the required pharmacophore features, (iv) docking and clustering of the virtual compounds passing the first pharmacophore filter, and (v) ranking and manual selection of top compounds for the next in vitro validation. Finally, we purchased a subset of ∼90 compounds for the in vitro screening, taking under consideration the tradeoff between success rates and cost-effective procedures. This set of compounds covered the largest possible chemical space, while still addressing the desired physiochemical criteria to ensure optimal binding.

**Btk Kinase Domain Expression and Purification.** To test the selected set of small molecules that passed the previously described computational filters, we set up orthogonal in vitro assays. For this purpose, we expressed the recombinant Btk kinase domain-containing residues 387−569 with cleavable N-terminus six-histidine and maltose binding protein (MBP) tags, for purification and increased solubility, respectively. Figure 1B shows the purification process of the protein. The left SDS-PAGE gel shows the purification of the protein using a nickel column. Purified MBP-tagged Btk kinase domain was eluted from the nickel column. Following cleavage by PP, the eluted fraction was repassed through the nickel column, to separate the purified Btk kinase domain from the MBP and histidine tags.

Establishing a “3-Injections” Iso Thermal Calorimetry (ITC) Experimental Setup for the Detection of Btk Small Molecule Binders. Following expression of the recombinant Btk kinase domain, we sought to test the subset of compounds that were purchased at the end of the in silico screening for binding. To that end, we used ITC, which is capable of measuring the direct heat change occurring due to the interaction of a pair of molecules.38,39 ITC has certain advantages, such as the capability to detect a relatively broad range of binding affinities, being a label-free method, and providing a wide set of interaction parameters, such as the affinity, stoichiometry, enthalpy, and entropy of binding. However, ITC also has a few disadvantages that limit its suitability to only low-throughput screening applications, or as a secondary tool for binding validation. Moreover, a relatively large quantity of protein is required for a single experiment, and performing a complete binding curve is time consuming. To overcome these limitations, we used the 3-injections ITC experiment, as depicted in Figure 2.40 In this experimental scheme, the protein was placed in the ITC syringe and injected into the cell containing the compound. A binary decision regarding the binding of a specific molecule was then obtained based on the absolute and/or change in heat magnitude of the different injections. To set up the experimental conditions for the Btk screening, we tested the 3-injections ITC experiment on RN-486 and LFM-A13, reference molecules known to bind Btk with a dissociation constant of 5 nM and 1 μM, respectively. Figure 2B shows the thermographs obtained following the
injection of Btk into each of these compounds, as well as into the experimental buffer, used as a control. This data was produced by monitoring a series of 3 injections of Btk, 3 μL each, into the ITC cell containing the target molecule. By using only 9 μL of protein at a concentration of 300 μM, we were able to obtain a binary result regarding the binding event. Although a lower volume and concentration could have been used, this setup aimed at detecting molecules that may have higher $k_d$ values, as one would expect with preliminary hits from virtual screening. It can be seen (Figure 2B) that two factors were correlated with the

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**A. ‘3-injections’ ITC experiment used for binary binding evaluation**

![Diagram of 3-injection ITC experiment](image)

**B. Setup of the ‘3-injections’ ITC experiment on reference molecules**

![Graphs of RN-486, LFM-13, and buffer](image)

*Figure 2. “3-Injection” ITC assay. (A) Schematic representation of the 3-injection ITC experiment. In this setup, the protein was placed in the syringe and the small molecule was placed in the ITC cell. Three injections were executed and both the heat magnitude and trend were correlated to find preliminary positive protein–ligand interactions. (B) 3-injection ITC experiment with [left] RN-486, an optimized molecule binding to Btk with a dissociation constant of 5 nM, [middle] LFM-A13, with a dissociation constant of 1 μM, and [right] with the experimental buffer as control.*
binding of Btk to the reference molecules. The first was the magnitude of the heat in $\mu$cal/s that was observed with each injection. The second was the change in heat that can be observed between two consecutive injections. Given that some of the molecules are already bound to Btk following the first injection, the lower number of binding events in the ITC cell in the second and in each of the following injections result in lower heat dissipation.

In Vitro Screening of Candidate Compounds for Binding to Btk Kinase Domain Using the 3-Injections ITC Experiment. The candidate small molecules were tested for binding to the Btk kinase domain using the described biophysical assay. Each experiment consisted of three sequential injections of 300 $\mu$M Btk kinase domain into 30 $\mu$M compound separated by a 120 s delay. To obtain a binary decision regarding the binding of the different molecules, we monitored the change in released or absorbed heat. Figure 3A shows the raw data obtained for 81 3-injection ITC experiments. The raw data, including the peak height and shape, was used to provide an initial assessment of the binding data. Indeed, whereas most mixtures show a low exothermic or endothermic reaction with approximately the same magnitude of energy change, a few panels show a potentially ITC detected binding event. In addition to the manual inspection of the data set, we also calculated a numerical value of the energy change for each of the molecules.
injections, as described elsewhere. Therefore, we normalized all curves to a zero baseline, by means of an auxiliary fitting procedure (applying vertical translations and rotations). Curves with negative energy peaks (exothermic reactions) were mirrored around the horizontal axis. Following integration of the heat peaks, we ran the least absolute deviations curve-fitting procedure to fit the peaks to a function of the form 

$$f_{K_{eff}}(x) = 1 - \left[ P_L K_{eff} \right]$$

where $[P_L K_{eff}]$ is the protein–ligand concentration in each injection according to the solution of a single site binding model

$$[P_L] = 0.5 \times \left( L_0 + P_0 + K_{eff} \right)$$

$$- \sqrt{(L_0 + L_0 + K_{eff})^2 - 4L_0P_0}$$

where $L_0$ and $P_0$ are the initial ligand and protein concentrations, and $K_{eff}$ is the effective dissociation constant being sought. Figure 3B shows a bar plot of the raw $K_{eff}$ values attained by fitting the different experiments. Naturally, in the majority of mixtures, no binding event occurred, which is reflected by the scattered values around the zero baseline. An exception is observed in compound 77, wherein the supposed binding event produced a large heat difference between each injection. To validate and better evaluate the initial affinity of the small molecule compound that showed binding to Btk in the 3-injection assay, we measured the full ITC binding curve, as depicted in Figure 4A. For this purpose, we performed a full ITC titration experiment employing a series of 14 injections of 2.5 μL each. The fitting of the raw data ITC curve to a single binding-site model yielded an association constant of $2.71 \times 10^5$ M$^{-1}$ ($k_d \sim 3.4 \mu$M). Figure 4B shows the chemical structure of the molecule identified as a binder of the Btk kinase domain.

**Focused Structure-Similarity Activity Study.** In addition to ITC, which serves as an excellent biophysical tool to determine the binding of a molecule to the target protein, it is crucial to test additional compounds with similar structures. Although a common course of action is usually to perform a structure–activity relationship (SAR) study in which a set of analogues with chemical modifications is synthesized, the required resources for...
Figure 5. Structure-similarity study by ITC. The panels show the results of ITC experiments conducted on six different compounds (L1–L6). Compounds L2–L4 showed effectively no or very weak binding. Compounds L1 and L5 had a dissociation constant of 5 and 1 μM, respectively. Compound L6 had the best affinity toward Btk with a dissociation constant of 0.25 μM.
that purpose are high. An alternative approach that we have taken here is to search for structurally similar compounds and devise a set of relevant analogues. Each of the structurally similar compounds is then subjected to docking and ranking using the same principal parameters used for the virtual screening process, and potential binders are then purchased and tested as described below. Looking at the chemical structure of the compound depicted in Figure 4B reveals several distinctive functional groups, such as a purine, phenyl, and morpholine ring. A study of structurally similar chemical analogues can thus expand our understanding of the functional moieties that best contribute to the binding of Btk. To this end, we tested compounds that exhibited structural similarities to our initial hit. Figure 5 shows six different molecules, each exhibiting different chemical substitutions, which were tested for binding to the Btk kinase domain by ITC. Compound L3, which underwent multiple modifications compared to the initial hit detected by the computational screen, showed no binding to Btk. We then explored specific modifications. Compound L1 has two methoxy groups on the benzene ring that substituted the fluorine atom of the original compound in Figure 4B. It showed relatively weak affinity toward Btk that was on the same order of magnitude as the initial hit. On the basis of the compounds tested, the benzene functional group seems crucial for binding whilst both fluorine and methoxy can serve as functional groups. Compound L2 had a morpholine to pyrrolidine substitution. Unlike the previous case, this single chemical modification diminished the interaction of the molecule with Btk. This demonstrated that the size or shape of the morpholine ring, or possibly the physiochemical properties of the oxygen, are important for the interaction with Btk. Nevertheless, as compound L4, containing the methoxy-benzene substitution but lacking the morpholine ring, did show weak signs of binding toward Btk, we put more emphasis on methoxy-benzene for obtaining high affinity toward Btk. Indeed, compounds L5 and L6, which exhibited two oxygen-containing functional groups on the benzene, showed medium and high affinity toward Btk, respectively. Between the latter two compounds, L6, which has two benzene-attached dioxane rings, showed prominent binding to Btk with $k_d = 250$ nM. The dissociation constants of molecules L1–L6 toward Btk are summarized in Table 1.

Table 1. Dissociation Constants of Molecules L1–L6 toward Btk Kinase Domain

| index | $k_d$ toward Btk (nM) |
|-------|----------------------|
| L1    | 5000                 |
| L2    | N/A                  |
| L3    | N/A                  |
| L4    | N/A                  |
| L5    | 1000                 |
| L6    | 250                  |

In addition to the ITC experiment, which demonstrated direct binding of compound L6 to Btk kinase domain, we tested binding in an orthogonal biophysical assay using differential scanning fluorimetry (DSF). In this approach, the temperature dependence of the 350–330 nm tryptophan fluorescence ratio is correlated. On the basis of this ratio, the melting temperature ($T_m$) of the protein in the free and bound forms can be extracted. Figure S2 shows the melting curves and the calculated $T_m$ of Btk with and without compound L6. It is important to note that in a previous study, a similar molecule was found to bind BRAF kinase with approximately 1 order of magnitude lower potency (a dissociation constant of $k_d = 5200$ nM). This significant difference in binding may be explained by a relatively low homology (identity of 27.6% and similarity of 56.7%) between the kinase domain of BRAF (residues 457–717) and Btk (residues 402–655), as is depicted in Figure S4. In addition, the improved activity toward Btk inhibition is further supported by Table 2.

Inhibition Validation by Biochemical and Cellular Assays. To validate the ability of compound L6 to bind Btk kinase domain and to interfere with its phosphorylation activity, we designed a simple yet direct in vitro assay. Specifically, we monitored the phosphorylation of the peptide NH$_2$-KKVVA-LYDYMPPMN-COOH corresponding to residues 217–229 in the full-length Btk, which included the autophosphorylated tyrosine, and served as an established in vitro substrate for Btk. Figure 6A shows western blot (WB) analysis following the kinase in vitro assay in the absence and in the presence of 50 μM molecule L6. As a positive control, and given its superior affinity, we used the known Btk inhibitor RN-486 in a concentration of 1 μM. We found that compound L6 demonstrated significant inhibition of Btk kinase activity. We next aimed to validate the inhibitory effect of the small molecule in the cellular setting. We specifically tested the ability of the identified compounds to inhibit Btk in U937 human monocyte cells. The latter were incubated with RN-486 and the compounds L1–L6 in a concentration of 1 and 20 μM, respectively. Cells were further activated by the phosphatase inhibitor pervanadate. Figure 6B shows WB analysis of the cellular extract following treatment with the compounds using phospho-Btk, phospho-PLCy2, and α-Tubulin antibodies. It can be seen that the small molecules interfered with Btk autophosphorylation as well as with the downstream phosphorylation of its main substrate PLCy2. In the last step, we characterized the EC50 of molecule L6. Figure 6C shows WB analysis of the dose–response effect of molecule L6 within the cellular environment. On the basis of the bands’ magnitude, it can be estimated that the molecule L6 shows a cellular EC50 of about 5 μM.

L6 Specificity Panel. To assess the specificity of L6 molecule toward Btk in comparison with other kinases, we performed a single-point activity assay toward a focused list of 40 kinases, which were mostly part of the TK family. Screening was executed at a compound concentration of 10 μM. Table 2 shows the kinase name versus the percent activity score given relative to a control test and fold activity relative to Btk. Although the score is not an affinity measurement, it represents the hit probability. Roughly speaking, hits less than 1% suggest that the dissociation constant ($k_d$) is most likely less than 1 μM. It can be seen that among the kinases in the list, only three exhibit such probability, including Btk. Moreover, in about 90% of the tested kinases, there is at least a 2-fold decrease in activity potency and in more than 60% of the tested kinases, the difference is of more than an order of magnitude.

**DISCUSSION**

Btk serves as an important therapeutic target, mainly in conditions that are associated with B-cell regulation, such as inflammation and autoimmune diseases. Indeed, the non-reversible covalent inhibitor Ibrutinib, a first-in-class small molecule drug, shows effective activity in a range of B-cell related clinical disorders. Yet, there is still immense need for the discovery of new inhibitors that present different pharmacological patterns. In this study, we described the discovery, via
demonstrated that combining virtual screening with biophysical methods identified compound L6 as a promising inhibitor and an excellent starting point for studying its pharmacological properties and selectivity. Our current understanding of the SARs and the promising cellular activity of compound L6 pave the way for the development of an anti-Btk clinically approved small molecule drug.

### Table 2. % Activity of L6 Molecule toward Selected Kinases

| kinase name             | % activity | fold activity/Btk | kinase name             | % activity | fold activity/Btk |
|-------------------------|------------|-------------------|-------------------------|------------|-------------------|
| BLK                     | 0.4        | 0.6               | CSK                     | 18         | 27.7              |
| BTK                     | 0.65       | 1.0               | FRK                     | 22         | 33.8              |
| TYK2 (JH1domain-catalytic) | 0.8     | 1.2               | LYN                     | 32         | 49.2              |
| ABL1-phosphorylated      | 1.2        | 1.8               | EPHA1                   | 46         | 70.8              |
| SRC                     | 1.8        | 2.8               | CTK                     | 49         | 75.4              |
| SYK                     | 2          | 3.1               | ALK                     | 51         | 78.5              |
| ABL1-nonphosphorylated   | 2.1        | 3.2               | FLT1                    | 51         | 78.5              |
| LCK                     | 2.1        | 3.2               | JAK1 (JH1domain-catalytic) | 52     | 80.0              |
| CLK1                    | 2.2        | 3.4               | FAK                     | 58         | 89.2              |
| PYK2                    | 2.3        | 3.5               | FES                     | 62         | 95.4              |
| JAK1 (JH2domain-pseudokinase) | 2.5     | 3.8               | IGFIR                   | 62         | 95.4              |
| TEC                     | 2.5        | 3.8               | ZAP70                   | 62         | 95.4              |
| EGFR                    | 3.5        | 5.4               | NDR1                    | 73         | 112.3             |
| ITK                     | 5.1        | 7.8               | ZAK                     | 76         | 116.9             |
| FER                     | 5.7        | 8.8               | DDR1                    | 77         | 118.5             |
| TXK                     | 8          | 12.3              | p38-α                   | 90         | 138.5             |
| FYN                     | 8.7        | 13.4              | BRAF                    | 92         | 141.5             |
| BMX                     | 9.7        | 14.9              | ERK1                    | 94         | 144.6             |
| VEGFR2                  | 11         | 16.9              | RAF1                    | 95         | 146.2             |
| TNK1                    | 14         | 21.5              | SGK                     | 100        | 153.8             |

rational drug design, and characterization of a new small molecule inhibitor of Btk, namely, compound L6, with a dissociation constant ($k_d$) of 250 nM. Generally speaking, protein kinases share a conserved ATP binding site located between two lobes that are connected by the hinge domain, as depicted in Figure 7A on the Btk model. Kinase inhibitors targeting the ATP site usually bind this region.46 Thus, selectivity toward a specific kinase should foster additional interactions that are unique to the kinase of interest. In this context, the Gly-rich loop and activation loop of Btk form a hydrophobic pocket that is distinctive to Btk and was targeted in our screening. The general chemical scaffold of the inhibitor that can be extracted from the selected compounds has a central purine of which the C2 and C6 are substituted by NH-flanked by two benzene-attached dioxane rings connected via an amine bond. The purine central group forms a hydrogen bond with the backbone of Btk Met477 located within the hinge domain, as depicted in Figure 7B. In this model, the C2-substituted NH and N3 of the pyrimidine form two hydrogen bonds with the amide and carboxyl backbone of Ser477 located on the hinge domain of Btk. In addition, one of the dioxane rings is located at the entrance of the unique hydrophobic pocket of Btk and forms a hydrogen bond with Lys343. The latter may contribute toward the superior selectivity of the compound to Btk. A full map of interactions between the L6 compound and Btk kinase domain is shown in Figure 7A. In our in vitro results are supported by the cellular activity assays depicted in Figure 6B. The strong correlation between the in vitro binding and the cellular inhibition of Btk and PLCγ2 highlights the mechanism of action of the compounds found herein. Although L6 is not yet considered to be a lead molecule, the biophysical data and the relatively high specificity toward Btk, as demonstrated in Table 2, mark compound L6 as a promising inhibitor and an excellent candidate for further development.

Within the challenging task of drug development, we demonstrated that combining virtual screening with biophysical tools that can measure direct binding affinities provides a powerful platform for the discovery of new bioactive molecules. Specifically, we used ITC as an in vitro screening technique secondary to in silico methods. The presented 3-injections ITC method provides a biophysical high-throughput tool to measure direct binding affinities, which allows direct chemical optimization toward the development of potent inhibitors. The strong correlation between ITC-derived data ($k_d$) and the biochemical and cellular assays points to the validity and potential of this approach. The initial affinity of compound L6 is an excellent starting point for studying its pharmacological properties and selectivity. Our current understanding of the SARs and the promising cellular activity of compound L6 pave the way for the development of an anti-Btk clinically approved small molecule drug.

### EXPERIMENTAL SECTION

#### Computational. Molecular Dynamics (MD) Simulations.

The MD cascade protocol included 5000 minimization steps followed by 500 ps of system heating to 300 K and an additional 500 ps of equilibration steps. The final production run (out of which snapshots for analysis were obtained) of 500 ns was distributed on a 120 core HPC server (4 HP ProLiant SL230s Gen8 servers with dual Intel Xeon E5-2670 CPUs). root-mean-square deviation and energy profile analysis along the simulation trajectory were done to verify convergence of the simulation and its correctness.

Generating a Three-Dimensional (3D) Model Used for Docking. An average structure based on all available Btk kinase domain crystal structures was generated and subsequent MD simulations were carried out with the program CHARMM47 to refine the model. Weak harmonic constraints were used to limit Btk backbone movements during the entire simulation. On the basis of the results of this analysis, we generated a single average model that was used for molecular docking.

Library Size Reduction Based on Profiling and Crude Pharmacophore Filtering. Library profiling was based on analysis of the interaction pattern between known small molecule binders and Btk. The fitting procedure was initiated by rapid
filtering of compounds based on the basic molecular features, followed by a rigid matching in the 3D space to a "crude" pharmacophore model consisting of only 3–4 required features. This process was repeated for each molecule’s conformer to filter out molecules that were unable to satisfy the pharmacophore requirements. Pharmacophore hypothesis and filtering were done using the search DB algorithm in Catalyst program.48,49 Small molecules that fulfilled all required features within the geometrical constraints of the pharmacophore model were then ranked by “fitting value”—a calculated score based on the distance between all features in the model, and the chemical groups’ actual position in the small molecule. The result of this step was a reduction of the library size to approximately 500,000 compounds.

**Molecular Docking and Final Compound Selection.** Molecular docking of the profiled library was done using the GOLD program,50,51 with preset genetic algorithm settings optimized for virtual screening. A post-docking scoring function was applied to rank each docking pose. A cutoff value was used to filter out compounds that did not satisfy specific location constraints and also to account for putative ligand interacting groups in addition to the structure-based complementary ones.

**Insect Cell Culture.** Insect cells sf9 and *Trichoplusia ni* (Tni) together with culture media (cat# 96-001) were purchased from Expression Systems Llc.
Protein Expression and Purification. The human Btk kinase domain, which includes residues 387–569, was produced as a cleavable MBP fusion protein and HISx6 tag containing a PP recognition site based on the Bac-to-Bac baculovirus expression system. More specifically, the Btk kinase domain gene was cloned into pFastBAC1 donor plasmid. Thereafter, pFastBAC1, carrying the gene of interest, was transformed into DH10Bac cells and recombinant bacmid was then used to generate the baculoviral stock. In the first step, sf9 cells were transfected with the latter viral suspension and Btk-related virus was produced. Then, Tni cells were transfected with 4% v/v of the previous viral suspension and grown for 72 h at 29 °C. To extract the protein, the cells were harvested and resuspended in 30 mL of 50 mM Tris–HCl (pH = 8), 300 mM NaCl, 30 mM imidazole, 5 mM ßME, and 0.5% glycerol. The suspended cells were then disrupted by sonication and the insoluble fraction was removed by centrifugation for 20 min at 15 000 g. The supernatant was applied to a 5 mL column of nickel beads. After washing the resin with 40 mL of 50 mM Tris–HCl (pH = 8), 300 mM NaCl, 30 mM imidazole, 5 mM ßME, and 5% glycerol, the protein was eluted with the same buffer containing 300 mM imidazole. The elution fraction was concentrated to 5–10 mL using 10 000-MWC membrane ultrafiltration. PP was added to the concentrated elution fraction, and the solution was dialyzed with 1 L of Tris with 10 mM imidazole. The digested solution
was applied to the nickel column again and the flow-through containing Btk was collected.

ITC. The purified protein was placed in a 6–8 MWCO dialysis bag in 30 mM phosphate buffer, 30 mM NaCl, 1 mM TCEP, and 5% dimethyl sulfoxide. Sequential buffer exchange dialyses were executed for 12 h each at 4 °C until dilution of >10,000 was achieved. ITC binding constant estimation was calculated using the calorimetric analysis program in the Origin suite. Extraction of $K_a$ based on the 3-injections ITC technique was conducted in MATLAB.

Biochemical Assay. For the in vitro kinase assay, Btk-derived peptide was suspended in the kinase assay buffer (20 mM Tris, pH 7.5, 10 mM MgCl$_2$, and 10 mM MnCl$_2$, and 500 mM ATP) in the presence of the different compounds with indicated concentration in the text. Reactions were performed at 25 °C for 30 min. The kinase reactions were terminated by the addition of SDS sample buffer followed by boiling for 5 min. All samples were separated by SDS-PAGE and transferred to WB analysis to monitor phosphorylation levels with antibody Phospho-Btk Y223 analysis (Abcam Cat. Ab68217).

Cellular Assay. AML cell line U937 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified incubator at 37 °C in 5% CO$_2$. WB analysis was conducted for protein levels of phospho-Btk Y223 (Abcam Cat. AB68217), phospho-PLCγ2 (Cell Signaling Cat. 3874), and α-tubulin (Santa Cruz Cat. SC8035). Cells were treated with the compounds for 18 h and activated with 100 nM pervenadate for 20 min. Cellular lysate was analyzed with the indicated Abs.

Small Molecule Chemistry. All molecules were purchased or synthesized by Akos GmbH or Enamine Ltd.

Kinome Scan. Selectivity profiling was executed by DiscoverX using KinomeScan Technology.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00576.

Compound structures in smile format (CSV)
Protein Data Bank details (PDB)
Figure S1, virtual screening cascade; Figure S2, DSF binding validation of L6 compound to Btk; Figure S3, interaction map of L6 compound with Btk; Figure S4, alignment between BRAF and Btk kinases domain (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. E.R. and I.B. designed and performed the experiments, and analyzed the data. I.B. performed the virtual screening and modeling, and wrote the paper. M.N. and N.R. performed the cellular WB-related experiments and the cellular assays. E.C. supported the computational study. M.L. and N.D. wrote the paper, assisted in data analysis, and gave critical review.

Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
Btk, Bruton’s tyrosine kinase; ITC, iso thermal calorimetry; WB, western blot; DSF, differential scanning calorimetry; MBP, maltose binding protein

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