Published in final edited form as:

J Med Chem. 2018 September 13; 61(17): 7573–7588. doi:10.1021/acs.jmedchem.8b00040.

Thiourea-Based Inhibitors of the B-Cell Lymphoma 6 (BCL6) BTB Domain via NMR-Based Fragment Screening and Computer-Aided Drug Design

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Abstract

Protein-protein interactions (PPI) between the transcriptional repressor B-cell lymphoma 6 (BCL6) BTB domain (BCL6^{BTB}) and its co-repressors have emerged as a promising target for anti-cancer therapeutics. However, identification of potent, drug-like inhibitors of the BCL6^{BTB} has remained challenging. Using NMR-based screening of a library of fragment-like small molecules, we have identified a thiourea compound (7CC5) that binds to the BCL6^{BTB}. From this hit, the application of computer-aided drug design (CADD), medicinal chemistry, NMR spectroscopy, and X-ray crystallography has yielded an inhibitor **15f**, which demonstrated over 100-fold improved potency for the BCL6^{BTB}. This gain in potency was achieved by a unique binding mode that mimics the binding mode of the corepressor SMRT into the “aromatic” and the “HDCH” sites. The structure-activity relationship based on these new inhibitors will have a significant impact on the rational design of novel BCL6 inhibitors, facilitating the identification of therapeutics for the treatment of BCL6-dependent tumors.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publication websites.

¹H and ¹³C NMR spectra of new compounds, crystallographic data for compounds 7CC5, **15a**, and **15f**, and molecular formula strings and some data.

PDB INFORMATION

Authors will release the atomic coordinates and experimental data upon article publication. Compound 7CC5: PDB ID, 6C3N

Compound **15a**: PDB ID, 6CQ1

Compound **15f**: PDB ID, 6C3L

GRAPHICAL ABSTRACT

INTRODUCTION

B-Cell lymphoma 6 (BCL6) is an oncoprotein associated with multiple cancers.¹ As the master regulator of the germinal center (GC) reaction, BCL6 is constitutively expressed in diffuse large B-cell lymphomas (DLBCL).^{2,3} It is one of the most upregulated genes in BCR-ABL positive B-cell precursor acute lymphoblastic leukemia (B-ALL) cells after exposure to BCR-ABL tyrosine kinase inhibitors (TKIs).⁴ BCL6 is also upregulated by mixed lineage leukemia (MLL) fusion oncoproteins in B-ALLs with MLL translocation.⁵ Moreover, patients with blast-phase chronic myeloid leukemia (BP-CML) cannot be cured by BCR-ABL TKIs because CML stem cells are not fully dependent on BCR-ABL.⁶ However, CML stem cells express high levels of BCL6, and targeting BCL6 eliminated these cells.⁶ Furthermore, the BCL6 locus is often amplified in breast cancers, and BCL6 maintains the survival of aggressive triple-negative breast cancer (TNBC) cells.⁷ Therefore BCL6 has broad oncogenic roles in many cancer subtypes.

BCL6 mediates tumorigenesis through its repression of DNA damage sensing genes such as *ATR*, and cell death and cell cycle checkpoint genes such as *CHEK1*, *TP53*, *CDKN1A*, *CDKN2A*, *P14ARF*, *CDKN1B*, and *EP300*.^{8–14} Blockade of BCL6 repressor activity induces expression of these genes, resulting in rapid cell death.^{10,15–18} Although different types of cancers are heterogeneous, a majority of them are BCL6-dependent.¹⁹

BCL6 has an *N*-terminal Broad-complex, Tramtrack, and Brick-a-brac (BTB) domain (BCL6^{BTB}) that mediates transcriptional repression, a *C*-terminal C2H2 zinc finger responsible for DNA binding and inflammatory reactions,²⁰ and a linker region containing a second repression domain.²¹ BCL6^{BTB} forms an obligate homodimer. The interface of the dimer forms a lateral groove (LG) that is employed in the recruitment of the corepressors silencing mediator of retinoid and thyroid hormone receptor (SMRT), nuclear receptor corepressor (NCOR), and BCL6 corepressor (BCOR).^{22,23} This unique BCL6^{BTB} LG/corepressor interaction is essential for the repression activity of BCL6.^{24,25}

BCL6 knockout is lethal and most knockout mice die within 4–6 weeks of a severe inflammatory syndrome.²⁶ This phenotype raises concern regarding potential side effects of BCL6 inhibitors. However, exposure to BCL6^{BTB} LG peptide inhibitors¹⁶ did not induce inflammation or other toxic effect.¹⁶ Moreover, mice expressing BCL6 with point mutations that abrogate corepressor binding lived healthy lives without inflammatory syndrome.²⁷ Taken together, the BCL6^{BTB} LG function is only essential in GC B-cells and in tumors, but is dispensable for the other functions of BCL6. Indeed the effect of BCL6 in macrophages appears to be linked to its zinc fingers competing for DNA binding with STAT proteins.²⁷

Because BCL6^{BTB} LG-targeting inhibitors do not affect BCL6 DNA-binding, its function in macrophages is preserved. Suppression of GC B-cells, especially in a transient manner, would likewise be non-toxic in humans. Therefore, BCL6^{BTB} LG inhibitors have favorable safety profiles and will likely be better tolerated than currently approved anti-cancer therapies.

Small molecule inhibitors of the BCL6^{BTB} are known (Figure 1). For instance, Takeda developed inhibitors using fragment-based screening, which disrupted BCL6 interaction with a BCOR peptide at micromolar range.^{28,29} Astra Zeneca developed macrocycles that could bind the BCL6^{BTB} LG with nanomolar affinity but had no effect on DLBCL proliferation.³⁰ Note that no target engagement studies were performed in either study so it is not known whether these compounds could actually block BCL6-mediated formation of repression complexes, or for how long this might occur. Recently, Boehringer developed two small molecules that bind to the BCL6^{BTB} LG with nanomolar activity.³¹ One of the compounds resulted in degradation of BCL6 in a DNA-binding dependent manner, while the non-degrading compounds had weak activity against BCL6 in cells.³¹ Our laboratories reported the discovery of inhibitors 79–6 and FX1.³² FX1 binds to the BCL6^{BTB} LG with improved potency (K_D 7 μ M) compared to 79–6 (K_D 129 μ M) and even SMRT (K_D 30 μ M) in the microscale thermophoresis (MST) assay.³² It maintains high specificity for BCL6^{BTB} over other BTB-containing proteins,³² and shows good specificity towards BCL6-dependent lymphoma cell lines over BCL6-independent cells.³² Results from *in vivo* studies show that FX1 is nontoxic at high concentrations and demonstrated excellent efficacy in both GCB- and ABC-type DLBCL xenograft models.³² Compounds 79–6 and FX1 exhibit promising biological profiles, yet these inhibitors also have potential limitations. First, they only bind to the top aromatic pocket and leave the rest of the LG unoccupied. We speculate that new inhibitors with extended structures that occupy a larger portion of the LG will have improved inhibitory activities. Moreover, the rhodanine-based scaffold of inhibitors 79–6 and FX1 can potentially be problematic for further development of as clinical candidates.^{33,34}

Herein we report the identification and optimization of a novel series of thiourea-based inhibitors of the BCL6^{BTB}. The scaffold was first identified by screening a fragment-like small molecule library using protein-observed NMR spectroscopy. New inhibitors were subsequently designed using computer-aided drug design (CADD) strategies. This study presents the design strategy, chemical synthesis and experimental characterization of this series of compounds.

RESULTS AND DISCUSSION

Identification and Characterization of Hit 7CC5.

To identify small molecules binding to the BCL6^{BTB}, we began by screening an in-house library of approximately 1500 chemically-diverse, fragment-like small molecules by protein-observed NMR spectroscopy. Recombinant, isotopically-labeled BCL6^{BTB} was used for two-dimensional ¹H-¹⁵N HSQC NMR experiments. Identification of binders was based on ¹H and ¹⁵N chemical shift perturbations on the ¹H-¹⁵N HSQC NMR spectra. Screening was initially performed with 10 compounds per sample at 500 μ M final concentration of each

compound, followed by deconvolution experiments on individual compounds to identify hits. Through this screen, we discovered compound 7CC5 (1-phenethyl-3-(pyridin-3-yl) thiourea) (Figure 2A). To determine the affinity of this compound we performed NMR titration experiments and estimated the K_D of 7CC5 to be 3.2 mM (Figure 2B). Despite their relatively weak binding affinities, fragment-like compounds with mM affinities are usually suitable candidates for further optimization.³⁵

To map the binding site of hit 7CC5 on BCL6^{BTB}, we analyzed chemical shift perturbations, and found that binding of 7CC5 results in several chemical shift perturbations for residues in the BCL6^{BTB} LG (Figure 2C). These chemical shift perturbations are consistent with those observed for FX1, suggesting the same binding site.¹ To accurately establish the binding mode of compound 7CC5, we determined a crystal structure of compound 7CC5 in complex with BCL6^{BTB} at 2.5 Å resolution (Figure 2D). The structure reveals that compound 7CC5 binds in a well-defined pocket in the BCL6^{BTB} LG. The sulfur atom of the thiourea group occupies a hydrophobic site comprised of Asn21, Leu25, Met51, Ala52, and Tyr58. One of the thioamide hydrogens forms a hydrogen bond (2.59 Å) with backbone carbonyl of Met51. Similar interactions have been observed with other BCL6^{BTB} inhibitors.^{17,28–32} The pyridine ring binds in a site above the α -helix with one ring carbon in contact distance with the CG and CD1 atoms of Tyr58.

New Inhibitor Design.

We used the site identification by ligand competitive saturation (SILCS) approach^{36,37} to model the binding orientations and relative affinities of new compounds. SILCS accounts for both protein flexibility and protein/functional group desolvation contributions, as well as protein-functional group interactions, by running Grand Canonical Monte Carlo/Molecular Dynamics (GCMC/MD) simulations of the protein in an aqueous solution of small molecules representing different functional groups.³⁸ Three-dimensional (3D) probability distributions of the functional groups, or FragMaps, are obtained from the simulations. These are normalized by the solute bulk probability distributions and converted into free energies based on a Boltzmann distribution, yielding grid free-energy (GFE) FragMaps for both qualitative and quantitative use.³⁸ The SILCS approach was successfully used in the rational design of FX1.³²

We designed new compounds based on the SILCS FragMaps of BCL6^{BTB} along with the orientation of the thiourea and pyridine groups of 7CC5 (Figure 3). Specifically, the 3-substituted pyridine was replaced by a 6-methyl-1*H*-indole. Using the indole nitrogen as a handle, a fragment was built to expand the interaction of inhibitors with FragMaps (e.g., apolar, H-bond donor and acceptor, and negative donor) present in the HDCH (H14-D17-C53-H116) site of BCL6^{BTB} (Figure 3). A piperazine substituent was added to the 3-position of the indole ring to interact with the positive FragMaps in the region above the HDCH site. In addition, the phenethyl group of 7CC5, which forms stacking interactions with symmetry-related molecules in the crystal (Figure S1, Table S1), was replaced by a second indole ring for several reasons. First, similar rings have been successfully used in inhibitors 79–6 and FX1. Second, indole moieties could increase the polarity of the NMR hits (estimated logP values of 2.0 or above). Finally, indole rings could improve permeability

of 7CC5. The 4DBA values (4-dimensional bioavailability, a scalar indicator of bioavailability associated with Lipinski's rule of 5³⁹) of NMR hits were -2.7 or less, indicating potential cell penetration issues.

Chemistry.

The synthesis of inhibitors **7a-i** is detailed in Scheme 1. Alkylation of the indole nitrogen of the commercially available 6-nitroindole (**1**) using methyl bromoacetate in the presence of K_2CO_3 generated compound **2** in good yields. Catalytic hydrogenation of the nitro group in compound **2** using Pd/C provided amino compound **3** in modest yields. The amino compound **3** was converted into the isothiocyanate **4** using 1,1'-thiocarbonylbis(pyridine-2(1*H*)-one) in high yields. The resulting isothiocyanate group of compound **4** reacted with tryptamine to give the thiourea compound **5** in modest yields. Saponification of methylester **5** using $LiOH \cdot H_2O$ generated carboxylic acid **6**, which was submitted to a 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU)-mediated amide bond formation reaction to give the final inhibitors **7a-i** in modest yields.

The synthesis of inhibitors **15a-h** started with 6-nitro-1*H*-indole-3-carbaldehyde **8** (Scheme 2). Alkylation of the indole nitrogen of compound **8** using methyl bromoacetate in the presence of K_2CO_3 generated compound **9** in good yields. Reductive amination of aldehyde **9** with 1-methylpiperazine using $NaBH(OAc)_3$ generated compound **10** in modest yields. Reduction of the nitro group of compound **10** using catalytic hydrogenation provided compound **11** in modest yields. The amino compound **11** was converted into the isothiocyanate **12** using 1,1'-thiocarbonylbis (pyridine-2(1*H*)-one). The isothiocyanate group of compound **12** reacted with tryptamine to give thiourea **13** in modest yields. Saponification of methylester **13** using $LiOH \cdot H_2O$ generated carboxylic acid **14**, which was submitted to an HATU-mediated amide bond formation reaction to give the final inhibitors **15a-h** in modest yields.

Biological Evaluation.

To initially characterize binding and rank potency of new inhibitors, we used NMR. Because of relatively low solubilities, we were unable to perform full titrations to determine K_D values for some compounds. Instead, to rank compounds, we measured the sum of chemical shift perturbations of six selected amide (Thr62, Thr48, Phe61, Asn23, Arg28, and Val18) resonances (6PA) observed on the 1H - ^{15}N HSQC spectra (Table 1, Table S2). The 6PA value of hit 7CC5 was measured to be 49 Hz. The LGFE and ligand efficiency (LE, which is the LGFE value divided by number of heavy atoms) values of 7CC5 were calculated to be -32.2 kcal/mol and -1.79 kcal/mol, respectively. The LE is a computational metric that indicates the atom-based free energy contribution to binding.⁴⁰ Compound 7CC5-14, a commercial analog of 7CC5, indicated an increased 6PA value of 116 Hz, while its LE value remained similar to that of the hit. Keeping R1 as an indole group and replacing the Ph group of 7CC5-14 with a second indole, we synthesized compound **7a** with a 6PA value of 88 Hz and a slightly less favorable LE value. The urea analog of 7CC5-14 showed no obvious chemical shift perturbations (Figure S2), highlighting the importance of the thiourea group for this class. Next, using the nitrogen of the R2 indole as a handle, we synthesized compounds

7b-7i with various groups targeting the HDCH site (Figure 3). Considering the distance between the aromatic and HDCH sites, we chose a methylene amide linker for the new compounds. These substituted indole analogs indicated less favorable LE values. When *i*-Pr amine was used, compound **7b** showed a 3.7-fold improved 6PA value compared to compound **7a**. Interestingly however, when structurally similar *c*-Pr amine was used, the corresponding inhibitor **7c** indicated a much smaller 6PA value. The *n*-Pr analog **7d** had a 6PA value of 237 Hz while the branched *i*-Bu analog **7e** indicated a 6PA value of 166 Hz. These results indicated that the branching effect at the C β of the amide bond might play an important role for more favorable binding affinity. This result was further confirmed by inhibitor **7f** employing a longer aliphatic tail. The decrease of the 6PA values from inhibitor **7d** to inhibitors **7e** and **7f** indicated that the large flexible groups might not interact efficiently with the HDCH site of the BCL6^{BTB}. We were pleased to see that the morpholine analog **7g** indicated a large 6PA value. When a Ph group is included as a tail, the resulting compound **7h** indicated a significantly decreased 6PA value. Since the SILCS results also showed a negative charge donor FragMaps in the HDCH site, we synthesized and tested compound **7i** that employed a carboxylic acid tail. Indeed, this compound had a large 6PA value of 391 Hz as well as the most favorable LGFE score of the tested compounds.

One common limitation of inhibitors **7a-i** was the relatively poor aqueous solubility (< 200 μ M in aqueous solution containing 1% DMSO). To address this we synthesized new compounds **13** and **15a-h** with a methyl piperazine fragment built off the C3 position of the right arm indole (Table 2). While the ionizable nitrogen atoms of the piperazine ring can help the aqueous solubility of inhibitors, the terminal tertiary amine group may also form additional interactions with residue Glu115, as previously shown in BCL6^{BTB} inhibitors.²⁸⁻³¹ The 6PA value of compound **13** was 363 Hz, and the LGFE and LE values of this compound were calculated to be -39.5 kcal/mol and -1.07 kcal/mol, respectively. Compared to inhibitor **13**, the *n*-Pr analog **15a** showed a decreased 6PA value (Table 2). Further extension of the hydrophobic tail of the inhibitor gave compound **15b** that had an even lower 6PA value. Inhibitor **15c**, with a free carboxylic acid group to occupy the negative charge donor FragMaps in the HDCH site, showed a 6PA value of 323 Hz. When secondary amines were used, inhibitors **15d-15h** demonstrated significant improvements in their 6PA values. Specifically, inhibitor **15f** showed the largest 6PA value of 473 Hz. Note that with the additional 1-methylpiperazine substitution, new inhibitors **13** and **15a-h** showed significantly improved solubility (>1000 μ M in aqueous solution containing 1% DMSO).

Increased solubility of compounds **13**, **15a-h** permitted full NMR titration experiments to determine binding affinities. K_D values were calculated by fitting chemical shift perturbations as a function of compound concentration using a previously established model (Table 2).⁴¹ The majority of these compounds bind to BCL6 with K_D values ranging from 153 to 405 μ M and the most potent affinity was observed for **15f** (K_D = 44 μ M, Figure 4A, B). The increased affinity of **15f** is consistent with the most extensive perturbations on the ¹H-¹⁵N HSQC spectra (6PA value of 473 Hz, Figure S3). We have further validated the binding of compound **15f** using isothermal titration calorimetry (ITC) and obtained very similar affinity, K_D = 36 $\pm$ 5 μ M (Figure 4C). Moreover, compound **15f** represents an improvement in affinity for BCL6^{BTB} by two orders of magnitude relative to hit 7CC5.

In order to establish the binding modes of optimized BCL6 inhibitors, we selected **15a** ($K_D = 219 \mu\text{M}$) and the most potent, **15f**, and determined high-resolution crystal structures of complexes with BCL6^{BTB} (Figure 4D, E). In both structures, **15a** and **15f** occupy a significant part of the LG and the positions of the thiourea group are identical to fragment hit 7CC5 (Figure 4F). In addition, the piperazine moiety is in close proximity to Glu115 and forms a favorable electrostatic interaction. The R groups in **15a** and **15f** bind in the HDCH site, composed of the backbones of Ala52 and Cys53, and side chains of His14, Asp17, Val18 and Asn21. Affinity of **15f** represents a 6-fold improvement over **15a** and, based on the crystal structures, the morpholine group in **15f** extends more and therefore better occupies the HDCH site when compared to isopropyl group in **15a** (Figure 4F).

We have subsequently plotted 6PA against K_D values and found a very good correlation (Table S2, Figure S4). This correlation demonstrates that for compounds binding with fast-to-intermediate-exchange kinetics, it is possible to rank their affinities based on quantification of chemical shift perturbations. Such approach might be particularly valuable in cases where K_D s cannot be determined from NMR titration experiments due to limited solubility of the ligands (as, for example, compounds in Table 1). However, the limitation of using 6PA values renders its applicability only to ranking compounds with similar scaffold.

We further characterized the inhibitory activity of the top inhibitors, **7i**, **13**, **15a**, **15c**, **15e**, **15f** and **15g** in biochemical and cellular assays (Table 3). We established an AlphaLisa assay to test the inhibitory potency of compounds to compete with the binding of BCOR peptide to BCL6^{BTB}. The differential cell killing assays were then performed for inhibitors using a panel of four BCL6-dependent lymphoma cells (OCI-Ly1, OCI-Ly7, SUDHL4, and SUDHL6) along with three BCL6-independent lymphoma cells (K422, Toledo, and Ly1B50). The IC₅₀ of inhibitor **7i** was measured to be 170 μM , while this compound showed no effect for all seven lymphoma cell lines at the concentration of 500 μM . With the additional methylpiperazine fragment, inhibitor **13** indicated an IC₅₀ value of 80 μM , although with weak cellular activity only for the SUDHL6 cells. *i*-Pr-amide analog **15a** showed an IC₅₀ value of 142 μM , while the carboxylic acid compound **15c** showed an IC₅₀ value of 112 μM . Both of these two inhibitors showed no effects in the cell killing assays. Although the NMR-based K_D value of inhibitor **15e** was determined to be over 400 μM , it showed an IC₅₀ value of 34 μM in the AlphaLisa assay. The GI₅₀ (concentration to cause 50% reduction of cancer cell proliferation) of compound **15e** for BCL6-dependent lymphoma cells varies between 33 to 103 μM , while the same inhibitor indicated no effect for all tested BCL6-independent lymphoma cells at the concentration of 500 μM . The morpholine analog **15f** showed the best inhibitory potency for both the NMR-based K_D and the AlphaLisa assay. However, inhibitor **15f** only indicated weak potency against the OCI-Ly7 cells but no obvious activity to other tested BCL6-dependent lymphoma cells. By introducing a methyl group to the morpholine fragment, inhibitor **15g** indicated decreased NMR- and AlphaLisa-based potency compared to compound **15f**. Compound **15g** only showed activity in the OCI-Ly7 cell line. In general, the IC₅₀ values of tested inhibitors indicated the same trend as that of the NMR K_D values (Table 2, Figure S5), except for compound **15e**. The current series showed relatively weak cellular activity. We reasoned that these thioureas may have limited cell permeability due to the size, number of rotatable

bonds, and multiple ionizable functional groups of the inhibitor. The slightly improved cellular activity of the piperidine or morpholine amide analogs (**15e-g**) might be due to their increased rigidity.

Computational Explanation of the Binding of Best Inhibitors.

We compared the crystallographic orientation of inhibitor **15f** with the SILCS-MC predicted binding orientation (Figure 5). The two orientations are mostly similar, indicating the quality of the SILCS prediction. However, there are substantial differences in the ethylindole group, with the crystallographic orientation orientated away from the aromatic pocket, potentially due to the interactions with the symmetry-related protein molecule (Figure S6). In contrast, the SILCS-MC predicted orientation has the indole moiety occupying the aromatic site. This binding orientation is similar to that previously observed in SMRT (Figure S7) and the indolene moieties of 79-6 and FX1.³² The SILCS-MC predicted orientation is also consistent with the improved binding from the phenyl ring in 7CC5 to the indole in 7CC5-14 (Table 1).

Next we analyzed crystal contacts of the inhibitor with symmetry related molecules (Figure S6). Both the indole and methylpiperazine moieties of **15f** indicated symmetry related interactions. Specifically, interactions with indole involve a number of nonpolar contacts while the methylpiperazine forms an ionic interaction with residue Glu81 of the partner protein. The symmetry related interactions of BCL6^{BTB} inhibitors are commonly present in published structures (Table S1), though the presence of these interactions were not discussed previously.^{30,31}

We also performed correlation analysis to verify the predictive capability of the SILCS method. Figure 6 shows the correlation plots of the AlphaLisa activity at 50 μ M or NMR chemical shift perturbation 6PA data with LGFE scores. Good correlations and predictive indices⁴² are seen for both sets of experimental data verifying the good prediction ability of SILCS protocol used in BCL6^{BTB} inhibitor development.

Given the predictive ability of the SILCS FragMaps, we further analyzed the contributions of different functional groups to binding affinities. Table 4 summarizes the IC₅₀s, LGFEs and GFE contributions of functional groups of inhibitors **15a**, **15c**, **15e**, **15f** and **15g**. All selected compounds show favorable LGFE contributions for all functional groups. For more potent compounds **15e**, **15f** and **15g**, the 6-membered aliphatic ring from the amide in the HDCH site makes a significant favorable contribution to binding. This is consistent with the combination of apolar and acceptor FragMaps in that region. Compared to compound **15f**, the additional methyl group of **15g** does not significantly change the LGFE. This result suggests that while the HDCH can accommodate aliphatic groups there are limits on the size and/or spatial arrangement of the groups. Consistent with this is the less favorable GFE energy of the HDCH-interacting region of **15g** versus that of **15f**. The piperidine analog **15e** shows the most favorable contribution from the HDCH-interacting moiety, indicating that there are also limits on polar atoms in that region. Interestingly, the favorable interaction of **15e** with the HDCH site matches its cell viability results, suggesting that interactions with this site may contribute to specific cell killing. Similar SAR was observed for the two weak binders **15a** and **15c**. The increased polarity of the HDCH-targeting groups led to less

favorable GFEs in that region and higher IC₅₀s. The loss of binding in this region for **15a** is relatively small, but the contributions of other functional groups also become less favorable, indicating a shift in the overall orientation of the ligand in the binding site. For compound **15c** the presence of the acid moiety, designed to bind to the HDCH site, led to a decrease in the favorable interactions. This indicates that the acid group interactions cannot fully replace the non-polar interactions in that region, or the possibility that additional optimization of the linker length is required to properly exploit placing an acid group in the HDCH site. The presence of the acid group also perturbed the central indole to a less favorable contribution, further decreasing binding. In combination, the experimental/SILCS analysis indicates a model where occupancy of the HDCH site by the appropriate functional group can lead to enhanced affinity, with the spatial relationship of the functional group in the HDCH site and the remainder of the inhibitor also being important for binding affinity.

CONCLUSION

In conclusion, we have identified and optimized a series of thiourea inhibitors of the BCL6^{BTB}. We used NMR-based fragment screening to identify the initial hit. We took the advantage of the novel CADD SILCS method to direct the optimization of the scaffold. Our efforts led to the identification of compounds **15e** and **15f**. Compound **15f** is one of the most potent BCL6 inhibitors in our series and represents two orders of magnitude improvement when compared to the original fragment hit 7CC5. We have also tested binding of **15f** to the three related BTB domain proteins: KAISO, MIZ1 and LRF and found no interactions (Figure S8), which suggests that the thiourea scaffold is selective for BCL6. We have not observed potent activity in cell based assays for this class of BCL6 inhibitors, likely due to insufficient permeability of the compounds. Nevertheless, availability of the crystal structure and validation of the key role of the HDCH pocket for inhibitor binding identify this scaffold as valuable core structure for further modifications to develop potent BCL6 inhibitors. Investigations of new inhibitors, by replacing the thiourea fragment in the current series with high affinity scaffolds, are ongoing in our laboratories.

EXPERIMENTAL SECTION

General Information

All reagents were purchased without further purification unless otherwise noted. Reactions were monitored using thin-layer chromatography (TLC) on commercial silica gel plates (GF254). Visualization of the developed plates was performed under UV light (254 nm). Flash column chromatography was performed on silica gel (200–300 mesh). ¹H and ¹³C NMR spectra were recorded on a Varian INOVA 400 MHz NMR spectrometer at 25 °C. Chemical shifts (δ) are reported in ppm referenced to an internal tetramethylsilane standard, or the DMSO-*d*₆ residual peak (δ 2.50) for ¹H NMR. Chemical shifts of ¹³C NMR are reported relative to CDCl₃ (δ 77.0) or DMSO-*d*₆ (δ 39.5). The following abbreviations were used to describe peak splitting patterns when appropriate: br s = broad singlet, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Coupling constants, *J*, were reported in Hertz unit (Hz). High-resolution mass spectra (HRMS) were obtained on a JEOL AccuTOF with ESI/APCI ion sources coupled to an Agilent 1100 HPLC system. HPLC analysis was

performed on a Agilent 1100 series (DAD detector) fitted with a C-18 reversed-phase column (XTerra RP 18, 5 μ M, 4.6 $\times$ 250mm) with a flow rate of 0.8 mL/min using CH₃CN-H₂O mobile phase and detected under 254 nm UV light. The purity of final products is >95%. Compounds 7CC5 and 7CC5-14 were purchased from Maybridge and Vitas, respectively.

New Compound Synthesis and Characterization

Methyl 2-(6-nitro-1*H*-indol-1-yl)acetate (2).—To a mixture of 6-nitroindole (1.62 g, 10.0 mmol) and K₂CO₃ (2.07 g, 15.0 mmol) in DMF (20 mL) was added ethyl bromoacetate (1.1 mL, 11.0 mmol) dropwise at room temperature. The reaction mixture was allowed to stir at 60 °C overnight and then poured onto ice water and extracted with ethyl acetate (3 $\times$ 20 mL). The combined organic layers were washed with brine, dried over Na₂SO₄, and concentrated under vacuum. The resulting crude product was purified by flash chromatography to yield the title compound as a yellow solid (2.01 g, 86%). ¹H NMR (CDCl₃): δ 8.23 (s, 1 H), 8.04 (d, *J* = 9.2 Hz, 1 H), 7.68 (d, *J* = 8.8 Hz, 1 H), 7.38 (d, *J* = 3.2 Hz, 1 H), 6.67 (d, *J* = 2.4 Hz, 1 H), 4.95 (s, 2 H), 3.79 (s, 3 H) ppm; ¹³C NMR (CDCl₃): δ 168.2, 143.4, 135.2, 134.2, 133.5, 121.1, 115.5, 106.1, 103.6, 52.9, 47.7 ppm.

Methyl 2-(6-amino-1*H*-indol-1-yl)acetate (3).—To a round bottom flask containing methyl 2-(6-nitro-1*H*-indol-1-yl)acetate (2) (468 mg, 2.0 mmol) was added 10% palladium on active carbon (30 mg), MeOH (10 mL), and THF (10 mL). The reaction mixture was evacuated and flushed with H₂ for three times. The reaction mixture was allowed to stir vigorously under the atmosphere of H₂ overnight. The catalyst was removed by filtration through a pad of Celite and washed with MeOH. The filtrate was concentrated under vacuum. The resulting crude product was purified by flash chromatography to give the title compound (376 mg, 52%). ¹H-NMR (CDCl₃): δ 7.41 (d, *J* = 8.4 Hz, 1H), 6.89 (d, *J* = 3.2 Hz, 1H), 6.58 (d, *J* = 2.0 Hz, 1H), 6.52 (s, 1H), 6.45 (d, *J* = 2.8 Hz, 1H), 4.72 (s, 2H), 3.74 (s, 3H), 3.71 (s, 2H) ppm.

Methyl 2-(6-isothiocyanato-1*H*-indol-1-yl)acetate (4).—To a solution of 1,1'-thiocarbonyldipyridin-2(1*H*)-one (116 mg, 0.5 mmol) in dichloromethane (2 mL) at room temperature was added methyl 2-(6-amino-1*H*-indol-1-yl)acetate (3) (102 mg, 0.5 mmol). The reaction mixture was allowed to stir at room temperature for an additional 2 h and concentrated. The crude product was purified by flash chromatography to give the title compound (183 mg, 90%). ¹H-NMR (CDCl₃): δ 7.56 (d, *J* = 8.0 Hz, 1H), 7.14 (d, *J* = 3.2 Hz, 1H), 7.12 (s, 1H), 7.02 (d, *J* = 8.0 Hz, 1H), 6.56 (d, *J* = 2.4 Hz, 1H), 4.80 (s, 2H), 3.77 (s, 3H) ppm; ¹³C NMR (CDCl₃): δ 168.5, 136.1, 133.3, 130.5, 127.8, 125.1, 122.0, 118.3, 106.6, 103.1, 52.7, 47.7 ppm.

Methyl 2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)acetate (5).—To a solution of methyl 2-(6-isothiocyanato-1*H*-indol-1-yl)acetate (4, 123 mg, 0.5 mmol) in dichloromethane (2 mL) at room temperature was added tryptamine (80 mg, 0.5 mmol). The reaction mixture was allowed to stir at room temperature for 3 h and concentrated. The crude product was purified by flash chromatography to give the title compound (154 mg, 76%). ¹H-NMR (CDCl₃): δ 8.08 (s, 1H), 7.84 (s, 1H), 7.55 (d, *J* = 8.0 Hz, 1H), 7.47 (d, *J* = 7.6 Hz,

1H), 7.30–7.26 (m, 1H), 7.17–7.12 (m, 2H), 7.06–7.03 (m, 2H), 6.87 (s, 1 H), 6.75–6.71 (m, 2H), 6.55 (d, $J = 3.2$ Hz, 1H), 6.04 (s, 1H), 4.65 (s, 2 H), 3.89 (t, $J = 5.2$ Hz, 2H), 3.76 (s, 3H), 3.01 (t, $J = 5.2$ Hz, 2H) ppm; ^{13}C NMR (CDCl_3): δ 180.6, 169.0, 136.6, 136.2, 130.1, 127.9, 127.0, 122.4, 122.3, 122.0, 119.4, 118.7, 118.5, 112.3, 111.2, 107.1, 102.8, 52.8, 47.4, 45.2, 24.4 ppm; HRMS (ESI) m/z : calculated 407.1536 for $[\text{C}_{22}\text{H}_{22}\text{N}_4\text{O}_2\text{S}]^+$, found 407.1545.

2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)acetic acid (6).—To a solution of methyl 2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)acetate (**5**, 406 mg, 1.0 mmol) in MeOH:THF:H₂O (2 mL, 3:1:1) at room temperature was added LiOH·H₂O (63 mg, 1.5 mmol). The reaction mixture was allowed to stir at 70 °C for 3 h and then cooled. The reaction mixture was concentrated and neutralized using aqueous HCl (1 N). The resulting crude product was purified by flash chromatography to give the title compound (322 mg, 82%). ^1H -NMR (CD_3OD): δ 7.60 (d, $J = 8.0$ Hz, 1H), 7.45 (d, $J = 8.8$ Hz, 1H), 7.31 (d, $J = 8.0$ Hz, 1H), 7.19 (s, 1H), 7.09–7.04 (m, 2H), 6.98–6.95 (m, 2 H), 6.71 (d, $J = 8.0$ Hz, 1H), 6.46 (s, 1H), 4.81 (s, 2 H), 3.81 (t, $J = 6.4$ Hz, 2H), 3.01 (t, $J = 6.4$ Hz, 2H) ppm; ^{13}C NMR (CD_3OD): δ 176.8, 167.5, 133.3, 133.2, 127.3, 126.5, 124.1, 123.9, 118.8, 117.7, 117.5, 114.8, 114.7, 113.9, 108.2, 107.4, 103.1, 98.0, 43.2, 41.8, 20.8 ppm.

1-(2-(1*H*-indol-3-yl)ethyl)-3-(1*H*-indol-6-yl)thiourea (7a).—To a solution of 1,1'-thiocarbonyldipyridin-2(1*H*)-one (116 mg, 0.5 mmol) in dichloromethane (2 mL) was added tryptamine (80 mg, 0.5 mmol) at room temperature. The reaction mixture was allowed to stir at room temperature for 2 h and then added 1*H*-indol-6-amine (61 mg, 0.5 mmol) for an additional 10 h. The reaction mixture was concentrated and the crude product was purified by flash chromatography to give the title compound (70 mg, 42%). ^1H -NMR ($\text{DMSO}-d_6$): δ 11.09 (s, 1H), 10.83 (s, 1H), 9.50 (s, 1H), 7.66 (d, $J = 7.6$ Hz, 1H), 7.56 (s, 1H), 7.47 (d, $J = 7.6$ Hz, 1H), 7.46 (s, 1H), 7.36–7.34 (m, 2H), 7.16 (s, 1H), 7.07 (t, $J = 7.6$ Hz, 1H), 7.00–6.96 (m, 1H), 6.81 (d, $J = 8.8$ Hz, 1H), 6.41 (s, 1H), 3.77–3.75 (m, 2H), 2.97 (t, $J = 7.2$ Hz, 2H) ppm; ^{13}C -NMR ($\text{DMSO}-d_6$): δ 180.6, 136.7, 136.3, 132.5, 127.7, 126.2, 126.1, 125.8, 123.2, 121.4, 120.5, 119.0, 118.7, 117.0, 112.1, 111.8, 107.9, 101.4, 45.2, 25.3 ppm; HRMS (ESI) m/z : calculated 335.1325 for $[\text{C}_{19}\text{H}_{18}\text{N}_4\text{S}]^+$, found 335.1347; HPLC analysis: retention time = 4.24 min, peak area 99.2%, 80:20 CH₃CN-H₂O.

2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)-*N*-isopropylacetamide (7b).—To a mixture of 2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)acetic acid (**6**, 196 mg, 0.5 mmol), HATU (228 mg, 0.6 mmol), and *N,N*-diisopropylethylamine (130 μL , 0.7 mmol) in DMF (2 mL) was added isopropylamine (52 μL , 0.6 mmol). The reaction mixture was allowed to stir at room temperature overnight. The reaction mixture was poured onto water (10 mL) and extracted with ethyl acetate (3×10 mL). The combined organic layers were washed by brine, dried over Na₂SO₄, and concentrated. The resulting crude product was purified by flash chromatography to yield the title compound (145 mg, 67%). ^1H -NMR ($\text{DMSO}-d_6$): δ 10.82 (s, 1H), 9.55 (s, 1H), 8.14 (d, $J = 6.8$ Hz, 1H), 7.67 (d, $J = 8.0$ Hz, 1H), 7.50–7.48 (m, 2H), 7.38–7.32 (m, 3H), 7.14 (s, 1H), 7.08 (t, $J = 7.6$ Hz, 1H), 7.00–6.96 (m, 1H), 6.86 (d, $J = 8.0$ Hz, 1H), 6.43 (s, 1H), 4.73 (s, 2H), 3.88–3.83 (m, 1H), 3.77–3.75 (m, 2H), 2.97 (t, $J = 7.2$ Hz, 2H), 1.09 (d, $J = 6.8$ Hz, 6H) ppm; ^{13}C -NMR

(DMSO- d_6): δ 180.8, 166.7, 136.7, 132.6, 130.9, 127.7, 126.3, 123.1, 121.4, 120.9, 119.0, 118.6, 117.7, 112.1, 111.8, 106.9, 101.1, 49.1, 45.3, 41.2, 25.3, 22.8 (2C) ppm; HRMS (ESI) m/z : calculated 434.2009 for $[C_{24}H_{27}N_5OS]^+$, found 434.2029; HPLC analysis: retention time = 4.12 min, peak area 97.7%, 80:20 CH_3CN-H_2O .

Compounds **7c-h** were synthesized with procedures similar to that of compound **7b**.

2-(6-(3-(2-(1*H*-Indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)-*N*-

cyclopropylacetamide (7c).—Yield, 76%; 1H -NMR (DMSO- d_6): δ 10.81 (s, 1H), 9.53 (s, 1H), 8.33 (d, J = 3.2 Hz, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.48–7.46 (m, 2H), 7.33–7.30 (m, 3H), 7.13 (s, 1H), 7.08–7.04 (m, 1H), 6.98–6.95 (m, 1H), 6.85 (d, J = 7.2 Hz, 1H), 6.41 (s, 1H), 4.70 (s, 2H), 3.75–3.73 (m, 2H), 2.97–2.94 (m, 2H), 2.65–2.62 (m, 1H), 0.62–0.60 (m, 2H), 0.47–0.45 (m, 2H) ppm; ^{13}C -NMR (DMSO- d_6): δ 180.7, 168.8, 136.7, 132.6, 130.9, 127.7, 126.3, 123.1, 121.4, 120.9, 119.0, 118.6, 117.7, 112.1, 111.8, 106.7, 101.1, 44.9, 45.3, 25.2, 22.8, 6.1 (2C) ppm; HRMS (ESI) m/z : calculated 432.1853 for $[C_{24}H_{25}N_5OS]^+$, found 432.1855; HPLC analysis: retention time = 4.01 min, peak area 99.0%, 80:20 CH_3CN-H_2O .

2-(6-(3-(2-(1*H*-Indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)-*N*-propylacetamide

(7d).—Yield, 80%; 1H -NMR (DMSO- d_6): δ 10.82 (s, 1H), 9.55 (s, 1H), 8.15 (s, 1H), 7.66 (d, J = 7.6 Hz, 1H), 7.49–7.48 (m, 2H), 7.36–7.33 (m, 3H), 7.14 (s, 1H), 7.07 (t, J = 8.0 Hz, 1H), 6.99–6.95 (m, 1H), 6.86 (d, J = 8.8 Hz, 1H), 6.43 (s, 1H), 4.77 (s, 2H), 3.75–3.74 (m, 2H), 3.05 (q, J = 6.0 Hz, 2H), 2.96 (t, J = 7.2 Hz, 1H), 1.43 (q, J = 7.2 Hz, 2H), 0.84 (t, J = 7.2 Hz, 3H) ppm; ^{13}C -NMR (DMSO- d_6): δ 180.8, 166.6, 136.7, 132.6, 130.9, 127.7, 126.4, 123.1, 121.4, 120.9, 119.0, 118.6, 117.7, 112.1, 111.8, 106.8, 101.2, 49.1, 45.3, 40.9, 25.2, 22.7, 11.8 ppm; HRMS (ESI) m/z : calculated 434.2009 for $[C_{24}H_{27}N_5OS]^+$, found 434.2017; HPLC analysis: retention time = 4.13 min, peak area 95.7%, 80:20 CH_3CN-H_2O .

2-(6-(3-(2-(1*H*-Indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)-*N*-isobutylacetamide

(7e).—Yield, 82%; 1H -NMR (DMSO- d_6): δ 10.82 (s, 1H), 9.55 (s, 1H), 8.18–8.15 (m, 1H), 7.66 (d, J = 7.6 Hz, 1H), 7.50–7.48 (m, 2H), 7.38–7.34 (m, 3H), 7.14 (s, 1H), 7.09–7.06 (m, 1H), 7.00–6.96 (m, 1H), 6.87 (d, J = 8.8 Hz, 1H), 6.43 (s, 1H), 4.80 (s, 2H), 3.76–3.75 (m, 2H), 2.98–2.92 (m, 4H), 1.72–1.69 (m, 1H), 0.84 (d, J = 6.4 Hz, 6H) ppm; ^{13}C -NMR (DMSO- d_6): δ 180.8, 166.7, 136.7, 132.6, 130.9, 127.7, 126.4, 123.1, 121.4, 120.9, 119.0, 118.6, 117.7, 112.1, 111.8, 106.9, 101.1, 49.1, 46.6, 45.3, 28.5, 25.3, 20.6 (2C) ppm; HRMS (ESI) m/z : calculated 448.2166 for $[C_{25}H_{29}N_5OS]^+$, found 448.2172; HPLC analysis: retention time = 4.24 min, peak area 98.9%, 80:20 CH_3CN-H_2O .

(±) 2-(6-(3-(2-(1*H*-Indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)-*N*-(2-methylbutyl)acetamide (7f).—Yield, 51%; 1H -NMR ($CDCl_3$): δ 8.83 (s, 1H), 8.00 (s, 1H), 7.56–7.50 (m, 2H), 7.39 (d, J = 8.0 Hz, 1H), 7.16 (t, J = 8.0 Hz, 1H), 7.06–7.03 (m, 2H), 6.93 (s, 1H), 6.78 (d, J = 6.8 Hz, 1H), 6.55 (d, J = 3.2 Hz, 1H), 6.21 (s, 1H), 5.50 (s, 1H), 4.48 (s, 2H), 3.90 (s, 2H), 3.15–3.09 (m, 2H), 3.04–2.97 (m, 1H), 1.41–1.38 (m, 1H), 1.27–1.24 (m, 1H), 1.21–1.19 (m, 1H), 1.02–0.99 (m, 1H), 0.85–0.79 (m, 3H), 0.71 (d, J = 7.2 Hz, 3H) ppm; ^{13}C -NMR (DMSO- d_6): δ 180.8, 166.7, 136.7, 132.6, 130.9, 127.7, 126.3, 123.1, 121.4, 120.9, 119.0, 118.6, 117.7, 112.1, 111.8, 106.8, 101.1, 49.1, 45.3, 44.8, 34.8,

26.9, 25.3, 22.8, 11.6 ppm; HRMS (ESI) m/z : calculated 462.2322 for $[C_{26}H_{31}N_5OS]^+$, found 462.2322; HPLC analysis: retention time = 4.42 min, peak area 96.8%, 80:20 CH_3CN-H_2O .

1-(2-(1*H*-Indol-3-yl)ethyl)-3-(1-(2-morpholino-2-oxoethyl)-1*H*-indol-6-yl)thiourea (7g).—Yield, 86%; 1H -NMR ($DMSO-d_6$): δ 10.81 (s, 1H), 9.51 (s, 1H), 7.64 (d, J = 8.0 Hz, 1H), 7.47 (d, J = 8.4 Hz, 1H), 7.45 (s, 1H), 7.34–7.32 (m, 2H), 7.27 (s, 1H), 7.13 (s, 1H), 7.08–7.04 (m, 1H), 6.98–6.95 (m, 1H), 6.86 (d, J = 8.8 Hz, 1H), 6.42 (s, 1H), 5.13 (s, 2H), 3.73 (s, 2H), 3.65 (s, 2H), 3.57 (s, 4H), 3.43 (s, 2H), 2.96–2.93 (m, 2H) ppm; ^{13}C -NMR ($DMSO-d_6$): δ 176.5, 162.4, 132.9, 132.5, 128.2, 126.7, 123.5, 122.1, 118.9, 118.7, 117.2, 116.6, 114.8, 114.4, 113.4, 107.9, 107.5, 102.9, 96.9, 62.2 (2C), 43.1, 41.0 (2C), 38.1, 21.0 ppm; HRMS (ESI) m/z : calculated 462.1958 for $[C_{25}H_{27}N_5O_2S]^+$, found 462.1967; HPLC analysis: retention time = 3.89 min, peak area 97.4%, 80:20 CH_3CN-H_2O .

2-(6-(3-(2-(1*H*-Indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)-*N*-phenylacetamide (7h).—Yield, 82%; 1H -NMR ($CDCl_3$): δ 8.56 (s, 1H), 8.35 (s, 1H), 7.96 (s, 1H), 7.48 (d, J = 8.0 Hz, 1H), 7.42 (d, J = 8.0 Hz, 2H), 7.26–7.20 (m, 2H), 7.09–7.08 (m, 2H), 6.98 (d, J = 7.2 Hz, 1H), 6.84 (s, 1H), 6.68 (d, J = 8.8 Hz, 1H), 6.63 (s, 1H), 6.51 (s, 1H), 6.12 (s, 1H), 4.56 (s, 2H), 3.81 (d, J = 4.4 Hz, 2H), 2.94–2.91 (m, 2H) ppm; ^{13}C -NMR ($CDCl_3$): δ 180.4, 166.4, 137.0, 136.5, 136.3, 130.4, 130.0, 129.0 (2C), 127.7, 127.0, 125.0, 122.5, 121.9, 120.3 (2C), 119.2, 118.5, 118.4, 112.0, 111.4, 106.8, 103.1, 49.5, 45.1, 24.3 ppm; HRMS (ESI) m/z : calculated 468.1853 for $[C_{27}H_{25}N_5OS]^+$, found 468.1855; HPLC analysis: retention time = 4.43 min, peak area 96.5%, 80:20 CH_3CN-H_2O .

(2-(6-(3-(2-(1*H*-Indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)acetyl)glycine (7i).—To a mixture of 2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)acetic acid (**6**, 196 mg, 0.5 mmol), HATU (228 mg, 0.6 mmol), and *N,N*-diisopropylethylamine (260 μ L, 1.4 mmol) in DMF (2 mL) was added glycine ethyl ester hydrochloride (84 mg, 0.6 mmol). The reaction mixture was allowed to stir at room temperature overnight. The reaction mixture was poured onto water (10 mL) and extracted with ethyl acetate (3×10 mL). The combined organic layers were washed using brine, dried over Na_2SO_4 , and concentrated. The resulting crude product was purified by flash chromatography to yield compound ethyl(2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)acetyl)glycinate (148 mg, 62%). Saponification of ethyl (2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-1*H*-indol-1-yl)acetyl)glycinate gave the title compound (78%). 1H -NMR ($DMSO-d_6$): δ 12.63 (br s, 1H), 10.81 (s, 1H), 9.55 (s, 1H), 8.46 (s, 1H), 7.66 (d, J = 7.2 Hz, 1H), 7.49 (d, J = 8.4 Hz, 1H), 7.35–7.32 (m, 2H), 7.14 (s, 1H), 7.08–7.04 (m, 1H), 6.99–6.95 (m, 1H), 6.87 (d, J = 8.0 Hz, 1H), 6.44 (s, 1H), 4.86 (s, 2H), 3.81 (d, J = 4.4 Hz, 1H), 3.73 (s, 2H), 2.97–2.93 (m, 2H) ppm; ^{13}C -NMR ($DMSO-d_6$): δ 180.6, 171.5, 168.3, 136.7, 132.5, 130.8, 127.7, 126.4, 123.1, 121.4, 121.0, 119.0, 118.6, 117.8, 112.1, 111.8, 106.9, 101.4, 48.8, 45.3, 41.2, 40.5, 39.3, 25.2 ppm; HRMS (ESI) m/z : calculated 450.1595 for $[C_{23}H_{23}N_5O_3S]^+$, found 450.1589; HPLC analysis: retention time = 3.28 min, peak area 95.4%, 80:20 CH_3CN-H_2O .

Methyl 2-(3-formyl-6-nitro-1*H*-indol-1-yl)acetate (9).—To a mixture of 6-nitro-1*H*-indole-3-carbaldehyde (**8**, 571 mg, 3.0 mmol) and K_2CO_3 (484 mg, 3.5 mmol) in DMF (5

mL) was added ethyl bromoacetate (312 μ L, 3.3 mmol) dropwise at room temperature. The reaction mixture was allowed to stir at 70 °C overnight and then poured onto ice water (20 mL) and extracted with ethyl acetate (3 $\times$ 20 mL). The combined organic layers were washed with brine, dried over Na₂SO₄, and concentrated. The resulting crude product was purified by flash chromatography to yield the title compound as a yellow solid (621 mg, 79%). ¹H-NMR (CDCl₃): δ 10.09 (s, 1H), 8.44 (d, J = 8.4 Hz, 1H), 8.26–8.22 (m, 2H), 8.00 (s, 1 H), 5.03 (s, 2 H), 3.85 (s, 3H) ppm.

Methyl 2-(3-((4-methylpiperazin-1-yl)methyl)-6-nitro-1*H*-indol-1-yl)acetate (10).

—To a mixture of methyl 2-(3-formyl-6-nitro-1*H*-indol-1-yl)acetate (**9**, 134 mg, 0.5 mmol) and NaBH(OAc)₃ (159 mg, 0.75 mmol) in dichloromethane (2 mL) was added methylpiperazine (312 μ L, 3.3 mmol) dropwise at room temperature. The reaction mixture was stirred vigorously overnight. The reaction was quenched using saturated NH₄Cl and extracted using dichloromethane (3 $\times$ 20 mL). The combined organic layers were washed with brine, dried over Na₂SO₄, and concentrated. The resulting crude product was purified by flash chromatography to yield the title compound (101 mg, 58%). ¹H-NMR (CDCl₃): δ 8.18 (d, J = 1.6 Hz, 1H), 8.01 (dd, J = 8.8, 1.6 Hz, 1H), 7.77 (d, J = 8.8 Hz, 1H), 7.31 (s, 1 H), 4.90 (s, 2 H), 3.77 (s, 3H), 3.73 (s, 2H), 2.62 (br s, 8H), 2.37 (s, 3H) ppm; ¹³C NMR (CDCl₃) δ 168.2, 143.5, 135.5, 133.7, 133.0, 120.0, 115.3, 113.0, 106.1, 54.1 (2C), 52.9, 52.7, 51.6 (2C), 47.5, 44.7 ppm.

Methyl 2-(6-amino-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-1-yl)acetate (11).

—To a round bottom flask containing methyl 2-(3-((4-methylpiperazin-1-yl)methyl)-6-nitro-1*H*-indol-1-yl)acetate (**10**) (173 mg, 0.5 mmol), 10% palladium on active carbon (30 mg), MeOH (5 ml) was evacuated and flushed using H₂ for three times. The reaction mixture was evacuated and flushed with H₂ for three times. The reaction mixture was allowed to stir vigorously under the atmosphere of H₂ overnight. The catalyst was removed by filtration through a pad of Celite and washed with MeOH. The filtrate was concentrated under vacuum. The resulting crude product was purified by flash chromatography to give the title compound (114 mg, 72%). ¹H-NMR (CD₃OD): δ 7.41 (d, J = 8.0 Hz, 1H), 6.95 (s, 1 H), 6.62 (d, J = 8.8 Hz, 1H), 6.60 (d, J = 1.2 Hz, 1H), 4.83 (s, 2 H), 3.77 (s, 2H), 3.71 (s, 3H), 2.66 (br s, 8H), 2.37 (s, 3H) ppm.

Methyl 2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-1-yl)acetate (13).

—To a solution of 1,1'-thiocarbonyldipyridin-2(1*H*)-one (2.1 g, 8.9 mmol) in dichloromethane (30 mL) at room temperature was added methyl 2-(6-amino-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-1-yl)acetate (**11**, 2.8 g, 8.9 mmol). The reaction mixture was allowed to stir at room temperature for 4 h and then was added tryptamine (1.4 g, 8.9 mmol). The reaction was stirred for an additional 16 h at room temperature. The reaction solution was concentrated under vacuum. The resulting crude product was purified by flash chromatography to give the title compound (1.5 g, 33%). ¹H-NMR (CDCl₃): δ 9.24 (s, 1H), 7.75 (s, 1H), 7.54 (d, J = 7.6 Hz, 1H), 7.50 (d, J = 7.2 Hz, 1H), 7.29 (d, J = 8.0 Hz, 1H), 7.15 (dd, J = 8.0, 6.8 Hz, 1H), 7.10 (s, 1 H), 7.05 (dd, J = 8.0, 7.2 Hz, 1H), 6.88 (s, 3H), 6.66 (d, J = 8.4 Hz, 1H), 5.89 (s, 1H), 4.67 (s, 2H), 3.88–3.84 (m, 2H), 3.81 (s, 2H), 3.76 (s, 3H), 2.96 (t, J = 6.4 Hz, 2H),

2.74 (br s, 8H), 2.37 (s, 3H) ppm; ^{13}C NMR (CDCl_3): δ 180.9, 168.7, 137.0, 136.5, 130.4, 130.2, 127.8, 126.8, 122.5, 121.9, 121.0, 119.2, 118.8, 118.7, 111.6, 111.2, 110.7, 107.6, 54.0 (2C), 52.8, 52.4, 51.6 (2C), 47.4, 45.0, 44.6, 24.4 ppm; HRMS (ESI) m/z : calculated 519.2537 for $[\text{C}_{28}\text{H}_{34}\text{N}_6\text{O}_2\text{S}]^+$, found 519.2544; HPLC analysis: retention time = 5.47 min, peak area 98.0%, 65:35 $\text{CH}_3\text{CN}-\text{H}_2\text{O}$.

2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-1-yl)acetic acid (14).—To a solution (2 mL, $\text{MeOH}:\text{THF}:\text{H}_2\text{O} = 3:1:1$) of methyl 2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-1-yl)acetate (**13**, 130 mg, 0.25 mmol) at room temperature was added $\text{LiOH}\cdot\text{H}_2\text{O}$ (21 mg, 0.5 mmol). The reaction mixture was stirred at room temperature for 3 h. The reaction solution was concentrated and neutralized with aqueous HCl (1 N). The resulting crude product was purified by flash chromatography to give the title compound (101 mg, 80%). ^1H -NMR ($\text{DMSO}-d_6$): δ 10.87 (s, 1H), 9.68 (s, 1H), 7.70 (s, 1H), 7.64 (d, $J = 8.0$ Hz, 1H), 7.55 (d, $J = 8.0$ Hz, 1H), 7.33 (d, $J = 8.8$ Hz, 1H), 7.28 (s, 1H), 7.24 (s, 1H), 7.14 (s, 1H), 7.06 (t, $J = 7.2$ Hz, 1H), 6.98–6.91 (m, 2H), 4.75 (s, 2H), 3.73 (s, 2H), 3.69 (s, 2H), 2.95 (t, $J = 7.2$ Hz, 2H), 2.54 (br s, 8H), 2.27 (s, 3H) ppm.

2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-1-yl)-*N*-isopropylacetamide (15a).—To a mixture of 2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-1-yl)acetic acid (**14**, 100 mg, 0.2 mmol), HATU (76 mg, 0.2 mmol) and *N,N*-diisopropylethylamine (87 μL , 0.5 mmol) in DMF (1 mL) was added isopropylamine (20 mg, 0.3 mmol). The reaction mixture was stirred at room temperature overnight and poured into water and extracted with ethyl acetate (3×10 mL). The combined organic layers were washed using brine, dried over Na_2SO_4 , and concentrated. The resulting crude product was purified by flash chromatography to give the title compound (52 mg, 48%). ^1H -NMR ($\text{DMSO}-d_6$): δ 10.86 (s, 1H), 9.63 (s, 1H), 8.19 (d, $J = 8.8$ Hz, 1H), 7.66–7.64 (m, 2H), 7.59 (d, $J = 8.8$ Hz, 1H), 7.37 (s, 1H), 7.34 (d, $J = 7.6$ Hz, 1H), 7.28 (s, 1H), 7.15 (s, 1H), 7.06 (t, $J = 7.2$ Hz, 1H), 6.97 (t, $J = 7.2$ Hz, 1H), 6.90 (d, $J = 8.8$ Hz, 1H), 4.70 (s, 1H), 3.87–3.76 (m, 3H), 2.96 (t, $J = 7.2$ Hz, 2H), 2.75 (br s, 8H), 2.43 (s, 3H), 1.91 (s, 1H), 1.08 (d, $J = 7.2$ Hz, 6H) ppm; ^{13}C -NMR ($\text{DMSO}-d_6$): δ 180.9, 166.6, 137.0, 136.7, 133.3, 130.9, 127.7, 126.0, 123.1, 121.4, 119.7, 119.0, 118.6, 117.5, 112.1, 111.8, 106.6, 53.6, 52.4, 50.9, 49.0, 45.2, 44.3, 41.5, 41.0, 25.2, 22.8 (2C) ppm; HRMS (ESI) m/z : calculated 546.3010 for $[\text{C}_{30}\text{H}_{39}\text{N}_7\text{OS}]^+$, found 546.3016; HPLC analysis: retention time = 12.68 min, peak area 98.8%, 50:50 $\text{CH}_3\text{CN}-\text{H}_2\text{O}$.

Compounds **15b-h** were synthesized using a similar procedure to that of compound **15a**.

Ethyl 2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-1-yl)acetyl)glycinate (15b).—Yield, 38%; ^1H -NMR ($\text{DMSO}-d_6$): δ 10.82 (s, 1H), 9.56 (s, 1H), 8.56–8.53 (m, 1H), 7.65 (d, $J = 7.6$ Hz, 1H), 7.56 (d, $J = 8.4$ Hz, 1H), 7.54 (s, 1H), 7.33 (d, $J = 7.6$ Hz, 1H), 7.27 (s, 1H), 7.22 (s, 1H), 7.13 (s, 1H), 7.06 (t, $J = 7.6$ Hz, 1H), 6.96 (t, $J = 8.0$ Hz, 1H), 6.87 (d, $J = 7.6$ Hz, 1H), 4.81 (s, 2H), 4.06 (q, $J = 7.6$ Hz, 2H), 3.86 (d, $J = 5.6$ Hz, 2H), 3.73 (s, 2H), 3.60 (s, 2H), 2.95 (t, $J = 7.2$ Hz, 2H), 2.48–2.35 (m, 8H), 2.18 (s, 3H), 1.14 (t, $J = 7.2$ Hz, 3H) ppm; ^{13}C -NMR ($\text{DMSO}-d_6$): δ

180.7, 170.0, 168.5, 137.1, 136.7, 132.8, 130.0, 127.7, 126.3, 123.1, 121.4, 120.0, 119.0, 118.6, 117.5, 112.1, 111.8, 111.0, 106.7, 61.0, 54.9, 53.2, 52.5, 48.7, 45.8, 45.3, 41.3, 25.2, 14.5 ppm; HRMS (ESI) m/z : calculated 590.2908 for $[C_{31}H_{39}N_7O_3S]^+$, found 590.2901; HPLC analysis: retention time = 5.99 min, peak area 97.3%, 50:50 CH_3CN-H_2O .

(2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-1-yl)acetyl)glycine (15c).—Yield, 65%; 1H -NMR (CD_3OD): δ 7.61 (d, J = 8.4 Hz, 1H), 7.53 (d, J = 8.0 Hz, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.15 (s, 1H), 7.08–7.04 (m, 2H), 6.96–6.94 (m, 2H), 6.78 (d, J = 8.4 Hz, 1H), 4.68 (s, 2H), 3.80 (s, 2H), 3.75 (s, 2H), 3.67 (s, 2H), 3.01 (t, J = 6.8 Hz, 2H), 2.51 (br s, 8H), 2.25 (s, 3H) ppm; ^{13}C -NMR (CD_3OD): δ 180.2, 174.7, 168.7, 137.0, 136.6, 131.6, 130.2, 127.5, 127.1, 122.4, 121.0, 119.9, 118.3, 117.4, 111.8, 111.0, 110.1, 106.5, 53.8 (2C), 51.9, 51.3 (2C), 48.4, 45.3, 44.1, 43.2, 24.3 ppm; HRMS (ESI) m/z : calculated 562.2595 for $[C_{29}H_{35}N_7O_3S]^+$, found 562.2611; HPLC analysis: retention time = 2.79 min, peak area 96.1%, 65:35 CH_3CN-H_2O .

2-(6-(3-(2-(1*H*-indol-3-yl)ethyl)thioureido)-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-1-yl)-*N,N*-diethylacetamide (15d).—Yield, 51%; 1H -NMR ($DMSO-d_6$): δ 10.83 (s, 1H), 9.57 (s, 1H), 7.64 (d, J = 8.0 Hz, 1H), 7.54 (d, J = 8.8 Hz, 1H), 7.33 (d, J = 8.4 Hz, 1H), 7.27 (s, 1H), 7.18 (s, 1H), 7.13 (s, 1H), 7.06 (t, J = 8.0 Hz, 1H), 6.96 (t, J = 8.0 Hz, 1H), 6.86 (d, J = 8.0 Hz, 1H), 5.02 (s, 2H), 3.74–3.70 (m, 2H), 3.60 (s, 2H), 3.30–3.17 (m, 4H), 2.94 (t, J = 7.2 Hz, 2H), 2.48–2.35 (m, 8H), 2.18 (s, 3H), 1.18 (t, J = 7.2 Hz, 3H), 1.02 (t, J = 7.2 Hz, 3H) ppm; ^{13}C -NMR ($DMSO-d_6$): δ 180.6, 166.6, 137.4, 136.7, 132.8, 130.3, 127.7, 126.1, 123.1, 121.4, 119.8, 119.0, 118.6, 116.9, 112.1, 111.8, 110.6, 106.3, 55.0, 53.3, 52.5, 47.3, 45.8, 45.2, 41.1, 25.2, 14.6, 13.4 ppm; HRMS (ESI) m/z : calculated 560.3166 for $[C_{31}H_{41}N_7OS]^+$, found 560.3173; HPLC analysis: retention time = 8.45 min, peak area 99.5%, 50:50 CH_3CN-H_2O .

1-(2-(1*H*-indol-3-yl)ethyl)-3-(3-((4-methylpiperazin-1-yl)methyl)-1-(2-oxo-2-(piperidin-1-yl)ethyl)-1*H*-indol-6-yl)thiourea (15e).—Yield, 47%; 1H -NMR ($DMSO-d_6$): δ 10.81 (s, 1H), 9.53 (s, 1H), 7.64 (d, J = 8.0 Hz, 1H), 7.54 (d, J = 8.8 Hz, 1H), 7.49 (s, 1H), 7.33 (d, J = 7.6 Hz, 1H), 7.26 (s, 1H), 7.16 (s, 1H), 7.13 (s, 1H), 7.06 (t, J = 8.0 Hz, 1H), 6.96 (t, J = 8.0 Hz, 1H), 6.85 (d, J = 8.8 Hz, 1H), 5.04 (s, 2H), 3.72–3.70 (m, 2H), 3.60 (s, 2H), 3.48 (s, 4H), 2.94 (t, J = 7.2 Hz, 2H), 2.48–2.35 (m, 8H), 2.18 (s, 3H), 1.56 (s, 4H), 1.43 (s, 2H) ppm; ^{13}C -NMR ($DMSO-d_6$): δ 180.7, 165.9, 137.4, 136.7, 132.7, 130.3, 127.7, 126.1, 123.1, 121.4, 119.8, 119.0, 118.6, 117.2, 112.1, 111.8, 110.6, 106.7, 55.0, 53.3, 52.5, 47.4, 45.8, 45.2, 42.9, 26.3, 25.8, 25.2, 24.4 ppm; HRMS (ESI) m/z : calculated 572.3166 for $[C_{32}H_{41}N_7OS]^+$, found 572.3176; HPLC analysis: retention time = 12.72 min, peak area 97.1%, 50:50 CH_3CN-H_2O .

1-(2-(1*H*-indol-3-yl)ethyl)-3-(3-((4-methylpiperazin-1-yl)methyl)-1-(2-morpholino-2-oxoethyl)-1*H*-indol-6-yl)thiourea (15f).—Yield, 54%; 1H -NMR ($CDCl_3$): δ 9.30 (s, 1H), 7.71 (s, 1H), 7.53–7.50 (m, 2H), 7.28 (s, 1H), 7.17 (s, 1H), 7.13 (t, J = 8.0 Hz, 1H), 7.04 (d, J = 7.2 Hz, 1H), 6.91 (s, 1H), 6.68 (d, J = 8.4 Hz, 1H), 6.36 (s, 1H), 5.90 (s, 1H), 4.72 (s, 2H), 3.85–3.83 (m, 4H), 3.74–3.70 (m, 4H), 3.61 (s, 2H), 3.48 (s, 2H), 2.92 (t, J = 6.4 Hz, 2H), 2.90–2.54 (m, 10H), 2.37 (s, 3H) ppm; ^{13}C -NMR (CD_3OD): δ

180.3, 167.1, 137.4, 136.7, 131.3, 130.5, 127.4, 127.2, 122.2, 121.0, 119.8, 117.3, 111.6, 110.9, 110.0, 106.9, 66.2, 66.1, 54.0 (2C), 52.1, 51.7 (2C), 47.0, 46.7, 45.2, 45.1, 44.3, 42.2, 24.5 ppm; HRMS (ESI) *m/z*: calculated 574.2959 for $[C_{31}H_{39}N_7O_2S]^+$, found 574.2955; HPLC analysis: retention time = 4.04 min, peak area 96.3%, 65:35 CH_3CN-H_2O .

(±) 1-(2-(1*H*-Indol-3-yl)ethyl)-3-(1-(2-(2-methylmorpholino)-2-oxoethyl)-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-6-yl)thiourea (15g).—Yield, 32%; 1H -NMR (DMSO- d_6): δ 10.82 (s, 1H), 9.52 (s, 1H), 7.64 (d, J = 8.0 Hz, 1H), 7.54 (d, J = 8.0 Hz, 1H), 7.48 (s, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.28 (s, 1H), 7.15 (s, 1H), 7.13 (s, 1H), 7.04 (t, J = 8.0 Hz, 1H), 6.96 (t, J = 8.0 Hz, 1H), 6.85 (d, J = 8.8 Hz, 1H), 5.22–5.14 (m, 1H), 5.00–4.96 (m, 1H), 4.15–4.09 (m, 1H), 3.92–3.72 (m, 5H), 3.58 (s, 2H), 3.21–3.18 (m, 1H), 2.94 (t, J = 7.2 Hz, 2H), 2.89–2.83 (m, 1H), 2.48–2.35 (m, 9H), 2.14 (s, 3H), 1.11–1.05 (m, 3H) ppm; ^{13}C -NMR (DMSO- d_6): δ 180.7, 166.5, 137.5, 136.7, 132.6, 130.2, 127.7, 126.2, 123.1, 121.4, 119.9, 119.0, 118.6, 117.3, 112.1, 111.8, 111.0, 106.9, 71.7, 66.1, 55.2, 53.4, 52.8, 50.8, 47.8, 47.3, 46.1, 45.3, 44.6, 41.7, 25.2, 19.0, 18.8 ppm; HRMS (ESI) *m/z*: calculated 588.3115 for $[C_{32}H_{41}N_7O_2S]^+$, found 588.3117; HPLC analysis: retention time = 7.94 min, peak area 96.8%, 50:50 CH_3CN-H_2O .

1-(2-(1*H*-Indol-3-yl)ethyl)-3-(1-(2-(4-methylpiperazin-1-yl)-2-oxoethyl)-3-((4-methylpiperazin-1-yl)methyl)-1*H*-indol-6-yl)thiourea (15h).—Yield, 37%; 1H -NMR (DMSO- d_6): δ 10.81 (s, 1H), 9.55 (s, 1H), 7.64 (d, J = 7.2 Hz, 1H), 7.53 (d, J = 8.4 Hz, 1H), 7.50 (s, 1H), 7.33 (d, J = 8.8 Hz, 1H), 7.28 (s, 1H), 7.14 (s, 1H), 7.12 (s, 1H), 7.06 (t, J = 8.0 Hz, 1H), 6.96 (t, J = 7.6 Hz, 1H), 6.85 (d, J = 8.0 Hz, 1H), 5.06 (s, 2H), 3.73–3.72 (m, 2H), 3.58 (s, 2H), 3.53 (s, 2H), 2.96–2.92 (m, 2H), 2.35–2.35 (m, 14H), 2.17 (s, 3H), 2.15 (s, 3H) ppm; ^{13}C -NMR (DMSO- d_6): δ 180.7, 166.3, 137.5, 136.7, 132.7, 130.1, 127.7, 126.2, 123.3, 121.4, 119.8, 119.0, 118.6, 117.2, 112.1, 111.8, 111.9, 106.8, 55.1, 55.0, 54.6, 53.4, 52.8, 52.6, 47.3, 46.0, 45.9, 45.2, 44.6, 41.9, 25.2, 25.0 ppm; HRMS (ESI) *m/z*: calculated 587.3275 for $[C_{32}H_{42}N_8OS]^+$, found 587.3285; HPLC analysis: retention time = 15.3 min, peak area 95.1%, 25:75 CH_3CN-H_2O .

BCL6^{BTB}-BCoR AlphaLisa Assay

We designed and optimized an Alphascreen assay using BCL6^{BTB}-His6 protein, biotinylated BCOR peptide (Biotin-Ahx-RSEIISTAPSSWVPGP-COOH, Biosynthesis) and Alphascreen acceptor and donor beads (AlphaLisa Histidine detection kit, PerkinElmer). In this optimized low volume assay, we incubated the recombinant protein diluted in AlphaLisa immunoassay buffer with different concentrations of the small molecules for 30 min. Then, we added the acceptor and donor beads, together with the BCOR peptide. After 1h of incubation in the dark and agitation, we measured the luminescence produced by the acceptor beads after excitation of the donor beads with a laser. This setup produced a signal/background ratio of 20 and a z' Score of 0.8 that was calculated as follows:

$$Z' \text{ score: } 1 - \left[\frac{(3 * (SD \text{ negative control} + SD \text{ positive control}))}{(AV \text{ negative control} - AV \text{ positive control})} \right]$$

Using 0.2% DMSO as a negative control and 80 μ M of SMRT Peptide (H_2N -LVATVKEAGRSIHEIPR-CO₂H, Biosynthesis) as a positive control.

Expression and Purification of Recombinant Proteins

cDNA encoding BCL6^{BTB} (1–129; C8Q, C67R, C84N, and E99V) was cloned into a pet32a vector containing an *N*-terminal thioredoxin-His6 tag with an *N*-terminal Thrombin cleavage site. Cells were grown in either LB or ¹⁵N-labeled M9 medium with ampicillin selection, and protein was expressed with 0.25 mM IPTG for 16 hours at 18 °C. *E. Coli* cells were lysed in a buffer containing 50 mM Tris, pH 7.5, 300 mM NaCl, 30 mM imidazole, 1 mM TCEP, and 0.5 mM PMSF. Protein was purified using Ni-NTA resin, eluted with lysis buffer containing 300 mM imidazole. The eluate was proteolytically cleaved with Thrombin, and applied to Ni-NTA to remove the thioredoxin-His6 tag. In the final step, protein was dialyzed to 20 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM TCEP, and frozen at –80 °C. For protein-observed NMR experiments, cDNA encoding BCL6^{BTB} (1–129; C67R, C84N, E99V), LRF^{BTB} (1–130), KAISO^{BTB} (1–122), and Miz1^{BTB} (1–115) were cloned into a pet32a vector containing an *N*-terminal thioredoxin-His6 tag with an *N*-terminal PreScission cleavage site. Proteins were expressed and purified in similar manner as described above. Following affinity purification, proteins were cleaved with PreScission protease and thioredoxin-His6 tag was removed using Ni-NTA resin. Final BCL6^{BTB}, Miz1^{BTB}, and KAISO^{BTB} proteins were dialyzed to 50 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM TCEP, and used in NMR experiments. LRF^{BTB} was dialyzed to 50 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP.

Fragment Screening by Protein-Observed NMR Spectroscopy

The fragment library used for screening was a combination of commercially available fragment-like small molecules and compounds synthesized in-house. Samples for fragment screening were prepared with 150 μ M ¹⁵N-labeled BCL6^{BTB} (1–129; C67R, C84N, E99V) in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM TCEP, and 7.5% D₂O. Fragments were screened in mixtures of 10 compounds per sample at 500 μ M final concentration, in 5% DMSO. ¹H-¹⁵N HSQC spectra were acquired at 30 °C on a 600 MHz Bruker Avance III spectrometer equipped with cryoprobe, running Topspin version 2.1. Processing and spectral visualization was performed using NMRPipe and Sparky. Ranking of compound affinity was performed by calculating respective 6PA values measured at 500 μ M compound concentration for six of the most perturbed amide resonances on the ¹H-¹⁵N HSQC spectra of BCL6^{BTB}.

Calculation of 6PA Values

6PA values represent sum of ¹H and ¹⁵N chemical shift perturbations in Hz calculated for backbone amides of Thr62, Thr48, Phe61, Asn23, Arg28, and Val18 (Table S2, Figure S3). NMR samples for 6PA measurements contained 500 μ M compound (in 5% DMSO) and 250 μ M BCL6^{BTB} (1–129; C67R, C84N, E99V).

K_D Determination by Protein-Observed NMR Spectroscopy

^1H - ^{15}N HSQC experiments were performed at 30 °C with samples containing 150 μM BCL6^{BTB} (residues 1–129; C67R, C84N, E99V) in 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM TCEP, 7.5% D₂O, and 5% DMSO, by adding increasing concentrations of compound 7CC5 (1 mM, 2 mM, and 4 mM). For more potent compounds, the concentrations were varied from 150 to 1000 μM and 250 μM BCL6^{BTB} (1–129; C67R, C84N, E99V). Dissociation constants were determined from least-squares fitting of chemical shift perturbations as a function of ligand concentration

$$\delta_i = \frac{\{b - \sqrt{(b^2 - 4 \times a \times c)}\}}{2a}$$

with $a = (K_A/\delta_b) \times [P_t]$, $b = 1 + K_A([L_{ti}] + [P_t])$, and $c = \delta_b \times K_A \times [L_{ti}]$, where δ_i is the absolute change in chemical shift for each titration point, $[L_{ti}]$ is the total ligand concentration at each titration point, $[P_t]$ is the total protein concentration, $K_A = 1/K_D$ is the binding constant, and δ_b is the chemical shift of the resonance in question in the complex.⁴¹ K_D and δ_b were used as fitting parameters in analysis.

Isothermal Titration Calorimetry

BCL6^{BTB} was dialyzed repeatedly against 50 mM phosphate buffer, pH 7.5, 150 mM NaCl, and 1 mM TCEP (referred herein as ITC Buffer), at 4 °C. **15f** was dissolved in DMSO, and diluted to 1 mM in ITC Buffer containing 5% DMSO. Additionally, 5% DMSO was added to protein and buffer (reference) solutions. Titrations were performed using a VP-ITC titration calorimetric system (MicroCal) at 25 °C. The calorimetric cell, containing 100 μM BCL6^{BTB}, was titrated with **15f** at 1 mM, in 10 μL aliquots and at 300 second intervals. Data were analyzed by Origin 7.0 (OriginLab) to obtain the K_D and stoichiometry.

Crystallization of BCL^{BTB}-Inhibitor Complexes

Initial crystals of native-BCL6^{BTB} (1–129; C8Q, C67R, C84N, and E99V) were obtained through screening using the hanging-drop vapor diffusion technique. Crystals were further optimized using the sitting-drop technique, with equal volumes (1.5 μL) of protein (9 mg/mL in 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM TCEP) and precipitant solution (1.6 M ammonium formate, 0.1 M Tris, pH 7.5). Crystals formed overnight at 4 °C. For 7CC5, crystals were transferred to the mother liquor solution containing molar excess of 7CC5 and 2% DMSO to soak for two hours, and were then transferred to the mother liquor solution containing molar excess of 7CC5, 2% DMSO, and 30% glycerol for cryoprotection prior to freezing in liquid nitrogen. For compound **15a**, 3 mg/mL BCL6^{BTB} (1–129; C8Q, C67R, C84N, and E99V) was incubated with 2 mM compound (a 10-fold molar excess) for 6 h at room temperature. Crystals of BCL6^{BTB}-**15a** complex were obtained through screening using the hanging-drop vapor diffusion technique at 4 °C overnight in 0.2 M NaCl, 0.1 M sodium cacodylate, pH 6.0, and 8% (w/v) PEG-8,000. Before data collection, crystals were transferred into mother liquor containing 2 mM **15a**, 2% DMSO, and 30% glycerol for cryoprotection prior to freezing in liquid nitrogen. For compound **15f**, 6 mg/mL BCL6^{BTB} (1–129; C8Q, C67R, C84N, and E99V) was incubated with 2 mM compound for 6 h at room

temperature. Initial crystals were obtained through screening using the hanging-drop vapor diffusion technique. Crystals were further optimized using the sitting-drop technique, with equal volumes (1.5 μ L) of protein-inhibitor complex and precipitant solution (0.1 M sodium acetate pH 4.5, 27% (w/v) PEG-3,350). Crystals grew within one week at 4 °C. Before data collection, crystals were transferred to the mother liquor solution containing 2 mM **15f**, 2% DMSO, and 30% glycerol for cryoprotection prior to freezing in liquid nitrogen.

Crystallographic Data Collection and Structure Determination

Diffraction data for BCL6^{BTB}-inhibitor complexes were collected at the 21-ID-F and 21-ID-G beam lines at the Life Sciences Collaborative Access Team at the Advanced Photon Source. Data were integrated and scaled using HKL-2000,⁴³ and structures were solved by molecular replacement with MOLREP using the known native-BCL6^{BTB} structure for the search model. Refinement for structures was performed using REFMAC,⁴⁴ COOT,⁴⁵ the CCP4⁴⁶ program suite, and the PHENIX⁴⁷ program suite. The structures were validated using the MOLPROBITY⁴⁸ and ADIT.⁴⁹ Data collection and structure refinement statistics are presented in Table 5.

Computational Details

Simulations were initiated from the structure of the BCL6^{BTB} in the presence of SMRT peptide (PDB entry 1R2B), following removal of the peptide. Site identification by ligand competitive saturation (SILCS) simulations and analysis were performed using in-house programs and scripts^{36,50} along with the GROMACS simulation program.⁵¹ The setup of the SILCS simulations was similar to that used in our previously reported studies^{37,50} using an iterative Grand-Canonical Monte Carlo (GCMC) and molecular dynamics (MD) protocol in which GCMC involves the sampling of water and solutes with the subsequent MD simulation allowing for sampling of protein conformational dynamics as well as of the solutes and water.³⁷ All calculations used the CHARMM36 protein force field⁵² with the CHARMM TIP3P water model⁵³ and the CHARMM General Force Field (CGenFF) for the solute molecules and the thiourea inhibitors.^{54,55} To dock the thiourea inhibitors to the BCL6^{BTB} homodimer, the Monte Carlo based SILCS (SILCS-MC) protocol⁵⁰ was adopted to predict inhibitor-binding modes based on the SILCS GFE FragMaps as the scoring function. In the present study FragMaps based on generic apolar (benzene, propane), generic H-bond donor (methanol O, formamide N, imidazole (NH)) and acceptor (methanol O, formamide O, imidazole N, acetaldehyde O), negatively charged (acetate) and positively charged (methylammonium) were used for visualization and scoring. The sulfur atoms in the ligands were treated as apolar groups. For each compound, five independent SILCS-MC runs were performed with each run including multiple SILCS-MC cycles performed in a two-step fashion.⁵⁶ The first portion of each cycle is 10,000 Metropolis MC steps that sample a wide range of different binding poses with steps sizes of 180° for overall rotations, 1 Å for translations and 180° for dihedral angle rotations at a temperature of 298 K, which is followed by 40,000 steps of MC simulated annealing (SA) designed to find a local minimum with the acceptance criteria defined by the Ligand Grid Free Energies (LGFE).⁵⁶ SA involved maximum ranges of steps sizes of 9° for overall rotations, 0.2 Å for translations and 9° for dihedral angle rotations with gradual cooling from 298 to 0 K. The five SILCS-MC runs involved multiple MC cycles each initiated with a different random seed, that were

repeated until the three most-favorable LGFE scores were within 0.5 kcal/mol of each other, with a minimum of 50 cycles and a maximum of 250 cycles performed. The docking pose with the most favorable LGFE was reported as the predicted binding mode from which the LGFE and atom-based GFE scores were obtained.

Cell Lines

Sources: Human embryonic kidney 293T cells, and human DLBCL cells Toledo, SUDHL-4 and SUDHL-6, cells were purchased in ATCC. Human DLBCL cell lines OCI-Ly1 and OCI-Ly7 were from the DSMZ German collection of Microorganisms and cell cultures. OCI-Ly1-B50 are OCI-Ly1 cells that were grown in the presence of increasing concentrations of RI-BPI and grow independently of BCL6 inhibitors. DLBCL cell lines, TMD8 and HBL-1 were kindly provided by Dr. Louis Staudt, National Institute of Health (NIH, MD). All human cell lines were identified and authenticated by DNA genotyping previous to use by Biosynthesis (Lewisville, TX).

293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (Life Technologies Corp., Grand Island, NY) and 1% penicillin–streptomycin (Life Technologies Corp., Grand Island, NY). DLBCL cell lines OCI-Ly1, OCI-Ly1-B50 and OCI-Ly7 were grown in Iscove's medium (Life Technologies Corp., Grand Island, NY), 10% FBS and 1% penicillin–streptomycin. DLBCL cell lines Toledo, SUDHL-4, HBL-1, and SUDHL-6 were cultured in 90% RPMI medium (Life Technologies Corp., Grand Island, NY), 10% FBS, 2 mM glutamine, 10 mM Hepes (Life Technologies Corp., Grand Island, NY), and 1% penicillin–streptomycin. All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO₂.

Growth-Inhibition Determination

Exponentially growing DLBCL cell lines were treated in three replicates with small molecules for 48 hours. Cell viability was then determined with the fluorescent redox dye CellTiter-Blue (Promega, Madison, WI). Fluorescence was determined with the Synergy NEO microplate reader (BioTek, North Brunswick Township, NJ) and the percentage of cell viability of the drug-treated cells was normalized to their vehicle. The drug effect was calculated as 100 -percentage viability. Dose-effect curves were used to determine the drug concentration that inhibits the growth of cell lines by 50% compared with vehicle (GI₅₀) with the CompuSyn software (Biosoft, Cambridge, UK). Experiments were performed in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank the American Association for Cancer Research grant #00167155 to F.X., the Samuel Waxman Cancer Research Foundation, and the Leukemia Lymphoma Society for grant to A.M., A.M. F.X, and T.C.

ABBREVIATION USED

PPI	protein-protein interactions
BCL6	B-cell lymphoma 6
BTB	Broad-complex, Tramtrack, and Brick-a-brac
SMRT	silencing mediator of retinoid and thyroid hormone receptor
NCOR	nuclear receptor corepressor
BCOR	BCL6 co-repressor
CADD	computer-aided drug design
SILCS	site identification by ligand competitive saturation
DLBCL	diffuse large B-cell lymphomas
B-ALL	B-cell precursor acute lymphoblastic leukemia
MLL	mixed lineage leukemia
TKIs	tyrosine kinase inhibitors
BP-CML	blast-phase chronic myeloid leukemia
TNBC	triple-negative breast cancer
LG	lateral groove
MST	microscale thermophoresis
GCMC/MD	grand canonical monte carlo/molecular dynamics
3D	three-dimensional
GFE	grid free-energy
MC	monte carlo
LGFE	ligand grid free energy
HATU	1-[bis(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i>]pyridinium 3-oxid hexafluorophosphate
DMSO	dimethyl sulfoxide
DIPEA	<i>N,N</i> -diisopropylethylamine
DMF	dimethylformamide
THF	tetrahydrofuran
LE	ligand efficiency

6PA	sum of chemical shift perturbations of six selected amide resonances
GI₅₀	the concentration of compound to cause 50% reduction of cancer cell proliferation

REFERENCES

- (1). Cardenas MG; Oswald E; Yu W; Xue F; MacKerell AD; Melnick AM The expanding role of the BCL6 oncoprotein as a cancer therapeutic target. *Clin. Cancer Res* 2017, 23, 885–893. [PubMed: 27881582]
- (2). Ye BH; Cattoretti G; Shen Q; Zhang J; Hawe N; de Waard R; Leung C; Nouri-Shirazi M; Orazi A; Chaganti RS; Rothman P; Stall AM; Pandolfi PP; Dalla-Favera R The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nature genet* 1997, 16, 161–170. [PubMed: 9171827]
- (3). Fukuda T; Yoshida T; Okada S; Hatano M; Miki T; Ishibashi K; Okabe S; Koseki H; Hirosawa S; Taniguchi M; Miyasaka N; Tokuhisa T Disruption of the Bcl6 gene results in an impaired germinal center formation. *J. Exp. Med* 1997, 186, 439–448. [PubMed: 9236196]
- (4). Duy C; Hurtz C; Shojaee S; Cerchietti L; Geng H; Swaminathan S; Klemm L; Kweon SM; Nahar R; Braig M; Park E; Kim YM; Hofmann WK; Herzog S; Jumaa H; Koeffler HP; Yu JJ; Heisterkamp N; Graeber TG; Wu H; Ye BH; Melnick A; Muschen M BCL6 enables Ph⁺ acute lymphoblastic leukaemia cells to survive BCR-ABL1 kinase inhibition. *Nature* 2011, 473, 384–388. [PubMed: 21593872]
- (5). Geng H; Brennan S; Milne TA; Chen WY; Li Y; Hurtz C; Kweon SM; Zickl L; Shojaee S; Neuberg D; Huang C; Biswas D; Xin Y; Racevskis J; Ketterling RP; Luger SM; Lazarus H; Tallman MS; Rowe JM; Litzow MR; Guzman ML; Allis CD; Roeder RG; Muschen M; Paietta E; Elemento O; Melnick AM Integrative epigenomic analysis identifies biomarkers and therapeutic targets in adult B-acute lymphoblastic leukemia. *Cancer discov* 2012, 2, 1004–1023. [PubMed: 23107779]
- (6). Hurtz C; Hatzi K; Cerchietti L; Braig M; Park E; Kim YM; Herzog S; Ramezani-Rad P; Jumaa H; Muller MC; Hofmann WK; Hochhaus A; Ye BH; Agarwal A; Druker BJ; Shah NP; Melnick AM; Muschen M BCL6-mediated repression of p53 is critical for leukemia stem cell survival in chronic myeloid leukemia. *J. Exp. Med* 2011, 208, 2163–2174. [PubMed: 21911423]
- (7). Walker SR; Liu S; Xiang M; Nicolais M; Hatzi K; Giannopoulou E; Elemento O; Cerchietti L; Melnick A; Frank DA The transcriptional modulator BCL6 as a molecular target for breast cancer therapy. *Oncogene* 2015, 34, 1073–1082. [PubMed: 24662818]
- (8). Ranuncolo SM; Polo JM; Melnick A BCL6 represses CHEK1 and suppresses DNA damage pathways in normal and malignant B-cells. *Blood Cells Mol. Dis* 2008, 41, 95–99. [PubMed: 18346918]
- (9). Ci W; Polo JM; Cerchietti L; Shakhovich R; Wang L; Yang SN; Ye K; Farinha P; Horsman DE; Gascoyne RD; Elemento O; Melnick A The BCL6 transcriptional program features repression of multiple oncogenes in primary B cells and is deregulated in DLBCL. *Blood* 2009, 113, 5536–5548. [PubMed: 19307668]
- (10). Ranuncolo SM; Polo JM; Dierov J; Singer M; Kuo T; Grealley J; Green R; Carroll M; Melnick A Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. *Nature immunol* 2007, 8, 705–714. [PubMed: 17558410]
- (11). Phan RT; Saito M; Basso K; Niu H; Dalla-Favera R BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. *Nature immunol* 2005, 6, 1054–1060. [PubMed: 16142238]
- (12). Phan RT; Dalla-Favera R The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* 2004, 432, 635–639. [PubMed: 15577913]
- (13). Cerchietti LC; Hatzi K; Caldas-Lopes E; Yang SN; Figueroa ME; Morin RD; Hirst M; Mendez L; Shakhovich R; Cole PA; Bhalla K; Gascoyne RD; Marra M; Chiosis G; Melnick A BCL6

repression of EP300 in human diffuse large B cell lymphoma cells provides a basis for rational combinatorial therapy. *J. Clin. Invest* 2010, 120, 4569–4582. [PubMed: 21041953]

- (14). Parekh S; Polo JM; Shaknovich R; Juszczynski P; Lev P; Ranuncolo SM; Yin Y; Klein U; Cattorette G; Dalla Favera R; Shipp MA; Melnick A BCL6 programs lymphoma cells for survival and differentiation through distinct biochemical mechanisms. *Blood* 2007, 110, 2067–2074. [PubMed: 17545502]
- (15). Polo JM; Dell'Oso T; Ranuncolo SM; Cerchiatti L; Beck D; Da Silva GF; Prive GG; Licht JD; Melnick A Specific peptide interference reveals BCL6 transcriptional and oncogenic mechanisms in B-cell lymphoma cells. *Nat. Med* 2004, 10, 1329–1335. [PubMed: 15531890]
- (16). Cerchiatti LC; Yang SN; Shaknovich R; Hatzi K; Polo JM; Chadburn A; Dowdy SF; Melnick A A peptomimetic inhibitor of BCL6 with potent antilymphoma effects in vitro and in vivo. *Blood* 2009, 113, 3397–3405. [PubMed: 18927431]
- (17). Cerchiatti LC; Ghetu AF; Zhu X; Da Silva GF; Zhong S; Matthews M; Bunting KL; Polo JM; Fares C; Arrowsmith CH; Yang SN; Garcia M; Coop A; Mackerell AD, Jr.; Prive GG; Melnick A A small-molecule inhibitor of BCL6 kills DLBCL cells in vitro and in vivo. *Cancer Cell* 2010, 17, 400–411. [PubMed: 20385364]
- (18). Cerchiatti LC; Polo JM; Da Silva GF; Farinha P; Shaknovich R; Gascoyne RD; Dowdy SF; Melnick A Sequential transcription factor targeting for diffuse large B-cell lymphomas. *Cancer Res* 2008, 68, 3361–3369. [PubMed: 18451163]
- (19). Ci W; Polo JM; Melnick A B-cell lymphoma 6 and the molecular pathogenesis of diffuse large B-cell lymphoma. *Curr. Opin. Hematol* 2008, 15, 381–390. [PubMed: 18536578]
- (20). Ye BH BCL-6 in the pathogenesis of non-Hodgkin's lymphoma. *Cancer Invest* 2000, 18, 356–365. [PubMed: 10808372]
- (21). Zhang H; Okada S; Hatano M; Okabe S; Tokuhisa T A new functional domain of Bcl6 family that recruits histone deacetylases. *Biochim. Biophys. Acta* 2001, 1540, 188–200. [PubMed: 11583814]
- (22). Huynh KD; Bardwell VJ The BCL-6 POZ domain and other POZ domains interact with the corepressors N-CoR and SMRT. *Oncogene* 1998, 17, 2473–2484. [PubMed: 9824158]
- (23). Huynh KD; Fischle W; Verdin E; Bardwell VJ BCoR, a novel corepressor involved in BCL-6 repression. *Genes. Dev* 2000, 14, 1810–1823. [PubMed: 10898795]
- (24). Ahmad KF; Melnick A; Lax S; Bouchard D; Liu J; Kiang CL; Mayer S; Takahashi S; Licht JD; Prive GG Mechanism of SMRT corepressor recruitment by the BCL6 BTB domain. *Mol. Cell* 2003, 12, 1551–1564. [PubMed: 14690607]
- (25). Ghetu AF; Corcoran CM; Cerchiatti L; Bardwell VJ; Melnick A; Prive GG Structure of a BCOR corepressor peptide in complex with the BCL6 BTB domain dimer. *Mol. Cell* 2008, 29, 384–391. [PubMed: 18280243]
- (26). Bunting KL; Melnick AM New effector functions and regulatory mechanisms of BCL6 in normal and malignant lymphocytes. *Curr. Opin. Immunol* 2013, 25, 339–346. [PubMed: 23725655]
- (27). Huang C; Hatzi K; Melnick A Lineage-specific functions of Bcl-6 in immunity and inflammation are mediated by distinct biochemical mechanisms. *Nature Immunol* 2013, 14, 380–388. [PubMed: 23455674]
- (28). Yasui T; Yamamoto T; Sakai N; Asano K; Takai T; Yoshitomi Y; Davis M; Takagi T; Sakamoto K; Sogabe S; Kamada Y; Lane W; Snell G; Iwata M; Goto M; Inooka H; Sakamoto JI; Nakada Y; Imaeda Y Discovery of a novel B-cell lymphoma 6 (BCL6)-corepressor interaction inhibitor by utilizing structure-based drug design. *Bioorg. Med. Chem* 2017, 25, 4876–4886. [PubMed: 28760529]
- (29). Kamada Y; Sakai N; Sogabe S; Ida K; Oki H; Sakamoto K; Lane W; Snell G; Iida M; Imaeda Y; Sakamoto J; Matsui J Discovery of a B-cell lymphoma 6 protein-protein interaction inhibitor by a biophysics-driven fragment-based approach. *J. Med. Chem* 2017, 60, 4358–4368. [PubMed: 28471657]
- (30). McCoull W; Abrams RD; Anderson E; Blades K; Barton P; Box M; Burgess J; Byth K; Cao Q; Chuaqui C; Carbajo RJ; Cheung T; Code E; Ferguson AD; Fillery S; Fuller NO; Gangl E; Gao N; Grist M; Hargreaves D; Howard MR; Hu J; Kemmitt PD; Nelson JE; O'Connell N; Prince DB; Raubo P; Rawlins PB; Robb GR; Shi J; Waring MJ; Whittaker D; Wylot M; Zhu X Discovery of

pyrazolo[1,5-a]pyrimidine B-cell lymphoma 6 (BCL6) binders and optimization to high affinity macrocyclic inhibitors. *J. Med. Chem* 2017, 60, 4386–4402. [PubMed: 28485934]

- (31). Kerres N; Steurer S; Schlager S; Bader G; Berger H; Caligiuri M; Dank C; Engen JR; Ettmayer P; Fischerauer B; Flotzinger G; Gerlach D; Gerstberger T; Gmaschitz T; Greb P; Han B; Heyes E; Jacob RE; Kessler D; Kolle H; Lamarre L; Lancia DR; Lucas S; Mayer M; Mayr K; Mischerikow N; Muck K; Peinsipp C; Petermann O; Reiser U; Rudolph D; Rumpel K; Salomon C; Scharn D; Schnitzer R; Schrenk A; Schweifer N; Thompson D; Traxler E; Varecka R; Voss T; Weiss-Puxbaum A; Winkler S; Zheng X; Zoephel A; Kraut N; McConnell D; Pearson M; Koegl M Chemically induced degradation of the oncogenic transcription factor BCL6. *Cell Rep* 2017, 20, 2860–2875. [PubMed: 28930682]
- (32). Cardenas MG; Yu W; Beguelin W; Teater MR; Geng H; Goldstein RL; Oswald E; Hatzi K; Yang SN; Cohen J; Shaknovich R; Vanommeslaeghe K; Cheng H; Liang D; Cho HJ; Abbott J; Tam W; Du W; Leonard JP; Elemento O; Cerchietti L; Cierpicki T; Xue F; MacKerell AD, Jr.; Melnick AM Rationally designed BCL6 inhibitors target activated B cell diffuse large B cell lymphoma. *J. Clin. Invest* 2016, 126, 3351–3362. [PubMed: 27482887]
- (33). Baell JB; Holloway GA New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem* 2010, 53, 2719–2740. [PubMed: 20131845]
- (34). Mendgen T; Steuer C; Klein CD Privileged scaffolds or promiscuous binders: a comparative study on rhodanines and related heterocycles in medicinal chemistry. *J. Med. Chem* 2012, 55, 743–753. [PubMed: 22077389]
- (35). Erlanson DA; Fesik SW; Hubbard RE; Jahnke W; Jhoti H Twenty years on: the impact of fragments on drug discovery. *Nature Res. Drug Discov* 2016, 15, 605–619.
- (36). Guvench O; MacKerell AD, Jr Computational fragment-based binding site identification by ligand competitive saturation. *PLoS Comp. Biol* 2009, 5, e1000435.
- (37). Lakkaraju SK; Yu W; Raman EP; Hershfild AV; Fang L; Deshpande DA; MacKerell AD Mapping functional group free energy patterns at protein occluded sites: nuclear receptors and G-protein coupled receptors. *J. Chem. Inf. Model* 2015, 55, 700–708. [PubMed: 25692383]
- (38). Raman EP; Yu W; Lakkaraju SK; MacKerell AD, Jr. Inclusion of multiple fragment types in the site identification by ligand competitive saturation (SILCS) approach. *J. Chem. Inf. Model* 2013, 53, 3384–3398. [PubMed: 24245913]
- (39). Oashi T; Ringer AL; Raman EP; MacKerell AD Automated selection of compounds with physicochemical properties to maximize bioavailability and druglikeness. *J. Chem. Inf. Model* 2011, 51, 148–158. [PubMed: 21142079]
- (40). Hopkins AL; Keseru GM; Leeson PD; Rees DC; Reynolds CH The role of ligand efficiency metrics in drug discovery. *Nature Rev. Drug Discov* 2014, 13, 105–121. [PubMed: 24481311]
- (41). Tugarinov V; Kay LE Quantitative NMR studies of high molecular weight proteins: application to domain orientation and ligand binding in the 723 residue enzyme malate synthase G. *J. Mol. Biol* 2003, 327, 1121–1133. [PubMed: 12662935]
- (42). Pearlman DA; Charifson PS Are free energy calculations useful in practice? A comparison with rapid scoring functions for the p38 MAP kinase protein system. *J. Med. Chem* 2001, 44, 3417–3423. [PubMed: 11585447]
- (43). Otwinowski Z; Minor W [20] Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 1997, 276, 307–326.
- (44). Murshudov GN; Vagin AA; Dodson EJ Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr. D Biol. Crystallogr* 1997, 53, 240–255. [PubMed: 15299926]
- (45). Emsley P; Cowtan K Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr* 2004, 60, 2126–2132. [PubMed: 15572765]
- (46). The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D Biol. Crystallogr* 1994, 50, 760–763. [PubMed: 15299374]
- (47). Adams PD; Afonine PV; Bunkoczi G; Chen VB; Davis IW; Echols N; Headd JJ; Hung LW; Kapral GJ; Grosse-Kunstleve RW; McCoy AJ; Moriarty NW; Oeffner R; Read RJ; Richardson DC; Richardson JS; Terwilliger TC; Zwart PH PHENIX: a comprehensive Python-based system

for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr* 2010, 66, 213–221. [PubMed: 20124702]

- (48). Davis IW; Leaver-Fay A; Chen VB; Block JN; Kapral GJ; Wang X; Murray LW; Arendall WB, 3rd; Snoeyink J; Richardson JS; Richardson DC MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res* 2007, 35, W375–383. [PubMed: 17452350]
- (49). Yang H; Guranovic V; Dutta S; Feng Z; Berman HM; Westbrook JD Automated and accurate deposition of structures solved by X-ray diffraction to the Protein Data Bank. *Acta Crystallogr. D Biol. Crystallogr* 2004, 60, 1833–1839. [PubMed: 15388930]
- (50). Raman EP; Yu WB; Lakkaraju SK; MacKerell AD Inclusion of multiple fragment types in the site identification by ligand competitive saturation (SILCS) approach. *J. Chem. Inf. Model* 2013, 53, 3384–3398. [PubMed: 24245913]
- (51). Hess B; Kutzner C; van der Spoel D; Lindahl E GROMACS 4: algorithms for highly efficient, load-balanced, and scalable molecular simulation. *J. Chem. Theory Comput* 2008, 4, 435–447. [PubMed: 26620784]
- (52). MacKerell AD; Bashford D; Bellott M; Dunbrack RL; Evanseck JD; Field MJ; Fischer S; Gao J; Guo H; Ha S; Joseph-McCarthy D; Kuchnir L; Kuczera K; Lau FT; Mattos C; Michnick S; Ngo T; Nguyen DT; Prodhom B; Reiher WE; Roux B; Schlenkrich M; Smith JC; Stote R; Straub J; Watanabe M; Wiorkiewicz-Kuczera J; Yin D; Karplus M All-atom empirical potential for molecular modeling and dynamics studies of proteins. *Journal Phys. Chem. B* 1998, 102, 3586–3616. [PubMed: 24889800]
- (53). Neria EF,S; Karplus M *J. Chem. Phys* 1996, 105, 1902–1921.
- (54). Vanommeslaeghe K; Hatcher E; Acharya C; Kundu S; Zhong S; Shim J; Darian E; Guvench O; Lopes P; Vorobyov I; Mackerell AD, Jr. CHARMM general force field: A force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields. *J. Comput. Chem* 2010, 31, 671–690. [PubMed: 19575467]
- (55). Yu W; He X; Vanommeslaeghe K; MacKerell AD, Jr. Extension of the CHARMM general force field to sulfonyl-containing compounds and its utility in biomolecular simulations. *J. Comput. Chem* 2012, 33, 2451–2468. [PubMed: 22821581]
- (56). Heinzl GA; Huang W; Yu W; Giardina BJ; Zhou Y; MacKerell AD, Jr.; Wilks A; Xue F Iminoguanidines as allosteric inhibitors of the iron-regulated heme oxygenase (HemO) of *Pseudomonas aeruginosa*. *J. Med. Chem* 2016, 59, 6929–6942. [PubMed: 27353344]

Figure 1.
Chemical structures of previously reported inhibitors for the BCL6^{BTB}.

Figure 2.

(A) Chemical structure of hit 7CC5; (B) NMR titration experiment to determine K_D value for binding of 7CC5 and BCL6^{BTB}; (C) Superposition of the ¹H-¹⁵N HSQC spectra of 150 μM BCL6^{BTB} with 5% DMSO (black) and 500 μM 7CC5 (red); (D) Crystal structure of BCL6^{BTB}-7CC5 complex (PDB 6C3N). The mFo-DFc electron density map contoured at 3.0 sigma is shown for 7CC5 and selected protein residues involved in the interactions with 7CC5 are shown as sticks.

Figure 3.

The crystal binding mode of 7CC5 overlaid with the SILCS apolar (green), H-bond donor (blue), H-bond acceptor (red), negative (orange) and positive (cyan) FragMaps. FragMaps are shown at contour levels of -1.0 kcal/mol for the generic apolar H-bond donor and H-bond acceptor maps and at -1.5 kcal/mol for the aromatic, aliphatic, negative and positive maps.

Figure 4. Characterization of the binding of 15a and 15f to BCL6^{BTB}.

(A) Chemical structures of **15a** and **15f**; (B) NMR titration experiment to determine K_D values for binding of **15a** and **15f** to BCL6^{BTB}; (C) Binding affinity of **15f** to BCL6^{BTB} determined using isothermal titration calorimetry; (D) Crystal structure of BCL6^{BTB}-**15a** complex (PDB 6CQ1). The 2mFo-DFc electron density map contoured at 1.0 sigma is shown for **15a** and selected protein residues involved in the interactions with **15a** are shown as sticks; (E) Crystal structure of BCL6^{BTB}-**15f** complex (PDB 6C3L). The 2mFo-DFc electron density map contoured at 1.0 sigma is shown for **15f** and selected protein residues involved in the interactions with **15f** are shown as sticks; (F) Superposition of compounds 7CC5 (magenta), **15a** (yellow), and **15f** (cyan) from BCL6^{BTB}-complex crystal structures. HDCH site is labeled.

Figure 5. SILCS predicted (magenta) and X-ray crystal (yellow) binding orientations of compound **15f** overlaid on the SILCS FragMaps and the BCL6^{BTB} crystal structure. Apolar (green), H-bond donor (blue) and acceptor (red), and positively charged (cyan) SILCS FragMaps are shown. H-bond donor and H-bond acceptor FragMaps are set to a cutoff of -0.6 kcal/mol, while Apolar and positively charged FragMaps are set to a cutoff of -1.5 kcal/mol.

Figure 6.

Correlation plots for (A) Lg($\alpha\%$) with LGFE scores and (B) NMR perturbation data with LGFE scores. Correlation coefficient R^2 and predictive index (PI)⁴² value are shown. Lg($\alpha\%$) represents the common logarithm (log10) value of remaining AlphaLisa signal (in percentage) in the presence of an inhibitor (50 μ M).

^aReagents and conditions: (a) methyl bromoacetate, K_2CO_3 , DMF, 60 °C, overnight, 86%; (b) Pd/C , H_2 , $\text{MeOH}:\text{THF} = 1:1$, r.t., overnight, 52%; (c) 1,1'-thiocarbonylbis(pyridine-2(1*H*)-one), CH_2Cl_2 , r.t., 2 h, 90%; (d) tryptamine, CH_2Cl_2 , r.t., 3 h, 76%; (e) $\text{LiOH}\cdot\text{H}_2\text{O}$, $\text{MeOH}:\text{THF}:\text{H}_2\text{O} = 3:1:1$, 70 °C, 3 h, 82%; (f) amines, HATU, DIPEA, DMF, r.t., overnight, 42-86%.^b The synthesis of **7a** and **7i** were slightly different from compounds **7b-h**; synthesis of compound **7a** and **7i** were detailed in the experimental section.

Scheme 1.
Synthesis of compounds **7a-i**.^a

^aReagents and conditions: (a) methyl bromoacetate, K₂CO₃, DMF, 70 °C, overnight, 79%; (b) 1-methylpiperazine, NaBH(OAc)₃, CH₂Cl₂, r.t., overnight, 58%; (c) Pd/C, H₂, MeOH, r.t., 3 h, 72%; (d) 1,1'-thiocarbonylbis(pyridine-2(1*H*)-one), CH₂Cl₂, r.t., 4 h; (e) tryptamine, CH₂Cl₂, r.t., overnight, 33% for two steps; (f) LiOH·H₂O, MeOH:THF:H₂O = 3:1:1, r.t., 3 h, 80%; (g) amines, HATU, DIPEA, DMF, r.t., overnight, 32-65%.

Scheme 2.
Synthesis of inhibitors **13**, **15a-h**.^a

Table 1.

Structures, 6PA, LGFE, and LE Values of Inhibitors 7CC5, 7CC5-14, and 7a-i

$\text{R}_1\text{---CH}_2\text{---CH}_2\text{---NH---C(=S)---NH---R}_2$					
Cmpd	R1	R2	6PA (Hz)	LGFE (kcal/mol)	LE (kcal/mol)
7CC5			49	-32.2	-1.79
7CC5-14			116	-30.8	-1.47
7a			88	-33.7	-1.41
7b			323	-34.6	-1.12

Cmpd	R1	R2	6PA (Hz)	LGFE (kcal/mol)	LE (kcal/mol)
7c			185	-35.9	-1.16
7d			237	-37.5	-1.21
7e			166	-34.7	-1.09

$\text{R}_1\text{---CH}_2\text{---CH}_2\text{---NH---C(=S)---NH---R}_2$					
Cmpd	R1	R2	6PA (Hz)	LGFE (kcal/mol)	LE (kcal/mol)
7f			72	-36.2	-1.10
7g			380	-35.9	-1.09
7h			95	-40.7	-1.20

Cmpd	R1	R2	6PA (Hz)	LGFE (kcal/mol)	LE (kcal/mol)
7i			391	-40.8	-1.28

Table 2.

Chemical Structures, 6PA, LGFE, and LE Values of Inhibitors 13 and 15a-h

Cmpd	R	6PA (Hz)	LGFE (kcal/mol)	LE (kcal/mol)	NMR K_D (μM)
13		363	-39.5	-1.07	191 $\pm$ 14
15a		288	-41.8	-1.07	219 $\pm$ 32
15b		277	-42.5	-1.01	283 $\pm$ 19
15c		323	-40.7	-1.02	226 $\pm$ 17

Cmpd	R	6PA (Hz)	LGFE (kcal/mol)	LE (kcal/mol)	NMR K_D (μ M)
15d		390	-44.2	-1.11	245 $\pm$ 37
15e		316	-46.0	-1.12	405 $\pm$ 32
15f		473	-44.5	-1.08	44 $\pm$ 16
15g		385	-44.6	-1.06	153 $\pm$ 22
15h		320	-44.6	-1.06	221 $\pm$ 15

Table 3.

Top Inhibitors Activities using AlphaLisa and Cellular Viability Assays

Cmpd	AlphaLisa IC ₅₀ (μM)	GI ₅₀ BCL6 dependent cell lines (μM)				GI ₅₀ BCL6 independent cell (μM)		
		OCI-Ly1	OCI-Ly7	SUDHL4	SUDHL6	K422	Toledo	Ly1B50
7i	170 ± 4	>500	>500	>500	>500	>500	>500	>500
13	80 ± 3	>500	>500	>500	100 ± 2	>500	>500	>500
15a	142 ± 7	>500	>500	>500	>500	>500	>500	>500
15c	112 ± 5	>500	>500	>500	>500	>500	>500	>500
15e	34 ± 7	100 ± 1	33 ± 4	103 ± 18	60 ± 1	>500	>500	>500
15f	27 ± 4	>500	193 ± 12	>500	>500	>500	>500	>500
15g	62 ± 17	>500	116 ± 13	>500	>500	>500	>500	>500

Table 4.IC₅₀s, LGFEs and Functional Group GFE Contributions for Selected Inhibitors^a

Cmpd	IC ₅₀ (μ M)	LGFE (kcal/mol)	Functional group GFE contributions (kcal/mol)				
			indole1 ^b	thiourea	indole2 ^c	piperazine	HDCH tail
15e	34 $\pm$ 7	-46.0	-22.1	-8.69	-2.24	-2.67	-10.30
15f	27 $\pm$ 4	-44.5	-21.1	-8.77	-3.00	-2.64	-8.89
15g	62 $\pm$ 17	-44.6	-21.2	-8.80	-3.01	-2.65	-8.68
15a	142 $\pm$ 7	-41.8	-20.5	-8.38	-2.53	-2.30	-8.16
15c	112 $\pm$ 5	-40.7	-21.2	-8.84	-1.89	-2.59	-5.42

^aThree best inhibitors **15f**, **15f** and **15g**, and two worst inhibitors **15a** and **15c** were selected for comparison. Functional group GFE contributions are calculated as the sum of the atomic GFE scores for the SILCS classified atoms in the respective functional groups.

^bThe indole moiety on the left side of the chemical structures.

^cThe indole moiety in the center of the chemical structures.

Table 5.

Crystallographic Data Collection and Refinement Statistics

	BCL6^{BTB}-7CC5	BCL6^{BTB}-15f	BCL6^{BTB}-15a
PDB Code	6C3N	6C3L	6CQ1
Space group	P212121	P1	P1
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	34.1 85.1 117.7	30.9 39.6 55.2	31.0, 39.6, 55.2
Cell dimensions α β γ (deg)	90 90 90	83.7 73.8 67.0	83.7, 73.6, 67.0
Resolution (Å)	2.53 (2.62 – 2.53)	1.46 (1.51 – 1.46)	1.70 (1.76 – 1.70)
Unique reflections	11920 (1054)	38467 (3801)	22269 (2398)
<i>R</i> _{merge}	0.116 (0.438)	0.066 (0.316)	.062 (.347)
<i>I</i> / σ <i>I</i>	16.6 (4.4)	11.4 (2.1)	11.5 (2.5)
Completeness (%)	84.4 (87.5)	95.9 (93.6)	86.6 (93.6)
Redundancy	6.3 (6.8)	2.0 (2.0)	2.0 (1.9)
	Refinement		
<i>R</i> _{work} / <i>R</i> _{free} (%)	20.7/26.7	19.9/22.4	18.1/21.7
No. atoms			
Protein	1937	1974	1991
Water	86	286	242
Mean B-factors (Å ²)	45.33	29.62	32.58
RMS deviations			
Bond lengths (Å)	0.003	0.002	.012
Bond angles (deg)	0.52	0.48	1.26
	Ramachandran plot		
Most favored regions (%)	97.10	98.36	98.35
Additional allowed regions (%)	2.90	1.64	1.65