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Discovery of pyrazolo[1,5-*a*]pyrimidine B-cell lymphoma 6 (BCL6) binders and optimization to high affinity macrocyclic inhibitors

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KEYWORDS

BCL6; macrocycle; PPI inhibitor, fragment; virtual screen, DLBCL, pyrazolo[1,5-*a*]pyrimidine; off-rate, NMR conformational analysis

ABSTRACT

Inhibition of the protein-protein interaction between B-cell lymphoma 6 (BCL6) and corepressors has been implicated as a therapeutic target in diffuse large B-cell lymphoma (DLBCL) cancers and profiling of potent and selective BCL6 inhibitors are critical to test this hypothesis. We identified a pyrazolo[1,5-*a*]pyrimidine series of BCL6 binders from a fragment screen in parallel with a virtual screen. Using structure based drug design, binding affinity was increased 100,000-fold. This involved displacing crystallographic water, forming new ligand-protein interactions and a macrocyclization to favor the bioactive conformation of the ligands. Optimization for slow off-rate constant kinetics was conducted as well as improving selectivity against an off-target kinase, CK2. Potency in a cellular BCL6 assay was further optimized to afford highly selective probe molecules. Only weak anti-proliferative effects were observed across a number of DLBCL lines and a multiple myeloma cell line without a clear relationship to BCL6 potency. As a result we conclude that the BCL6 hypothesis in DLBCL cancer remains unproven.

Introduction.

B-cell lymphoma 6 (BCL6) is a transcriptional repressor required for germinal center (GC) formation and maintenance during the humoral immune response but also has been implicated to play a role in lymphomas derived from GC B-cells. BCL6 was originally identified as a target of chromosomal translocations in diffuse large B-cell lymphoma (DLBCL) (~40%) and follicular lymphoma (5-10%) cancers.¹ Furthermore, in gain-of-function and loss-of-function studies, transgenic I μ HA-BCL6 mice developed lethal DLBCL-like tumors² and BCL6 shRNA induced lethality in DLBCL cell lines,³ respectively. Published studies from a BCL6 mutant knock-in mouse model provided insight on the physiological effects of targeting BCL6 but in the context of normal GC development.⁴ Small molecule BCL6 inhibitors based on a promiscuous inhibitor class⁵ and peptido-mimetic⁶ inhibitors have been reported to lead to an anti-proliferative phenotype in DLBCL. Recently, a high affinity BCL6-binding peptide has been reported.⁷ BCL6 has been proposed as an attractive cancer therapeutic target⁸ but there is a need for potent and selective tools to improve understanding of the BCL6 inhibition phenotype in DLBCL. A homodimer of the *N*-terminal Broad-Complex, Tramtrack and Bric a brac (BTB) domain of BCL6 forms an extended groove which acts as a binding site for the corepressors silencing mediator for retinoid or thyroid-hormone receptors (SMRT), nuclear receptor corepressor (NCOR) and BCL6 corepressor (BCOR).^{9, 10} The resulting protein-protein interaction (PPI) leads to the repression of genes through which the survival and proliferation effects of GC B-cells is allowed to progress. PPI inhibition provides a significant challenge within medicinal chemistry,¹¹ potentially explaining the lack of BCL6 inhibitors published to date. In this manuscript we describe how we identified and optimized inhibitors of the PPI between BCL6 and its corepressors to assess the impact of such inhibition on the proliferation of DLBCL cell lines.

Results and Discussion

Our lead generation strategy followed two parallel workstreams. Firstly, inspection of the structure of the BCL6 BTB domain homodimer in complex with the corepressors BCOR¹² and SMRT¹³ revealed what we considered to be a druggable binding pocket along the binding groove. In the case of SMRT binding, Ile1428 and His1426 sidechains occupied this pocket but these interactions did not seem to fill the entirety of the available space. Thus, a virtual screen to identify compounds that could bind to this pocket was conducted. The first step was to dock 3000 fragment-sized molecules with molecular weight less than 250 Daltons (Schrödinger, Glide). These were used to map out affinity interactions in the binding site. Several top-scoring fragment poses were identified and combined into a single ‘virtual molecule’ that was used to define the desired ligand shape and pharmacophore. The next step was to use this for a shape based virtual screen (OpenEye, ROCS) of the AstraZeneca compound collection. Approximately 8000 compounds were selected from this virtual screening exercise for screening in a BCL6 fluorescence resonance energy transfer (FRET) affinity assay. Despite numerous false positives, one hit from this screen, pyrazolo[1,5-*a*]pyrimidine **1** was confirmed to competitively displace SMRT peptide in a 2D NMR experiment, and had a FRET IC₅₀ affinity of 61 μ M (Figure 1). Secondly, a surface plasmon resonance (SPR) affinity screen of about 3500 fragments identified pyrazolo[1,5-*a*]pyrimidine **2** with a K_d of 689 μ M which also competitively displaced SMRT peptide in a 2D NMR experiment. Both initial hits **1** and **2** were crystallized with BCL6 BTB domain homodimer and found to occupy similar positions for the pyrazolo[1,5-*a*]pyrimidine bicyclic ring system deeply bound into the cleft formed between the BCL6 BTB domain homodimers (Figure 2).

Figure 1. Initial hits from virtual and fragment screen

Figure 2. Overlay of crystal structures of **1** (green, PDB code 5N20) and **2** (turquoise, PDB code 5N1X) in complex with BCL6 BTB domain homodimer (selected residues shown from the former only).

Despite the weaker affinity of **2**, it had a higher ligand efficiency¹⁴ (LE) of 0.26 than **1** (LE = 0.17). However, since the large di-substituted aryl group at the 5-position of **1** was not as buried as the rest of the molecule in the protein, this group could be removed to give **3** which attained affinity and LE in the same region as **2** (Table 1). Inspection of the crystal structure of **2** revealed that Arg24 appeared to form a cation- π interaction with the 5-pyridyl group and thus we postulated that a less planar ring system with a carboxylic acid group at this position could bind with higher affinity through an ionic interaction. The non-planar pyrrolidine analogue **4** was inactive in FRET but addition of a carboxylic acid in **5** did achieve measurable binding affinity. Likewise homologated acid **6** was measured with FRET affinity of 55 μ M

and although the LE is less than 0.3, an alternative measure of compound quality, ligand lipophilicity efficiency (LLE)¹⁵ was significantly increased due to reduced lipophilicity. Our aim in subsequent optimization was to increase FRET affinity while maintaining LE around 0.3 and LLE above 5.

Table 1. Initial SAR exploration at R1.

Cpd	R1	BCL6 FRET IC ₅₀ (μM) ^{a,b}	LE ^c	LLE ^d
3	H	>99 [SPR <i>K</i> _d = 319]	0.33 ^e	2.4 ^e
4		>99	-	-
5		45	0.26	5.7
6		64	0.24	4.7

^aMean of at least 2 independent measurements unless otherwise stated. ^bpIC₅₀ SEM <0.18. ^cLE = 1.4*pIC₅₀ (FRET)/heavy atom count. ^dLLE = pIC₅₀ (FRET)-log*D*_{7.4}. ^eusing SPR p*K*_d instead of FRET pIC₅₀.

Further inspection of the crystal structures of **1** and **2** in BCL6 revealed that the ligands only partially occupied the binding pocket (Figure 2). No solvent molecules were resolved in the remainder of the pocket for these structures, therefore 3D-RISM solvent analysis¹⁶ (CCG, MOE) was used to predict and characterize the positions of solvent molecules within the site. This analysis showed a network of unstable water molecules in the upper half of the pocket, one of which formed a hydrogen bond with the NH backbone of Glu115. This water was predicted to have high occupancy (0.79) and low stability

($\Delta G_{\text{bind}} = 2.5$ kcal/mol) suggesting that displacement would result in an increase in ligand affinity. We rationalized that increasing the size of the 7-cyclopropylamino group and adding a hydrogen bond forming moiety to interact with Glu115 could increase affinity through both Van der Waals and hydrogen bonding interactions. A bicyclic lactam group was selected to fulfill these criteria and resulted in **7** with a 150-fold increase in binding affinity (Table 2). The co-crystal structure of **7** and BCL6 revealed that the bicyclic lactam traversed the binding pocket and the lactam carbonyl formed a clear hydrogen bond with the Glu115 backbone NH as predicted (Figure 3). The carboxylic acid of **7** was proximal to Arg24 but did not appear to have the most favorable directionality for an ionic interaction and the interaction appeared to be *via* a structural water molecule. Consequently, the smaller alcohol **8** was prepared in the expectation that a less ionic but still polar hydrogen bonding interaction could be realized and indeed affinity was maintained at 0.37 μM . The importance of either the acid in **7** or alcohol in **8** was confirmed by pyrrolidine **9** which lacked these groups and had approximately 10-fold reduced affinity.

Figure 3. Crystal structure of **7** (PDB code 5N21) in complex with BCL6 BTB domain homodimer

Table 2. SAR for acyclic and macrocyclic lactam containing compounds

Cpd	R1	R2	BCL6 FRET IC ₅₀ (μM) ^{a,b}	LE ^c / LLE ^d	Cpd	R1 / R2	BCL6 FRET IC ₅₀ (μM) ^{a,b}	LE ^c / LLE ^d	Affinity fold- increase on macrocyclization
7		H	0.35	0.28 6.9	10		0.0017	0.32 9.1	206
8		H	0.37	0.30 4.1	11		0.0029	0.33 6.4	128
9		H	2.9	0.28 2.9	12		0.015	0.32 5.4	193
					13		0.21	0.27 5	14
					14		0.024	0.31 5.3	121

^aMean of at least 2 independent measurements. ^bpIC₅₀ SEM < 0.27. ^cLE = 1.4 * pIC₅₀ (FRET) / heavy atom count. ^dLLE = pIC₅₀ (FRET) - logD_{7.4}.

The next SAR breakthrough in our structure based drug design (SBDD) approach was realized through a macrocyclization strategy. The 3-position carbon within the pyrrolidine ring of **7** was measured to be 7 Å from the aryl carbon *meta* to the aniline when crystallized with BCL6. Only solvent, not protein, filled the space between these two atoms. This, coupled with the fact that the bound conformation of **7** is

not the lowest energy conformation of the isolated ligand, suggested that joining these two atoms with a linker could force the bioactive conformation of binding to BCL6 to be a more prevalent conformation. A bis-ether linker of six atoms in length was modelled to bridge the desired space and macrocycles **10**, **11** and **12** were prepared as the cyclized equivalents of **7**, **8** and **9** respectively. Gratifyingly, in all three cases, greater than 100-fold increase in affinity was realized through macrocyclization (Table 2). An additional SAR observation was that the (5*S*)-methanol substitution in **11** gave 5-fold affinity increase over 5*H* analogue **12**.

A crystal structure of **11** bound to BCL6 was obtained and a remarkably close overlay between the acyclic **7** and macrocyclic **11** was observed in what is presumed to be the bioactive conformation (Figure 4). The atoms in common between **11** and **7** overlay almost perfectly (RMSD = 0.96Å), with the exception of the substituent *alpha* to the pyrrolidine N which has switched sides due to the geometry enforced by the macrocyclic linker. The alcohol now forms hydrogen bonds to a structural water, Arg28 and back onto a pyrimidine N of the ligand. The linker is well-defined in the electron density and sits in a solvent-exposed region, on top of the protein surface, close to Tyr58, but making no polar interactions.

Figure 4. Overlay of crystal structures of **11** (magenta, PDB code 5N1Z) and **7** (orange, PDB code 5N21) in complex with BCL6 BTB domain homodimer

In order to evaluate the extent to which free molecules in solution are adopting the bioactive conformation, a NMR-conformational analysis study was conducted. This conformational analysis makes use of interproton distances derived from NOE based experiments¹⁷ and fits the NMR data to ensembles of conformers, selecting those that show the highest agreement with the experimental NMR.¹⁸ Following this approach, it was observed for compound **8** that the pyrrolidine hydrogens and the aromatic H from the pyrimidine presented broad signals in the ¹H NMR at rt, indicating the presence of a slow-intermediate exchange equilibrium between conformers affecting that part of the molecule, whereas the lactam ring showed sharp signals pointing to a fast rotation of the ring in solution. By heating the NMR sample to 70 °C, a fast exchange between conformations was achieved and all the NMR signals were sharpened up. NMR data for **8** was acquired at the latter temperature as it gave optimal spectra for the conformational analysis study (see supporting information). The analysis yielded two main conformations in solution (Figure 5) with a relative population of 60:40. The main conformation **8A** shows a good overall overlay with the X-ray structure of **11** except for the orientation of the lactam ring, which is rotated 180 degrees with respect to the bioactive conformation of **11**. The second conformer **8B** presents the lactam ring in the bioactive conformation, but shows a rotation of the pyrrolidine group compared to the X-ray structure. Therefore, the flexibility of the lactam and pyrrolidine rings prevent **8** from fully adopting the bioactive conformation.

Figure 5. NMR solution conformations and relative populations for compounds **8** and **11**. The X-ray structure of **11** as obtained bound to BCL6 (in green) is shown for comparison.

A similar NMR analysis was carried out for macrocycle **11**. In this case, the ^1H NMR at room temperature showed a pattern of signals and J -couplings indicative of a rigid linker and no experiments were needed at high temperature for the conformational study. Two main conformations were found to be populated in solution at a ratio 79:18. Both show a good agreement with the bioactive conformation of the same molecule (Figure 5), especially the most populated conformation **11A** (79%) which shows a nearly perfect match with the X-ray structure, indicating that the molecule has already adopted in solution the bioactive conformation, hence explaining the 128-fold affinity increase on macrocyclization of **8** to **11**.

It was expected that a variety of different linkers could enable the bioactive conformation to be obtained but that some linkers would have greater probability of achieving this. In order to try to predict promising linker structures a method was developed to examine the relationship between molecule conformation and affinity. There are various ways to computationally derive the conformational ensemble of a molecule. Standard rule-based methods tend to perform poorly with large rings as they fail to properly sample different conformations of the macrocycle.^{19, 20} Instead, a hybrid simulated annealing

/ large-scale low-mode sampling method (Macrocycle Conformational Sampling, Schrödinger) was performed. To ensure comprehensive sampling, 10,000 simulated annealing runs were conducted, each with 10,000 sampling search steps and the RMSD threshold for duplicate removal set to 0.001. All generated conformations were retained and sampling was assumed complete such that the conformational ensemble is representative of the real solution conformation of the molecule. Superimposition of each conformation onto the known bioactive conformation of **7** (not including the linker) was conducted and the RMSD of each individual conformation from the known bioactive conformation was calculated. The distribution of RMSD from bioactive for the entire conformational population was thus derived. It should be expected that compounds with a high proportion of conformations close to the bioactive conformation will have higher affinity than compounds that with a lower proportion of such similar conformations.

This predictive relationship between conformation ensembles and affinity was examined for five representative compounds from the existing set (**9**, **11**, **12**) and new linkers (**13** and **14**). For each compound, a distribution of RMSD values for the conformational ensemble was observed, with a tendency to show a higher proportion at lower RMSD for higher affinity compounds. The fraction of the conformation population with $\text{RMSD} < 0.5 \text{ \AA}$ to the bioactive was observed to have an approximately linear correlation with the affinity on a log scale (Figure 6). Subsequently, we used this model to help prioritize further linker designs for synthesis.

Figure 6. Plot of BCL6 FRET affinity as a function of conformation ensemble RMSD (a) where line is a linear fit, with depictions of RMSD distribution for compound **9** (b) and compound **11** (c) where line is a bimodal normal fit

The amide linker **13** was predicted to have a lower fraction of conformational ensembles matching the bioactive conformation and indeed only resulted in a 14-fold affinity increase on macrocyclization. In contrast, the fully saturated linker **14** was predicted to have a higher fraction of conformational ensembles matching the bioactive conformation and resulted in a 121-fold affinity increase. All these macrocycles met our desired LE and LLE criteria. It is worth noting that the bis-allyl compound **15** (Figure 7), which is the acyclic precursor to **11**, had a FRET affinity of 0.15 μM, indicating that addition of alkyl groups alone does not generate an affinity increase unless they are joined to form a macrocycle. Furthermore **8** and **11** are isolipophilic (measured $\log D_{7.4}$ of 2.3 and 2.2 for **8** and **11** respectively) thus a change in lipophilicity is not causing the increased affinity on macrocyclization.

Figure 7. Acyclic precursor to macrocycle **11**

In acyclic compounds such as **8**, free rotation of the pyrrolidine allows the chiral alcohol group freedom to occupy multiple conformations. Indeed, the enantiomer of **8** has a very similar binding affinity of 0.59 μM , indicating that either stereochemistry can adopt a conformation favourable to binding. When converted to a macrocycle such as **11**, the rotation of the pyrrolidine is restricted and the chiral alcohol occupies a limited range of conformations which would be expected to show different SAR. Consequently, we prepared a number of different chiral alcohol containing macrocycles to explore the effect on binding affinity for different positions of this group. This was completed in a simpler system without the lactam (Table 3). Unsubstituted pyrrolidine **16** is inactive ($> 100 \mu\text{M}$) in FRET affinity but addition of a chiral alcohol in **17** gives a measurable binding affinity of 24 μM . Macrocycle **18** contains pyrrolidine with only the macrocycle linker as a substituent and has affinity of 2.3 μM , consistent with previous increases in affinity due to macrocyclization (>43 -fold in this case). Macrocycle **19** showed that (2*R*)-methanol substitution reduced affinity 3.5-fold and (5*R*)-methanol substitution in **20** further reduced affinity. In contrast (5*S*)-methanol substitution in **21** increased affinity 6-fold which is a similar increase compared to that observed in the lactam analogues **11** and **12**. In an attempt to explain the origin of this increase, 3D-RISM solvent analysis was used to characterize solvent molecules. Analysis of the protein structure with **7**, which does not contain the methanol moiety, predicts a stable structural water adjacent to the pyrimidine N and forming a bridging interaction to Arg28 (occupancy = 0.71, $\Delta G_{\text{bind}} = -0.98$). The protein structure of **11** demonstrates that the (5*S*)-methanol group perfectly displaces this predicted water and replaces all these interactions, spanning both units of the BCL6 dimer, and thus provides an explanation for the observed increase in affinity.

Table 3. SAR for acyclic and macrocyclic compounds exploring effect of methanol substitution

Cpd	R1	R2	BCL6 FRET IC ₅₀ (μM) ^{a,b}	Cpd	R1 / R2	BCL6 FRET IC ₅₀ (μM) ^{a,b}
16		H	> 100	18		2.3
17		H	24	19		8.2
				20		> 10
				21		0.39

^aMean of at least 2 independent measurements. ^bpIC₅₀ SEM <0.18.

In our attempts to improve binding affinity to BCL6, we monitored the kinetics of binding with the aim of identifying compounds that had longer residence times and thus gave potential for longer lasting effects in cellular assays.²¹ A selection of kinetic data is shown in Table 4 to illustrate our findings. Gratifyingly, the binding affinity from SPR correlated very well with the affinity measured in FRET ($R^2 = 0.99$). As affinity was increased, the on and off-rate constants were not modulated equally (Figure 8). Addition of the lactam ring (purple arrow) gave a significant decrease in the off-rate constant and a smaller increase in on-rate constant. The transformation from acyclic to macrocycle (green arrows) gave a

substantially greater decrease in off-rate constant compared to increase in on-rate constant. Addition of the (5*S*)-methanol (or (5*S*)-acetic acid) to a macrocyclic compound gave a substantial decrease in off-rate constant (pink arrows). Thus, the affinity increase observed on macrocyclization of **7** to **10** and **8** to **11** can be understood as a combination of two factors. Firstly, an on-rate constant increase due to a more rigid linker giving the preorganized binding conformation and secondly, the delivery of the (5*S*)-methanol or (5*S*)-acetic acid to a favorable position for multiple hydrogen bonding interactions hence giving an off-rate constant decrease. Consequently, for the remainder of our optimization, the lactam ring, macrocycle and (5*S*)-methanol were retained.

Table 4. FRET affinity and binding kinetics for BCL6 of selected compounds

Cpd	BCL6 FRET IC ₅₀ (μM) ^{a,b}	BCL6 SPR K _d (μM) ^{a,c}	BCL6 SPR k _{on} (M ⁻¹ .s ⁻¹) ^{a,d}	BCL6 SPR k _{off} (s ⁻¹) ^{a,e}
5	45	60 ^f	-	-
7	0.35	1.1 ^f	705000 ^f	0.77 ^f
8	0.37	0.50	865000	0.44
10	0.0017	0.0034 ^f	4430000 ^f	0.015 ^f
11	0.0029	0.0065	1560000	0.0083
12	0.015	0.022	5530000	0.14
21	0.39	0.39	506000	0.24
25	0.0015	0.0050 ^f	3270000 ^f	0.016 ^f

^aMean of at least 2 independent measurements unless otherwise stated. ^bpIC₅₀ SEM <0.12. ^cpK_d SEM <0.06. ^dlogSEM <0.26.

^elogSEM <0.22. ^fn=1

Figure 8. On and off-rate constants for compounds in Table 4. Arrows connect matched molecular pairs: pink arrow indicates addition of (5*S*)-methanol or (5*S*)-acetic acid, green arrow indicates macrocyclization and purple arrow indicates addition of lactam ring.

Our original virtual screening hit **1** originated from an AstraZeneca project targeting the kinase CK2.²² Thus we profiled our lead BCL6 macrocycle **11** across a panel of 126 kinases to understand kinase selectivity (see supporting information). Since we had already performed significant optimization for BCL6, we anticipated that CK2 activity would have been reduced. The only kinase to exhibit greater than 60% inhibition at 1 μM across the panel was indeed CK2 and this translated into a CK2 IC₅₀ of 0.64 μM (220-fold margin vs BCL6). The original hit **1** had CK2 IC₅₀ of 0.012 μM in the same assay, thus the CK2 activity had been reduced 50-fold. However, since the pyrazolo[1,5-*a*]pyrimidine core of our BCL6 binders had been common to a series of AstraZeneca CK2 inhibitors, we sought to further develop SAR against CK2. A crystal structure of **11** bound to CK2 was obtained which showed that the aniline NH of **11** formed a key hydrogen bond in both proteins (Figure 9). In CK2, this interaction was with the hinge domain of the kinase (Val116) while for BCL6 a strong hydrogen bond to the carbonyl of Met51 was formed. While structurally quite different the binding pockets of BCL6 and CK2 exhibit similar interactions and **11** shows very similar bound conformations in each case: both show polar interactions for the lactam group and for the (5*S*)-methanol group, underlining the potential difficulty in obtaining selectivity over CK2.

Figure 9. Crystal structures of **11** bound to BCL6 (a, PDB code 5N1Z) and CK2 (b, PDB code 5N1V) showing nearly identical binding modes.

As the 3-cyano group appeared to form a key interaction with Asp175 (via a bridging water molecule) in CK2 with no equivalent interaction in BCL6, we probed the SAR at this position (Table 5). BCL6 affinity was highest for the 3-CN analogue **22** and when only a hydrogen was present at this position (**23**) BCL6 affinity was reduced 20-fold along with a loss of CK2 activity ($>10\ \mu\text{M}$), as anticipated from structural analysis. Changing the 3-CN to 3-Cl in **24** resulted in similar BCL6 affinity with 3.5-fold reduction in CK2 activity, presumably due to the 3-Cl being unable to make the water mediated polar interaction with Asp175 in CK2. This led us to prefer the use of 3-Cl in subsequent analogues.

Table 5. SAR exploration at R3.

Cpd	R3	BCL6 FRET IC ₅₀ (μM) ^{a,b}	CK2 IC ₅₀ (μM) ^{a,c}	BCL6: CK2 selectivity
22	CN	0.0024	0.40	166
23	H	0.055	>10	>181
24	Cl	0.0033	1.4	424

^aMean of at least 2 independent measurements. ^bpIC₅₀ SEM <0.22. ^cpIC₅₀ SEM <0.14.

Finally, the SAR around substitution of the lactam nitrogen (R4) was explored (Table 6). With high affinity BCL6 binders, we were able to demonstrate activity in a BCL6 cell reporter assay and develop cellular SAR. The unsubstituted analogue **25** exhibited high BCL6 affinity, weak CK2 activity and modest BCL6 cell activity (4.1 μM). The measured intrinsic permeability of **25** in a Caco-2 assay was measured to be high ($P_{app} = 46 \times 10^{-6} \text{cm} \cdot \text{s}^{-1}$) and in a bidirectional Caco-2 efflux assay the efflux ratio was measured to be 1.4. Aqueous solubility of **25** was measured as 31 μM thus it was difficult to attribute a physicochemical reason for the large drop-off from binding affinity to cellular activity against BCL6. We believed it was likely that although the FRET binding affinity is a direct measure of affinity to the BCL6 BTB domain homodimer, this assay may not be fully representative of the binding of compound to BCL6 within a complex cellular environment in the presence of endogenous corepressors. Consequently, although we wished to maintain high FRET affinity, our focus was to develop SAR and increased potency against the BCL6 cell reporter assay.

Lipophilic groups of increasing size at R4 maintained high affinity for BCL6 while maintaining CK2 activity above 1 μM (**26 -28**). Notably, the largest of these, containing the di-fluorocyclobutyl group (**28**), gave a 10-fold increase in BCL6 cell potency. Analysis of all BCL6 crystal structures could not rationalize a binding interaction between the di-fluorocyclobutyl group and protein as it would be expected to occupy a solvent accessible space. Encouraged by the cell potency of this group, carboxylic acid **29** with the same R4 was prepared but BCL6 affinity and cell potency decreased 3-fold and 6-fold respectively and CK2 activity was increased 13-fold. The acid side-chain of Glu115 exists on the BCL6 homodimer surface and compound **30** with a basic sidechain was designed to make an interaction with

this acid. High BCL6 affinity was observed for **30** but the cell activity remained above 1 μM . Thus we returned to lipophilic R4 groups and a range of benzylic compounds (**31-36**) were prepared. All these benzyl compounds had similar BCL6 binding affinity of around 0.01 μM and showed no activity against CK2 when a 3-Cl substituent was employed. Comparison of 3-Cl **32** with 3-CN **33** highlighted that even in these optimized compounds the 3-CN substituent still produced some weak CK2 activity. Sub-micromolar BCL6 cell potency with SAR was observed for all these benzyl compounds irrespective of the 3-substituent. Methoxy substitution at the *meta*-benzyl position in **32** displayed 0.10 μM cell potency and methyl substitution at the *ortho*-benzyl position in **34** gave 0.087 μM cell potency. Moving the methyl to the *para*-benzyl position in **35** reduced cell potency 10-fold to 0.83 μM . Aza-incorporation in pyridine **36** was tolerated and afforded 0.43 μM cell potency. For comparison, FX1, the most potent BCL6 small molecule inhibitor reported to date has BCL6 binding affinity of 37 μM in our FRET assay and a best measured cell potency of 26 μM (close to the 30 μM top concentration used) which is consistent with reported data.⁵

Table 6. SAR exploration at R4.

Cpd	R3	R4	BCL6 FRET IC ₅₀ (μM) ^{a,b}	CK2 IC ₅₀ (μM) ^{a,c}	BCL6 cell IC ₅₀ (μM) ^{a,d}
25	Cl	H	0.0015	7.3	4.1
26	Cl	Me	0.0049	7.0	6.1
27	Cl	<i>c</i> PrCH ₂	0.036	2.2	5
28	Cl	3,3-diFcBu CH ₂	0.0050	2.1	0.48

29	-	-	0.016	0.16	3.2
30	Cl	morpholino- CH ₂ CH ₂ CH ₂	0.00087	5.9	1.1
31	Cl	Bn	0.011	>10	0.20
32	Cl	m-MeOBn	0.012	>10	0.10
33	CN	m-MeOBn	0.011	1.2	0.14
34	Cl	o-MeBn	0.013	>10	0.087
35	Cl	p-MeBn	0.029	>10	0.83
36	Cl	4-pyridyl-CH ₂	0.0099	>10	0.43
FX1	-	-	49	0.69	>30 ^e

^aMean of at least 2 independent measurements. ^bpIC₅₀ SEM <0.26. ^cpIC₅₀ SEM <0.18. ^dpIC₅₀ SEM <0.25. ^ean effect was observed during 3 independent measurements with IC₅₀ = 26 μM obtained on one occasion and >30 μM on two other occasions.

All compounds tested in the BCL6 cell reporter assay were also tested in a PLZF cell reporter assay to rule out pan-BTB domain protein activity. All compounds were inactive (>30 μM) in the PLZF assay. Compound **31** was selected from our set of potent cell active BCL6 inhibitors and profiled across a panel of 398 kinases screened at 1 μM at ThermoFisher. No activity above 70% was detected for any kinase (see supporting information). Furthermore, *in vitro* pharmacological profiling²³ of **31** against a panel of over 30 diverse targets was conducted and exhibited a good selectivity profile (see supporting information) with only two targets showing activity below 1 μM (GABA receptor, *K_i* = 0.25 μM; 5-hydroxytryptamine receptor 1B, EC₅₀ = 0.57 μM). This profiling led us to conclude that we had achieved our aim to develop highly selective cell potent BCL6 inhibitors.

Antiproliferative activity

DLBCL cell lines have been classified as BCL6 dependent or independent on the basis of whether they are affected by BCL6 inhibition or knockdown.^{3, 5, 6} We sought to examine the antiproliferative activity of our BCL6 inhibitors in a range of DLBCL cell lines (both GCB and ABC subtypes) based on this classification of BCL6 dependent (e.g. OCI-Ly1, SUDHL-4, OCI-Ly3, SUDHL2, U-2932, OCI-Ly10, TMD8) and BCL6 independent (e.g. Karpas 422, OCI-Ly19) lines (Table 7). In addition, we included a

multiple myeloma (MM) cell line as a cell line that we expected to be insensitive to BCL6 inhibition given that MM lines derive from post GC-B cells and generally express weak or undetectable levels of BCL6.²⁴ FX1 displayed weak antiproliferative activity across the entirety of our cell panel. We hoped that as we tested increasingly more potent and selective BCL6 inhibitors, a clear pattern of inhibition dependent on sensitivity to BCL6 would emerge. However, as illustrated in Figure 10, weak antiproliferative activity was consistently observed across the entirety of the cell panel. Those DLBCL cell lines previously defined as BCL6 sensitive did not show any difference from those defined as insensitive and the pattern of activity in the MM line was also similar. This leads us to conclude that the inhibition of BCL6 is either not relevant for proliferation of these DLBCL lines or that the effect is too weak to be of significance. Consequently, we conclude that the BCL6 hypothesis as a means of treatment for DLBCL is still unproven and we have elected not to progress this series of BCL6 inhibitors further into development.

Table 7. BCL6 cell potency and cell proliferation data

Cpd	BCL6 cell pIC50 ^{a,b}	GCB Krpas422 pGI50 ^{a,c}	GCB OCI-Ly1 pGI50 ^{a,d}	GCB SUDHL4 pGI50 ^{a,e}	GCB OCI-Ly19 pGI50 ^{a,f}	ABC OCI-Ly3 pGI50 ^{a,g}	ABC SUDHL2 pGI50 ^{a,h}	ABC U-2932 pGI50 ^{a,i}	ABC OCI-Ly10 pGI50 ^{a,j}	ABC TMD8 pGI50 ^{a,k}	MM AMO-1 pGI50 ^{a,l}
11	5.7	5.1	5.0	5.1	5.2	5.4	5.5	5.2	5.3	5.1	4.8 ^m
14	4.8	5.1	5.1	5.0	5.1	5.4	5.0	5.0	5.1	5.3	5.0 ^m
22	5.2	4.7	4.6	4.6	4.8	4.9	4.9	4.8	5.0	4.8	4.7
23	5.0	4.9	4.9	4.7	4.9	4.8	5.0	4.8	4.9	5.0	4.9 ^m
24	5.4	5.3	5.2	5.0	5.8	5.5	5.4	5.3	5.6	5.5	5.4 ^m
25	5.4	5.3	5.4	5.2	5.7	5.7	5.4	5.6	5.8	5.6	5.5 ^m
26	5.2	5.3	5.2	5.2	5.3	5.2	5.5	5.3	5.2	5.4	5.3 ^m
27	5.3	<4.5	<4.5	<4.5	4.8	<4.5	4.7	5.0	4.7	4.6	<4.5 ^m
28	6.3	5.0	5.1	5.0	5.2	4.9	5.1	5.2	5.1	5.0	4.6
29	5.5	4.7	4.8	4.8	5.0	4.7	4.7	4.8	4.8	4.8	4.5
30	5.9	4.9	5.0	4.9	5.1	5.0	5.0	5.0	5.1	5.0	4.9
31	6.7	4.6	4.9	4.8	5.0 ^m	4.9 ^m	5.0 ^m	5.1 ^m	5 ^m	5.0	4.6
32	7.0	4.6	5.2	4.5 ^m	5.0	<4.5	5.2	4.8	5.2	4.7	4.8
33	6.9	5.3	5.5	5.2	5.3	5.2	5.6	5.4	5.4	5.4	5.0
34	7.1	<4.5	5.0	5.2	5.0	4.8	5.2	5.0	5.0	4.7	4.6
35	6.1	<4.5	5.1	4.7	5.0	4.8	5.2	5.0	4.9	4.8	4.7
36	6.4	4.9	5.0	5.0	4.9	4.9	5.1	5.0	5.0	5.0	4.7

FX1 | <4.5 | 4.9 4.7 5.0 5.0 | 4.7 4.9 4.9 4.9 5.0 | 4.7
^aMean of at least 2 independent measurements unless otherwise stated. ^bpIC₅₀ SEM <0.25. ^cpIC₅₀ SEM <0.24. ^dpIC₅₀ SEM <0.18. ^epIC₅₀ SEM <0.25. ^fpIC₅₀ SEM <0.35. ^gpIC₅₀ SEM <0.43. ^hpIC₅₀ SEM <0.25. ⁱpIC₅₀ SEM <0.27. ^jpIC₅₀ SEM <0.14. ^kpIC₅₀ SEM <0.25. ^lpIC₅₀ SEM <0.15. ^mn=1

Figure 10. Plot of proliferation pGI₅₀ variation with BCL6 cell pIC₅₀ for compounds in Table 7 (FX1 is marked by a diamond, AstraZeneca compounds by circles and each compound is a different colour)

Synthesis

Aromatic nucleophilic substitution of the corresponding 5,7-dichloropyrazolopyrimidines with nitrogen nucleophiles was a key reaction for efficient assembly of all BCL6 inhibitors prepared during this work. For example, in a simple one-pot procedure, dichloropyrazolopyrimidine **37** reacted first with 6-amino-3,4-dihydroquinolin-2(1*H*)-one then (*S*)-2-(pyrrolidin-2-yl)acetic acid to afford **7** in 41% yield (Scheme 1).

Scheme 1. Synthesis of acyclic pyrazolo[1,5-*a*]pyrimidines^a

^aReagents and conditions: (a) *c*-PrNH₂, EtOH, 99%; (b) Pd/C, H₂, EtOAc, 53%; (c) *c*-PrNH₂ or PhNH₂ or 6-amino-3,4-dihydroquinolin-2(1*H*)-one, DIPEA, NMP, rt-100 °C, then pyrrolidine-R6, μw , 65-130 °C, 25-64%; (d) 6-amino-3,4-dihydroquinolin-2(1*H*)-one, EtOH, 80 °C, 91%; (e) (*S*) or (*R*)-prolinol, DIPEA, NMP, 80 °C, 65-67%.

Initially, we utilized the ring-closure metathesis (RCM) reaction between two allyloxy groups to form the large rings of macrocyclic inhibitors. For instance, the bicyclic aniline **39** reacted with the dichloropyrazolopyrimidine **37** and then with 3-(allyloxy)pyrrolidine to afford bis-allyloxy derivative **40** which underwent RCM in the presence of Grubbs II catalyst to afford the macrocycle **12** (Scheme 2). The acid containing analogue **10** was prepared in a similar fashion from **39** and the disubstituted pyrrolidine **42**.

Scheme 2. Synthesis of macrocycles using RCM route^a

^aReagents and conditions: (a) 2M K₂CO₃, MeOH, rt; (b) allyl bromide, K₂CO₃, DMF, rt; (c) Zn, AcOH, 60 °C, 79% (3 steps); (d) **37**, DIPEA, EtOH, 100 °C, then (*S*)-3-(allyloxy)pyrrolidine hydrochloride, 100 °C, 54%; (e) Grubbs II catalyst, DCM, 40 °C, 40%; (f) (Boc)₂O, Et₃N, DCM, rt, 49%; (g) DIAD, PPh₃, 4-nitrobenzoic acid, THF, 0 °C; (h) NaOH, MeOH, THF, rt, 67% (2 steps); (i) NaOH, MeOH, rt, 91%; (j) allyl bromide, NaH, THF/DMSO, rt, 58%; (k) TMSCHN₂, PhMe, MeOH, rt, 68%; (l) HCl in dioxane, rt, 95%; (m) **39**, **37**, DIPEA, EtOH, 80 °C, 74%; (n) **42**, DIPEA, EtOH, 130 °C, 85%; (o) Grubbs II catalyst, DCM, 40 °C, 41%; (p) LiOH, H₂O, MeOH, 40 °C, 59%.

Alternatively, we developed a novel strategy for the construction of diverse macrocyclic motifs. Our new approach was based on the double aromatic nucleophilic substitution reaction between pre-assembled masked di-nucleophiles (*e.g.* **47**) with corresponding heterocyclic di-electrophiles (**37** or **48**, Scheme 3). In this sequence the nitro group in **47** was reduced to the corresponding aniline which subsequently reacted with 5,7-dichloropyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (**37**) by displacing the more reactive chlorine at the 7-position. Both trityl and Boc protecting groups were removed simultaneously under acidic conditions and the resulting secondary amine displaced the chlorine at the 5-position under thermal conditions to afford the macrocycle **11** in 46% overall yield. Using this double S_NAr attack (SNACK) strategy the macrocyclic linker could be easily varied and a number of diverse

macrocycles including **25**, **13**, **14** and **22-24** were obtained by cyclizing corresponding masked di-nucleophiles **47**, **50**, **53** and **56**, respectively (Scheme 3).

Scheme 3. Synthesis of macrocycles using double S_NAr (SNACK) route^a

^aReagents and conditions: (a) K_2CO_3 , MeOH, rt, 95%; (b) TrCl, Et_3N , DMAP_{cat}, DCM, rt, 66%; (c) (*E*)-1,4-dibromobut-2-ene, $n-Bu_4HSO_4$ cat, PhMe, $NaOH_{aq}$, rt, 65%; (d) K_2CO_3 , DMF, 80 °C, 96%; (e) Fe, NH_4Cl , EtOH, H_2O , 60 °C, 96%; (f) **37** or **48**, DIPEA, EtOH, 60 °C; (g) TFA, DCM, rt; (h) DIPEA, *n*-BuOH, μw , 130 °C, 46% for **11**, 7% for **25** (3 steps); (i) *tert*-butyl (2-

hydroxyethyl)carbamate, DIAD, Ph₃P, DCM, rt, 88%; (j) HCl, 1,4-dioxane, MeOH, 84%; (k) (*R*)-2-(1-(*tert*-butoxycarbonyl)pyrrolidin-2-yl)acetic acid, DIPEA, HATU, DMF, 81%; (l) Pd/C, H₂, MeOH, quant.; (m) **37**, DIPEA, EtOH, 70 °C; (n) HCl, 1,4-dioxane, rt; (o) DIPEA, *n*-BuOH, μ w, 120 °C, 11% (3 steps); (p) 1,4-dibromobutane, *n*-Bu₄HSO₄ cat, PhMe, NaOH_{aq}, rt, 88%; (q) **38**, K₂CO₃, DMF, 70 °C, 48%; (r) Pd/C, 1-methylcyclohexa-1,4-diene, EtOH, 80 °C, 100%; (s) **37**, DIPEA, EtOH, 80 °C; (t) HCl, 1,4-dioxane, rt; (u) DIPEA, *n*-BuOH, μ w, 120 °C, 42% (3 steps); (v)) TrCl, Et₃N, DMAP_{cat}, DCM rt, 66%; (w) 1,4-dibromobutane, *n*-Bu₄HSO₄ cat, PhMe, NaOH_{aq}, rt, 93%; (x) **44**, K₂CO₃, DMF, 80 °C, 99%; (y) Pd/C, HCOONH₄, EtOH, 80 °C, 100%; (z) **37** or **57** or **48**, DIPEA, EtOH, 80 °C; (aa) HCl, 1,4-dioxane, rt; (ab) DIPEA, *n*-BuOH, μ w, 120-150 °C, 14-28% (3 steps).

The substituent on the lactam nitrogen was introduced by alkylation of the corresponding 6-nitro bicyclic lactams (e.g. **47**) with alkyl/benzyl bromides or iodides (Scheme 4). R₄ as a pyridin-4-ylmethyl was introduced using Mitsunobu reaction of **47** with pyridin-4-ylmethanol. The macrocycle **29** was prepared from **60** employing RCM. Other analogues having a substituent on the lactam nitrogen (**26-28** and **30-36**) were synthesized from the aniline **62** using our preferred SNACK approach.

Scheme 4. Synthesis of R₄ substituted macrocycles^a

^aReagents and conditions: (a) 3-(bromomethyl)-1,1-difluorocyclobutane, Cs₂CO₃, DMF, 80 °C, 82%; (b) Fe, EtOH, H₂O, 80 °C, 92%; (c) **48**, DIPEA, EtOH, 80 °C, 82%; (d) **42**, DIPEA, EtOH, 130 °C; (e) TMSCHN₂, PhMe, MeOH rt, 21% (2 steps); (f) Grubbs II catalyst, DCM, 40 °C, 35%; (g) LiOH, H₂O, MeOH, 40 °C, 54%; (h) R4Br or R4I, Cs₂CO₃, DMF, rt-80 °C, 59-93%; (i) pyridin-4-ylmethanol, 2-(tributyl-15-phosphanylidene)acetonitrile, THF, μ w, 150 °C, 36%; (j) morpholine, *i*-PrOH, μ w, 100 °C, 97%; (k) Fe, NH₄Cl, THF, MeOH, H₂O, reflux, 54-100%; (l) **37** or **48**, DIPEA, EtOH, 60-80 °C; (m) HCl, 1,4-dioxane, rt or TFA, DCM, rt; (n) DIPEA, *n*-BuOH, μ w, 130-140 °C, 13-82% (3 steps).

Conclusion

Starting with a fragment screen and virtual screen we identified weak hits targeting the PPI interaction between BCL6 and its corepressors. Using SBDD, binding affinity was increased 100,000-fold. This involved displacing binding site water, forming new ligand-protein interactions and a macrocyclization to favour the bioactive conformation of the ligands. Optimization for slow-off rate constant kinetics was conducted as well as improving selectivity against an off-target kinase, CK2. Potency in a cellular BCL6 assay was further optimized to afford highly selective probe molecules. Anti-proliferative activity was assessed across a number of DLBCL lines, and a MM cell line. Despite increasing BCL6 potency, only

weak anti-proliferative effects were observed across the cellular panel with no clear relationship to BCL6 sensitive or insensitive lines. As a result, we conclude that the BCL6 hypothesis as a means of treatment for DLBCL remains unproven.

Experimental Section

General. All solvents and chemicals used were reagent grade. Anhydrous solvents THF, DCM and DMF were purchased from Aldrich. Flash column chromatography was carried out using prepacked silica cartridges (from 4 g up to 330 g) from RedisepTM or Silicycle and eluted using an Isco Companion system. Purity and characterization of compounds were established by a combination of liquid chromatography-mass spectroscopy (LC-MS), gas chromatography-mass spectroscopy (GC-MS) and NMR analytical techniques and was >95% for all test compounds. ¹H NMR were recorded on a Varian INOVA (600 MHz), Varian Gemini 2000 (300 MHz) or Bruker Avance DPX400 (400 MHz) and were determined in CDCl₃, DMSO-d₆ or MeOH-d₄. Chemical shifts are reported in ppm relative to TMS (0.00 ppm) or solvent peaks as the internal reference. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad peak. Elevated temperatures were used where necessary to sharpen broad NMR peaks due to rotamers and the temperature used is noted for such compounds. Merck precoated TLC plates (silica gel 60 F₂₅₄, 0.25 mm, art. 5715) were used for TLC analysis.

Synthesis of representative key examples (11, 31, 32 and 34)

6-Nitro-2-oxo-1,2,3,4-tetrahydroquinolin-8-yl acetate (38)

Fuming nitric acid (180 mL, 3.8 mol) was added slowly to a suspension of 2-oxo-1,2,3,4-tetrahydroquinolin-8-yl acetate (78 g, 0.38 mol) in AcOH (570 mL) cooled in a water bath. The addition was controlled to keep the internal temperature below 35 °C. After addition was complete, the solution was stirred (a precipitate started to form after 5 min) for a further 25 min. The suspension was poured into ice water (1.56 L) and stirred for 20 min. The precipitate was collected by filtration, washed with water

(400 mL), diethylether (200 mL) and dried under vacuum to afford the title compound (90 g, 95%) as a cream solid. ^1H NMR (400 MHz, DMSO- d_6) δ 2.32 (s, 3H), 2.52-2.57 (m, 2H), 3.04-3.09 (m, 2H), 7.97 (d, $J = 2.5$ Hz, 1H), 8.02-8.05 (m, 1H), 10.53 (br s, 1H); MS (ESI) $[\text{M}-\text{H}]^-$ m/z 249.

8-Hydroxy-6-nitro-3,4-dihydroquinolin-2(1H)-one (44)

A solution of K_2CO_3 (2M in water) (216 mL, 431 mmol) was added to **38** (90 g, 360 mmol) in MeOH (1 L) and the resulting mixture was stirred for 10 min. The mixture was diluted with water (1 L), acidified to pH 1 with 2M HCl and the resulting solid was collected by filtration, washed with water and dried under vacuum to afford the title compound (71 g, 95%) as a yellow solid. ^1H NMR (500 MHz, DMSO- d_6) δ 2.51-2.54 (m, 2H), 2.94-3.01 (m, 2H), 7.52 (d, $J = 2.5$ Hz, 1H), 7.63-7.69 (m, 1H), 9.51 (s, 1H), 10.72 (s, 1H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 25.07, 30.44, 108.59, 114.87, 125.45, 133.47, 141.92, 144.32, 170.23; HRMS (ES^+) for $\text{C}_9\text{H}_8\text{N}_2\text{O}_4$ ($\text{M}+\text{H}$) $^+$: calcd, 209.0557; found, 209.0558

(2S,4S)-tert-Butyl 4-hydroxy-2-((trityloxy)methyl)pyrrolidine-1-carboxylate

DMAP (1.57 g, 12.9 mmol) was added to a stirred solution of (2S,4S)-tert-butyl 4-hydroxy-2-(hydroxymethyl)pyrrolidine-1-carboxylate (**45**) (35 g, 129 mmol), trityl chloride (72 g, 256 mmol) and triethylamine (36 mL, 256 mmol) in DCM (400 mL). The mixture was stirred at 20 °C for 3 d. The mixture was concentrated to 75% of the initial volume. The reaction mixture was quenched with water (200 mL), extracted with diethylether/heptane (1:1) (2 x 1 L), the organic layer was dried over MgSO_4 , filtered and evaporated. The crude product was purified by flash silica chromatography, elution gradient 10-30% EtOAc in heptane to afford the title compound (39 g, 66%) as a white crystalline solid. ^1H NMR (400 MHz, DMSO- d_6 , 100 °C) δ 1.28 (s, 9H), 1.88-2 (m, 1H), 2.15 (ddd, $J = 6.2, 8.3, 14.2$ Hz, 1H), 2.98 (dd, $J = 4.4, 11.2$ Hz, 1H), 3.13-3.2 (m, 1H), 3.28 (dd, $J = 4.6, 8.4$ Hz, 1H), 3.54 (dd, $J = 5.9, 11.2$ Hz, 1H), 3.90 (tt, $J = 4.5, 8.5$ Hz, 1H), 4.13-4.21 (m, 1H), 4.54 (d, $J = 4.0$ Hz, 1H), 7.19-7.26 (m, 3H), 7.26-7.34 (m, 6H), 7.37 (dt, $J = 1.9, 8.4$ Hz, 6H); HRMS (ES^+) for $\text{C}_{29}\text{H}_{33}\text{NO}_4$ ($\text{M}+\text{Na}$) $^+$: calcd, 482.2307; found, 482.2296.

tert-Butyl (2S,4S)-4-(((E)-4-bromobut-2-en-1-yl)oxy)-2-((trityloxy)methyl)pyrrolidine-1-carboxylate (46)

50% Sodium hydroxide solution (10 mL) was added to a vigorously stirred solution of *tert*-butyl (2*S*,4*S*)-4-hydroxy-2-((trityloxy)methyl)pyrrolidine-1-carboxylate (2.06 g, 4.48 mmol), (*E*)-1,4-dibromobut-2-ene (4.31 g, 20.2 mmol) and tetrabutylammonium hydrogensulfate (76 mg, 0.22 mmol) in toluene (30 mL). The reaction mixture was stirred at rt for 18 h. The mixture was diluted with water (50 mL) and diethylether (150 mL). The organic phase was separated and washed with water (2 x 50 mL) and saturated brine (50 mL). The organic layer was dried over MgSO₄, filtered and evaporated to afford a residue which was purified by flash silica chromatography, elution gradient 0 to 30% EtOAc in heptane to afford the title compound (1.72 g, 65%) as a colourless oil. ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.30 (s, 9H), 2.03-2.21 (m, 2H), 3.01-3.06 (m, 1H), 3.11 (dd, *J* = 2.8, 11.7 Hz, 1H), 3.30 (dd, *J* = 4.7, 8.3 Hz, 1H), 3.55 (dd, *J* = 5.7, 11.8 Hz, 1H), 3.83-3.89 (m, 2H), 3.91-4.08 (m, 4H), 5.71-5.81 (m, 2H), 7.17-7.45 (m, 15H). ¹³C NMR (101 MHz, CDCl₃) δ 28.40, 31.90, 34.02, 51.88, 53.40, 56.26, 64.55, 68.21, 79.54, 86.34, 126.89, 127.75, 128.02, 128.76, 131.70, 144.21, 154.38; HRMS (ES⁺) for C₃₃H₃₈NO₄Br (M+Na)⁺: calcd, 614.1882; found, 614.1889.

(2*S*,4*S*)-*tert*-Butyl 4-(((*E*)-4-((6-nitro-2-oxo-1,2,3,4-tetrahydroquinolin-8-yl)oxy)but-2-en-1-yl)oxy)-2-((trityloxy)methyl)pyrrolidine-1-carboxylate (47)

A mixture of **44** (2.0 g, 14.5 mmol), **46** (1.83 g, 3.09 mmol) and DMF (15 mL) was stirred at 80 °C for 75 min. After cooling to rt the mixture was diluted with EtOAc (250 mL) and the solid was filtered off. The filtrate was washed with 1M citric acid (25 mL), water (2 x 25 mL) and saturated brine (25 mL). The organic layer was dried over Na₂SO₄, filtered and evaporated to afford a residue which was purified by flash silica chromatography, elution gradient 0 to 70% EtOAc in heptane to afford the title compound (2.14 g, 96%) as a pale yellow solid. ¹H NMR (400 MHz, DMSO-*d*₆, 100 °C) δ 1.31 (s, 9H), 2.23-2.04 (m, 2H), 2.54-2.61 (m, 2H), 3.00-3.10 (m, 3H), 3.12 (ddd, *J* = 0.8, 3.5, 11.7 Hz, 1H), 3.32 (dd, *J* = 4.7, 8.3 Hz, 1H), 3.58 (dd, *J* = 5.8, 11.7 Hz, 1H), 3.91 (dq, *J* = 1.3, 2.7 Hz, 2H), 3.98 (tt, *J* = 4.3, 8.1 Hz, 1H), 4.04 (tt, *J* = 3.6, 5.7 Hz, 1H), 4.70-4.76 (m, 2H), 5.85-5.90 (m, 2H), 7.21-7.24 (m, 2H), 7.25 (t, *J* = 1.4 Hz, 1H), 7.27-7.34 (m, 6H), 7.34-7.43 (m, 6H), 7.70 (d, *J* = 2.4 Hz, 1H), 7.78 (dd, *J* = 1.1, 2.3 Hz, 1H), 9.10 (br s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆, 100 °C) δ 25.17, 28.61, 30.33, 33.86, 52.50, 56.32, 65.17,

68.51, 69.87, 76.97, 79.04, 86.55, 107.27, 116.67, 125.16, 126.63, 127.29, 128.11, 128.77, 131.50, 134.65, 142.51, 144.54, 144.97, 153.99, 169.85; HRMS (ES⁺) for C₄₂H₄₅N₃O₈ (M+Na)⁺: calcd, 742.3099; found, 742.3098.

(2*S*,4*S*)-tert-Butyl 4-(((*E*)-4-((6-amino-2-oxo-1,2,3,4-tetrahydroquinolin-8-yl)oxy)but-2-en-1-yl)oxy)-2-((trityloxy)methyl)pyrrolidine-1-carboxylate

Iron powder (0.366 g, 6.56 mmol) was added to **47** (787 mg, 1.09 mmol) and ammonium chloride (41 mg, 0.77 mmol) in EtOH (15 mL) and water (5 mL). The resulting mixture was stirred at 60 °C for 4 h. The reaction mixture was evaporated to dryness, redissolved in EtOAc (100 mL), and washed sequentially with water (2 x 100 mL) and saturated brine (100 mL). The organic layer was dried with MgSO₄, filtered and evaporated to afford the title compound (727 mg, 96%) as a yellow foam. ¹H NMR (400 MHz, DMSO-d₆, 100 °C) δ 1.29 (s, 9H), 2.05-2.2 (m, 2H), 2.33-2.41 (m, 2H), 2.69-2.77 (m, 2H), 3.05 (t, *J* = 8.1 Hz, 1H), 3.11 (dd, *J* = 3.2, 11.7 Hz, 1H), 3.30 (dd, *J* = 4.7, 8.3 Hz, 1H), 3.56 (dd, *J* = 5.7, 11.7 Hz, 1H), 3.88 (d, *J* = 3.5 Hz, 2H), 3.96 (dq, *J* = 4.1, 4.5, 8.1 Hz, 1H), 4.02 (tt, *J* = 2.9, 5.8 Hz, 1H), 4.42-4.52 (m, 4H), 5.72-5.86 (m, 2H), 6.06 (d, *J* = 1.6 Hz, 1H), 6.20 (d, *J* = 2.2 Hz, 1H), 7.18-7.25 (m, 3H), 7.26-7.32 (m, 6H), 7.34-7.4 (m, 6H), 7.92 (s, 1H); HRMS (ES⁺) for C₄₂H₄₇O₆N₃ (M+H)⁺: calcd, 690.3538; found, 690.3534.

(13*S*,15*S*,26*Z*,7*E*)-15-(Hydroxymethyl)-42-oxo-41,42,43,44-tetrahydro-5,10-dioxo-3-aza-4(6,8)-quinolina-2(5,7)-pyrazolo[1,5-*a*]pyrimidina-1(1,3)-pyrrolidinacyclodecaphan-7-ene-23-carbonitrile (11**)**

To (2*S*,4*S*)-tert-butyl 4-(((*E*)-4-((6-amino-2-oxo-1,2,3,4-tetrahydroquinolin-8-yl)oxy)but-2-en-1-yl)oxy)-2-((trityloxy)methyl)pyrrolidine-1-carboxylate (0.242 g, 0.35 mmol) was added **37** (75 mg, 0.35 mmol) and DIPEA (0.183 mL, 1.05 mmol) in EtOH (3.5 mL). The resulting mixture was stirred at 60 °C for 4 h and then evaporated to dryness. The residue was dissolved in DCM (3.5 mL) and TFA (3.5 mL) was added slowly. The mixture was stirred for 3 h and then evaporated to dryness. The residue was dissolved in DCM (3.5 mL) and added to DIPEA (1.83 mL, 10.5 mmol) and *n*-butanol (35 mL) at 130 °C. The mixture was heated for 4 h and then cooled to rt. The reaction mixture was evaporated to dryness

and redissolved in EtOAc (25 mL), and washed sequentially with water (25 mL) and saturated brine (25 mL). The organic layer was dried with MgSO₄, filtered and evaporated to afford a residue which was purified by flash silica chromatography, elution gradient 0 to 5% MeOH in DCM to afford a yellow residue. This was triturated with *tert*-butyl methylether to give a solid which was collected by filtration and dried under vacuum to give the title compound as a yellow powder (78 mg, 46%). ¹H NMR (400 MHz, DMSO-d₆) δ 2.11-2.18 (m, 2H), 2.44-2.47 (m, 1H), 2.85 (hept, *J* = 7.3, 7.8 Hz, 2H), 3.16 (dd, *J* = 3.2, 11.7 Hz, 1H), 3.35-3.43 (m, 1H), 3.86 (d, *J* = 11.8 Hz, 1H), 3.93 (dd, *J* = 8.7, 15.3 Hz, 1H), 4.08-4.18 (m, 3H), 4.3-4.38 (m, 1H), 4.6-4.69 (m, 1H), 4.84 (dd, *J* = 4.0, 6.6 Hz, 1H), 4.93 (dd, *J* = 8.7, 15.1 Hz, 1H), 5.65-5.79 (m, 3H), 5.92 (dt, *J* = 9.8, 16.2 Hz, 1H), 6.72 (d, *J* = 1.5 Hz, 1H), 7.19 (d, *J* = 1.9 Hz, 1H), 8.35 (s, 1H), 9.04 (s, 1H), 9.70 (s, 1H); ¹³C NMR (126 MHz, DMSO-d₆) δ 25.03, 30.45, 34.65, 53.06, 58.72, 61.89, 66.40, 70.05, 75.38, 76.80, 79.22, 105.72, 115.09, 115.69, 124.81, 125.49, 127.92, 131.93, 134.00, 144.21, 145.39, 145.95, 151.62, 157.20, 169.45; HRMS (ES⁺) for C₂₅H₂₅N₇O₄ (M+H)⁺: calcd, 488.2046; found, 488.2045; [α]_D²⁴ -68.9 (*c* 2.1, CHCl₃).

***tert*-Butyl (2*S*,4*S*)-4-(((*E*)-4-((1-benzyl-6-nitro-2-oxo-1,2,3,4-tetrahydroquinolin-8-yl)oxy)but-2-en-1-yl)oxy)-2-((trityloxy)methyl)pyrrolidine-1-carboxylate (61, R₄ = Bn)**

Benzyl bromide (0.147 mL, 1.24 mmol) was added to a stirred solution of **47** (850 mg, 1.18 mmol) and cesium carbonate (380 mg, 1.17 mmol) in DMF (8 mL). The mixture was stirred at 80 °C for 30 min. The reaction mixture was evaporated to dryness and redissolved in EtOAc (100 mL), and washed sequentially with water (2 x 50 mL) and saturated brine (50 mL). The organic layer was dried with MgSO₄, filtered and evaporated to afford a residue which was purified by flash silica chromatography, elution gradient 0 to 60% EtOAc in heptane to afford the title compound (733 mg, 77%) as a yellow solid. ¹H NMR (400 MHz, DMSO-d₆, 100 °C) δ 1.29 (s, 9H), 2.03-2.19 (m, 2H), 2.59-2.64 (m, 2H), 2.94-2.99 (m, 2H), 3.06 (t, *J* = 8.1 Hz, 1H), 3.10 (dd, *J* = 3.5, 11.8 Hz, 1H), 3.30 (dd, *J* = 4.6, 8.3 Hz, 1H), 3.56 (dd, *J* = 5.8, 11.7 Hz, 1H), 3.83 (d, *J* = 4.6 Hz, 2H), 3.9-4.04 (m, 2H), 4.53-4.64 (m, 2H), 5.28 (s, 2H), 5.57-5.66 (m, 1H), 5.71-5.8 (m, 1H), 7.03-7.12 (m, 3H), 7.14-7.24 (m, 5H), 7.24-7.32 (m, 6H), 7.33-7.41 (m, 6H), 7.65 (d, *J* = 2.5 Hz, 1H), 7.75 (d, *J* = 2.5 Hz, 1H); ¹³C NMR (101 MHz, DMSO-d₆, 100 °C) δ 25.88, 28.61, 32.05,

33.88, 47.58, 52.51, 56.33, 65.15, 68.43, 69.97, 76.98, 79.06, 86.56, 108.64, 115.82, 126.14, 126.99, 127.06, 127.30, 128.11, 128.48, 128.79, 132.10, 133.21, 136.01, 138.67, 143.98, 144.56, 148.82, 154.02, 171.61; HRMS (ES⁺) for C₄₉H₅₁N₃O₈ (M+Na)⁺: calcd, 832.3568; found, 832.3569.

***tert*-Butyl (2*S*,4*S*)-4-(((*E*)-4-((6-amino-1-benzyl-2-oxo-1,2,3,4-tetrahydroquinolin-8-yl)oxy)but-2-en-1-yl)oxy)-2-((trityloxy)methyl)pyrrolidine-1-carboxylate (62, R₄ = Bn)**

Iron powder (397 mg, 7.11 mmol) was added to a stirred mixture of **61** (R₄ = Bn) (720 mg, 0.89 mmol), ammonium chloride (221 mg, 4.13 mmol), THF (10 mL), MeOH (10 mL) and water (4 mL). The resulting mixture was stirred at 70 °C for 60 min, cooled, diluted with EtOAc (125 mL), and washed sequentially with water (50 mL) and saturated brine (50 mL). The organic layer was dried with MgSO₄, filtered and evaporated to afford a residue which was purified by flash silica chromatography, elution gradient 30 to 70% EtOAc in heptane to afford the title compound (547 mg, 79%) as a yellow solid. ¹H NMR (400 MHz, DMSO-d₆, 100 °C) δ 1.29 (s, 9H), 2.04-2.2 (m, 2H), 2.39-2.45 (m, 2H), 2.60 (dd, *J* = 5.3, 8.2 Hz, 2H), 3.06 (t, *J* = 8.1 Hz, 1H), 3.11 (dd, *J* = 3.2, 11.7 Hz, 1H), 3.31 (dd, *J* = 4.7, 8.3 Hz, 1H), 3.56 (dd, *J* = 5.8, 11.7 Hz, 1H), 3.84 (d, *J* = 4.7 Hz, 2H), 3.9-4.05 (m, 2H), 4.36 (d, *J* = 4.6 Hz, 2H), 4.60 (s, 2H), 5.16 (s, 2H), 5.59-5.75 (m, 2H), 6.05 (d, *J* = 2.3 Hz, 1H), 6.13 (d, *J* = 2.3 Hz, 1H), 7.01-7.11 (m, 3H), 7.11-7.18 (m, 2H), 7.18-7.24 (m, 3H), 7.25-7.32 (m, 6H), 7.33-7.42 (m, 6H); HRMS (ES⁺) for C₄₉H₅₃N₃O₆ (M+H)⁺: calcd, 780.4013; found, 780.4017.

(13*S*,15*S*,26*Z*,7*E*)-41-Benzyl-23-chloro-15-(hydroxymethyl)-41,42,43,44-tetrahydro-5,10-dioxo-3-aza-4(6,8)-quinolina-2(5,7)-pyrazolo[1,5-*a*]pyrimidina-1(1,3)-pyrrolidinacyclodecaphan-7-en-42-one (31)

3,5,7-Trichloropyrazolo[1,5-*a*]pyrimidine (**48**) (78 mg, 0.35 mmol) was added to a stirred solution of **62** (R₄ = Bn) (273 mg, 0.35 mmol) and DIPEA (0.1 mL, 0.57 mmol) in EtOH (5 mL). The reaction mixture was stirred at 80 °C for 2.5 h. After cooling to rt, 4M HCl in 1,4-dioxane (5 mL, 20 mmol) was added and the mixture was stirred at rt for 1 h. The mixture was evaporated to dryness to afford the uncyclized product as gum which was transferred into a microwave vial and *n*-butanol (20 mL) and DIPEA (0.6 mL, 3.44 mmol) were added. The mixture was stirred at 140 °C within the microwave reactor for 3 h and

cooled to rt. The solvent was evaporated *in vacuo* to yield crude product which was purified by preparative HPLC (Waters XSelect CSH C18 column, 5 μ silica, 30 mm diameter, 100 mm length), using decreasingly polar mixtures of water (containing 1% NH₃) and acetonitrile as eluents to afford the title compound (36 mg, 18%) as a tan solid. ¹H NMR (500 MHz, DMSO-d₆) δ 2.02 (d, *J* = 12.6 Hz, 1H), 2.18 (ddt, *J* = 4.6, 9.2, 13.8 Hz, 1H), 2.57 (t, *J* = 7.4 Hz, 2H), 2.74-2.87 (m, 2H), 3.12 (dd, *J* = 3.2, 11.3 Hz, 1H), 3.35-3.44 (m, 1H), 3.81 (d, *J* = 11.6 Hz, 1H), 3.86 (dd, *J* = 9.4, 15.8 Hz, 2H), 4-4.14 (m, 3H), 4.27-4.35 (m, 1H), 4.44-4.52 (m, 1H), 4.79 (dd, *J* = 8.9, 15.1 Hz, 1H), 5.17-5.26 (m, 2H), 5.27 (dd, *J* = 3.6, 7.5 Hz, 1H), 5.59 (dd, *J* = 8.6, 15.9 Hz, 1H), 5.86 (dd, *J* = 8.7, 16.1 Hz, 1H), 6.77 (d, *J* = 2.2 Hz, 1H), 7.09-7.18 (m, 4H), 7.19-7.26 (m, 2H), 7.98 (s, 1H), 9.57 (s, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 26.58, 32.62, 38.37, 48.25, 53.79, 61.44, 67.12, 69.24, 71.20, 76.37, 79.17, 94.68, 104.78, 114.64, 126.56, 126.65, 126.98, 127.32, 128.13, 133.29, 133.54, 133.82, 138.89, 141.51, 144.21, 144.39, 148.58, 156.28, 172.01; HRMS (ES⁺) for C₃₁H₃₁ClN₆O₄ (M+H)⁺: calcd, 587.2174; found, 587.2159; [α]_D²⁵ -81.3 (*c* 0.56, CHCl₃).

***tert*-Butyl (2*S*,4*S*)-4-(((*E*)-4-((1-(3-methoxybenzyl)-6-nitro-2-oxo-1,2,3,4-tetrahydroquinolin-8-yl)oxy)but-2-en-1-yl)oxy)-2-((trityloxy)methyl)pyrrolidine-1-carboxylate (61, R₄ = *m*-MeO-Bn)**

1-(Bromomethyl)-3-methoxybenzene (0.128 mL, 0.91 mmol) was added to a stirred solution of **47** (626 mg, 0.87 mmol) and cesium carbonate (283 mg, 0.87 mmol) in DMF (8 mL). The mixture was stirred at 80 °C for 30 min. The reaction mixture was evaporated to dryness and redissolved in EtOAc (20 mL) and washed sequentially with water (2 x 20 mL) and saturated brine (20 mL). The organic layer was dried with MgSO₄, filtered and evaporated to afford crude product. The crude product was purified by flash silica chromatography, elution gradient 0 to 60% EtOAc in heptane to afford the title compound (550 mg, 75%) as a yellow solid. ¹H NMR (500 MHz, CDCl₃) δ 1.29 (s, 9H), 2.13-2.24 (m, 1H), 2.32-2.41 (m, 1H), 2.66-2.75 (m, 2H), 2.9-2.99 (m, 2H), 3.05-3.17 (m, 1H), 3.28 (dd, *J* = 3.5, 11.8 Hz, 1H), 3.35-3.44 (m, 1H), 3.62-3.68 (m, 1H), 3.69 (s, 3H), 3.85 (d, *J* = 5.1 Hz, 2H), 4.02 (s, 2H), 4.50 (d, *J* = 5.6 Hz, 2H), 5.36 (s, 2H), 5.57-5.68 (m, 1H), 5.75 (dt, *J* = 5.3, 15.7 Hz, 1H), 6.57-6.71 (m, 3H), 7.09 (t, *J* = 7.9 Hz, 1H), 7.20 (t, *J* = 7.3 Hz, 3H), 7.23-7.3 (m, 6H), 7.43 (d, *J* = 7.7 Hz, 6H), 7.60 (d, *J* = 2.5 Hz, 1H), 7.71 (d, *J* = 2.5 Hz, 1H); MS (ESI) [M + Na]⁺ *m/z* 862.

***tert*-Butyl (2*S*,4*S*)-4-(((*E*)-4-((6-amino-1-(3-methoxybenzyl)-2-oxo-1,2,3,4-tetrahydroquinolin-8-yl)oxy)but-2-en-1-yl)oxy)-2-((trityloxy)methyl)pyrrolidine-1-carboxylate (62, R4 = *m*-MeO-Bn)**

Iron powder (286 mg, 5.12 mmol) was added to a stirred mixture of **61** (R4 = *m*-MeO-Bn) (538 mg, 0.64 mmol), ammonium chloride (221 mg, 4.13 mmol), THF (8 mL), MeOH (8 mL) and water (4 mL). The resulting mixture was stirred at 70 °C for 60 min. The reaction mixture was diluted with EtOAc (125 mL) and washed sequentially with water (50 mL) and saturated brine (50 mL). The organic layer was dried with MgSO₄, filtered and evaporated to afford crude product. The crude product was purified by flash silica chromatography, elution gradient 40 to 70% EtOAc in heptane to afford the title compound (430 mg, 83%) as a yellow gum. ¹H NMR (500 MHz, CDCl₃) δ 1.29 (s, 6H), 1.44 (s, 3H), 2.12-2.22 (m, 1H), 2.3-2.38 (m, 1H), 2.56-2.61 (m, 2H), 2.68-2.74 (m, 2H), 3.06-3.17 (m, 1H), 3.27 (dd, *J* = 3.3, 11.8 Hz, 1H), 3.38 (dd, *J* = 4.1, 7.9 Hz, 1H), 3.49-3.57 (m, 2H), 3.63-3.69 (m, 4H), 3.8-3.88 (m, 2H), 3.97-4.06 (m, 2H), 4.34-4.38 (m, 2H), 5.25 (s, 2H), 5.56-5.71 (m, 2H), 6.05 (d, *J* = 2.4 Hz, 1H), 6.10 (d, *J* = 2.4 Hz, 1H), 6.64-6.67 (m, 2H), 6.68-6.71 (m, 1H), 7.04-7.1 (m, 1H), 7.20 (t, *J* = 7.3 Hz, 3H), 7.24-7.29 (m, 6H), 7.43 (d, *J* = 7.5 Hz, 6H); MS (ESI) [M-H]⁻ *m/z* 808.

(13*S*,15*S*,26*Z*,7*E*)-23-Chloro-15-(hydroxymethyl)-41-(3-methoxybenzyl)-41,42,43,44-tetrahydro-5,10-dioxa-3-aza-4(6,8)-quinolina-2(5,7)-pyrazolo[1,5-*a*]pyrimidina-1(1,3)-pyrrolidinacyclodecaphan-7-en-42-one (32)

3,5,7-Trichloropyrazolo[1,5-*a*]pyrimidine (**48**) (119 mg, 0.53 mmol) was added to a stirred solution of **62** (R4 = *m*-MeO-Bn) (432 mg, 0.53 mmol) and DIPEA (0.15 mL, 0.86 mmol) in EtOH (5 mL). The reaction mixture was stirred at 80 °C for 2.5 h. After cooling to rt, 4M HCl in 1,4-dioxane (8 mL, 32 mmol) was added. The mixture was stirred at rt for 60 min then evaporated to dryness to afford the uncyclized intermediate as a gum, which was transferred into a microwave vial and *n*-butanol (20 mL) and DIPEA (0.9 mL, 5.15 mmol) were added. The mixture was stirred at 140 °C within the microwave reactor for 3 h and cooled to rt. The solvent was evaporated *in vacuo* to yield crude product which was purified by preparative HPLC (Waters XSelect CSH C18 column, 5μ silica, 30 mm diameter, 100 mm length), using decreasingly polar mixtures of water (containing 1% NH₃) and acetonitrile as eluents to afford the title

compound (137 mg, 42%) as a tan solid. ^1H NMR (500 MHz, CDCl_3) δ 1.91 (d, $J = 14.0$ Hz, 1H), 2.44 (ddd, $J = 4.6, 10.3, 14.6$ Hz, 1H), 2.64-2.75 (m, 2H), 2.8-2.94 (m, 2H), 3.14 (dd, $J = 2.8, 11.4$ Hz, 1H), 3.73 (s, 3H), 3.76-3.94 (m, 3H), 3.98-4.09 (m, 2H), 4.33-4.4 (m, 1H), 4.44-4.55 (m, 2H), 4.57-4.65 (m, 1H), 5.31 (s, 2H), 5.52-5.6 (m, 1H), 5.71 (s, 1H), 5.82-5.91 (m, 1H), 6.50 (s, 1H), 6.61 (d, $J = 2.0$ Hz, 1H), 6.67-6.77 (m, 3H), 6.96 (d, $J = 2.0$ Hz, 1H), 7.16 (t, $J = 7.8$ Hz, 1H), 7.49 (s, 1H), 7.76 (s, 1H); ^{13}C NMR (101 MHz, CDCl_3) δ 26.58, 32.61, 38.37, 47.97, 53.79, 55.15, 61.45, 67.11, 69.24, 71.20, 76.38, 79.17, 94.61, 104.84, 111.70, 112.79, 114.68, 119.13, 126.90, 127.35, 129.15, 133.33, 133.60, 133.80, 140.50, 141.50, 144.24, 144.47, 148.57, 156.30, 159.56, 172.01; HRMS (ES^+) for $\text{C}_{32}\text{H}_{33}\text{ClN}_6\text{O}_5$ ($\text{M}+\text{H}$) $^+$: calcd, 617.2279; found, 617.2300; $[\alpha]_D^{25}$ -73.2 (c 2.01, CHCl_3).

***tert*-Butyl (2*S*,4*S*)-4-(((*E*)-4-((1-(2-methylbenzyl)-6-nitro-2-oxo-1,2,3,4-tetrahydroquinolin-8-yl)oxy)but-2-en-1-yl)oxy)-2-((trityloxy)methyl)pyrrolidine-1-carboxylate (61, R4 = *o*-Me-Bn)**

1-(Bromomethyl)-2-methylbenzene (0.158 mL, 1.18 mmol) was added to a stirred solution of **47** (532 mg, 1.12 mmol) and cesium carbonate (365 mg, 1.12 mmol) in DMF (8 mL). The mixture was stirred at 80 °C for 30 min. The reaction mixture was evaporated to dryness and redissolved in EtOAc (20 mL) and washed sequentially with water (2 x 20 mL) and saturated brine (20 mL). The organic layer was dried with MgSO_4 , filtered and evaporated to afford crude product. The crude product was purified by flash silica chromatography, elution gradient 0 to 60% EtOAc in heptane to afford the title compound (557 mg, 60%) as a yellow solid. ^1H NMR (500 MHz, CDCl_3) δ 1.28 (d, $J = 11.8$ Hz, 9H), 2.17 (s, 1H), 2.22 (s, 3H), 2.34 (d, $J = 13.5$ Hz, 1H), 2.69-2.77 (m, 2H), 3.01 (dd, $J = 5.5, 8.5$ Hz, 2H), 3.08 (t, $J = 8.4$ Hz, 1H), 3.27 (dd, $J = 3.6, 11.7$ Hz, 1H), 3.38 (d, $J = 6.6$ Hz, 1H), 3.6-3.69 (m, 1H), 3.74 (d, $J = 5.4$ Hz, 2H), 4.00 (d, $J = 31.3$ Hz, 2H), 4.34-4.41 (m, 2H), 5.21 (s, 3H), 5.57 (dt, $J = 5.5, 15.7$ Hz, 1H), 6.89 (d, $J = 7.3$ Hz, 1H), 7.02 (dd, $J = 5.1, 7.7$ Hz, 1H), 7.04-7.08 (m, 2H), 7.20 (t, $J = 7.3$ Hz, 3H), 7.23-7.31 (m, 6H), 7.42 (d, $J = 7.6$ Hz, 6H), 7.61 (d, $J = 2.5$ Hz, 1H), 7.75 (d, $J = 2.5$ Hz, 1H); MS (ESI) [$\text{M}+\text{formic-H}$] $^-$ m/z 868.

***tert*-Butyl (2*S*,4*S*)-4-(((*E*)-4-((6-amino-1-(2-methylbenzyl)-2-oxo-1,2,3,4-tetrahydroquinolin-8-yl)oxy)but-2-en-1-yl)oxy)-2-((trityloxy)methyl)pyrrolidine-1-carboxylate (62, R4 = *o*-Me-Bn)**

Iron powder (302 mg, 5.41 mmol) was added to a stirred mixture of **61** (R4 = *o*-Me-Bn) (557 mg, 0.68 mmol), ammonium chloride (325 mg, 6.08 mmol), THF (4 mL), MeOH (4 mL) and water (2 mL). The resulting mixture was stirred at 70 °C for 60 min. The reaction mixture was diluted with EtOAc (125 mL), and washed sequentially with water (50 mL) and saturated brine (50 mL). The organic layer was dried with MgSO₄, filtered and evaporated to afford the crude title compound (501 mg, 93%) as a yellow solid which was used without further purification. MS (ESI) [M + Na]⁺ *m/z* 816.

(13*S*,15*S*,26*Z*,7*E*)-23-Chloro-15-(hydroxymethyl)-41-(2-methylbenzyl)-41,42,43,44-tetrahydro-5,10-dioxo-3-aza-4(6,8)-quinolina-2(5,7)-pyrazolo[1,5-*a*]pyrimidina-1(1,3)-pyrrolidinacyclodecaphan-7-en-42-one (34)

3,5,7-Trichloropyrazolo[1,5-*a*]pyrimidine (**48**) (63.3 mg, 0.28 mmol) was added to a stirred solution of **62** (R4 = *o*-Me-Bn) (226 mg, 0.28 mmol) and DIPEA (0.099 mL, 0.57 mmol) in EtOH (5 mL). The reaction mixture was stirred at 80 °C for 2.5 h and then EtOH was evaporated. The reaction mixture was redissolved in 1,4-dioxane (2 mL), 4M HCl in 1,4-dioxane (3.6 mL, 14.2 mmol) was added and the reaction was stirred at rt for 1 h. The mixture was evaporated to dryness to afford the intermediate product as gum which was transferred into a microwave vial and *n*-butanol (5 mL) and DIPEA (0.15 mL, 0.85 mmol) was added. The mixture was stirred at 140 °C within the microwave reactor for 2 h and cooled to rt. The solvent was evaporated in vacuum to yield crude product. The crude product was purified by preparative HPLC (Waters XSelect CSH C18 column, 5μ silica, 30 mm diameter, 100 mm length), using decreasingly polar mixtures of water (containing 1% NH₃) and acetonitrile as eluents, then further purified by flash silica chromatography, elution gradient 1 to 5% MeOH in DCM to afford the title compound (32 mg, 19%) as a colourless gum. ¹H NMR (500 MHz, CDCl₃) δ 1.79-1.88 (m, 1H), 2.26 (s, 3H), 2.36 (ddd, *J* = 4.7, 10.3, 14.0 Hz, 1H), 2.58-2.75 (m, 2H), 2.79-2.95 (m, 2H), 3.05 (dd, *J* = 3.0, 11.4 Hz, 1H), 3.66 (dd, *J* = 9.1, 15.2 Hz, 1H), 3.73-3.86 (m, 2H), 3.91 (dd, *J* = 2.0, 11.5 Hz, 1H), 3.97 (d, *J* = 3.9 Hz, 1H), 4.19-4.46 (m, 4H), 5.12 (d, *J* = 16.2 Hz, 1H), 5.15-5.27 (m, 2H), 5.61 (s, 1H), 5.64-5.82 (m, 1H), 6.43 (d, *J* = 8.6 Hz, 1H), 6.58 (d, *J* = 2.3 Hz, 1H), 6.81-6.95 (m, 2H), 6.94-7.12 (m, 3H), 7.47 (s, 1H), 7.69 (s, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 19.22, 26.61, 32.57, 38.34, 46.64, 53.79, 61.42, 66.99, 69.20, 71.15,

76.33, 79.14, 94.60, 105.11, 114.94, 125.41, 125.56, 126.11, 127.15, 127.40, 129.96, 133.22, 133.36, 133.44, 134.48, 136.82, 141.49, 144.19, 144.61, 148.37, 156.27, 171.91; HRMS (ES⁺) for C₃₂H₃₃ClN₆O₄ (M+H)⁺: calcd, 601.2330; found, 601.2332; [α]_D²⁵ -65.5 (c 2.05, CHCl₃).

Biological Protocols:

Cloning, expression and purification of human BCL6 BTB domain

The gene sequence encoding BCL6 BTB domain (5-129)¹³ was customer-synthesized with N-terminal His₆ and thrombin protease cleavage site (Life Technologies, Carlsbad, CA). The synthetic gene was cloned into pET28b(+) (Novagen Biosciences, Madison, WI) to create plasmid pAZB1279. To reduce oxidation and aggregation, the mutations of C8Q, C57R and C84N were introduced during gene synthesis. The final construct was confirmed by sequencing, and the protein matched that of BCL6 BTB domain (Uniprot accession number P41182; residues 5-129 with the mutations of C8Q, C57R and C84N). This material was used in the FRET assay.

For structural studies, the same gene sequence encoding BCL6 BTB domain as pAZB1279 was customer-synthesized with N-terminal Thioredoxin-His₆, TEV protease cleavage site, AVI tag and thrombin cleavage site (Life Technologies, Carlsbad, CA). The synthetic gene was cloned into pET32a(+) (Novagen Biosciences, Madison, WI) to create plasmid pAZB1280. The final construct was confirmed by sequencing, and the protein matched that of BCL6 BTB domain (Uniprot accession number P41182; residues 5-129 with the mutations of C8Q, C57R and C84N). The plasmid was transformed into *E. coli* BL21(DE3) (Agilent Technologies). A single colony was inoculated into a 100 mL culture of LB containing 50 µg/ml kanamycin and grown overnight at 37 °C. The overnight culture was diluted to OD₆₀₀=0.1 in 2 x 1-liter of LB containing 50 µg/ml kanamycin and grown at 30 °C with aeration to mid-logarithmic phase (OD₆₀₀ = 0.6). The culture was incubated on ice for 30 min and transferred to 16 °C. IPTG was then added to a final concentration in each culture of 0.5 mM. The frozen cell paste from 6 L of cell culture was suspended in 50 mL of buffer A consisting of 25 mM Tris/HCl, pH 8.0, 0.5 M NaCl,

5% (v/v) glycerol, supplemented with 1 EDTA-free protease inhibitor cocktail tablet (Roche Molecular Biochemical, Indianapolis, IN). Cells were disrupted by French pressure at 18,000 psi twice at 4 °C, and the crude extract was centrifuged at 150,000 g (45Ti rotor, Bechman-Coulter, Brea, CA) for 30 min at 4 °C. The supernatant was applied at a flow rate of 2.0 mL/min onto a 5-ml HiTrap Ni²⁺ chelating column (GE Healthcare Life Sciences, Piscataway, NJ) pre-equilibrated with buffer A. The column was then washed with buffer A, and BCL6 was eluted by a linear gradient from 0 M to 0.5 M imidazole in buffer A. Fractions containing BCL6 were pooled, and the thioredoxin tag of BCL6 fusion protein was removed by thrombin protease. The BCL6 sample was applied at a flow rate of 1.5 mL/min to a 120-mL Superdex 200 (HR 16/60) (GE Healthcare Life Sciences) pre-equilibrated with buffer B consisting of 25 mM Tris/HCl, pH 8.5, 0.45 M NaCl, 1 mM TCEP, 10% (v/v) glycerol. The fractions containing BCL6 were pooled and concentrated by Amicon® Ultracel-10K (Millipore). The protein concentration was determined by Bradford method and characterized by SDS-PAGE analysis and analytical LC-MS. The protein was stored at -80 °C. For X-ray crystallography studies, the tag of BCL6 fusion protein was removed by thrombin protease.

¹⁵N-labeled BCL6 BTB domain: pAZB1280 was transformed into *E. coli* BL21 (Novagen, Madison WI) and plated on LB agar containing 50 µg/ml kanamycin and grown overnight at 37°C. Uniformly ¹⁵N-labeled protein was prepared by innoculating transformants from the above-described plate into M9 minimal media containing 50 µg/mL kanamycin and supplemented with 1 g/L [¹⁵N] NH₄Cl and 10 g/L glucose as the sole nitrogen and carbon sources. After growing at 37 °C with aeration to mid-logarithmic phase (OD₆₀₀ = 0.6), IPTG was added to a final concentration of 0.5 mM to induce expression. After 22 h at 16 °C, the cells were harvested by centrifugation at 5,000 x g for 15 min at 25 °C. The purification protocol was the same as non-labeled BCL6.

BCL6 SPR assay:

Biacore 4000 or T200 instruments (GE Healthcare) were used to monitor binding interactions *via* SPR. H6-BCL6 (5-129) was immobilized using NTA capture-coupling at a flow rate of 10 μ L/min in buffer containing 10 mM HEPES, 1 mM TCEP and 0.005% Tween-20, pH 7.5 at 25 °C. The sensor surface was activated with a 1 min injection of 0.5 mM NiCl₂ and a 7 min injection of 50 mM *N*-hydroxysuccinimide with 200 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. 50 μ g/mL of BCL6 protein was injected for 1 min. and the surface blocked with 1M ethanolamine, pH 8.5. Reference flow cells were prepared without protein. All binding measurements were performed in 25 mM HEPES, 150 mM NaCl, 1 mM TCEP, 0.005% (v/v) Tween-20, 2% DMSO, pH7.5 at 25 °C. Prior to analysis, solvent calibration and double referencing subtractions were made to eliminate bulk refractive index changes, injection noise and data drift. Affinity and binding kinetic parameters were determined by global fitting to a 1:1 binding model within the Biacore Evaluation Software (GE Healthcare).

BCL6 2D NMR assay

NMR spectra were acquired at 300 K with a 600 MHz NMR instrument (Bruker, Billerica MA) equipped with an AVANCE III console and a triple-resonance cryogenic probe. Samples were at pH 7.0 in a 50 mM sodium phosphate buffer containing 400 mM sodium chloride, and 5% deuterium oxide. The concentration of ¹⁵N labeled BCL6 was 0.09 mM. Two-dimensional [¹⁵N, ¹H] transverse relaxation optimized spectroscopy (TROSY) experiments were recorded with increasing ligand concentration.²⁵ Evolution times were approximately 53 ms in the ¹H dimension and 29 ms in the ¹⁵N dimension. The total acquisition time was 28 min per experiment.

BCL6 FRET assay

Compound binding to BCL6 was determined by a time resolved fluorescence resonance energy transfer assay. Assays were performed with 0.67 nM His BCL6 (5-129) C8Q, C67R & C84N, 0.125 nM Eu-labeled anti His Antibody (Perkin Elmer, Waltham, MA) and 30 nM Hilyte 647 SMRT2 peptide (1414-1430) H1426W¹² (Anaspec, Freemont, CA) in 40 mM Hepes pH 7, 50 mM NaCl, 0.005% Tween-20, 0.5

mM TCEP buffer. His tagged BCL6 (5-129) was mixed with Eu-anti-His antibody and added to white assay ready plates containing compound for a final assay concentration range of 100 μ M to 0.33 nM in 1% DMSO. Assay plates contained maximum signal controls (1% DMSO final) and minimum signal controls (50 nM unlabeled SMRT2 peptide 1414-1430 with H1426W mutation). BCL6, antibody and compound were incubated at rt for 30 min. The binding reaction was initiated by addition of Hilyte 647 SMRT2 peptide. After 60 min at rt fluorescence was measured with a Tecan M1000 plate reader using an excitation wavelength of 340 nm and emission wavelengths of 612 nm and 665 nm.

BCL6 and PLZF cell reporter assay

HEK 293T/17 cells were transfected using Lipfectamine 3000 (Invitrogen) with a luciferase reporter vector containing yeast GAL4 binding sites and a thymidine kinase (TK) promotor, (GAL4)5TK-Luc. Cells were also transfected with 160 ng/ml of plasmid expressing a GAL4 DNA binding domain fused to BCL6^{BTB}. Alternatively cell were transfected with 400 ng/mL plasmid expressing GAL4 DNA binding domain fused to PLZF^{BTB}. Plasmids were obtained through licensing agreements from the laboratory of Ari Melnick (Cornell Center for Technology). After transfection, cells were immediately distributed to 384 well plates at a density of 10,000 cells/well. Cells were incubated for 24 h and then treated in triplicate with multiple concentrations of inhibitor or DMSO. After additional 24 h incubation, cells were examined for luciferase activity using Bright-Glo luciferase assay system (Promega, Madison, WI). IC₅₀ values were determined relative to luciferase activity of reference compound **11** at 8 μ M, which compares to luciferase signal of (GAL4)5TK-Luc alone.

DLBCL/AML cell proliferation assays

Cells in their culture medium were directly dosed into 384-well flat bottom white microplates (Greiner, Monroe, NC) containing DMSO or multiple concentrations of compound (day 0), and then returned to the incubator for 72 h (day 3). Cell density was pre-determined for each cell line based on linear phase growth over a 96 h period. The number of viable cells on day 0 and 3 was determined using CellTiter-

Glo (Promega, Madison, WI). Quantification of signal was determined using a Tecan Infinite F500 (Männedorf, Switzerland). Percentage of net growth at day 3 (100%) relative to day 0 (0%) was calculated and the concentration of compound required to inhibit growth by 50% (GI₅₀) determined. The assays were conducted in duplicate across different plates.

Cell culture

All cell lines were obtained from ATCC (Manassas, VA) or DSMZ (Braunschweig, Germany) unless otherwise specified. OCI-Ly1 and TMD8 cells were obtained from Mark Minden (Ontario Cancer Institute) and Shuji Tohda (Tokyo Medical and Dental University), respectively, and OCI-Ly3 and OCI-Ly10 cells were obtained from Louis Staudt (National Cancer Institute). Cells were maintained under 37 °C humidified atmosphere containing 5% CO₂ in specified media supplemented with 2 mM L-glutamine. SUDHL2, SUDHL4, and U-2932 were grown in RPMI1640 medium with 10% fetal bovine serum (FBS), Karpas 422 with 15% FBS, and AMO-1 with 20% FBS. TMD8 and OCI-Ly19 were grown in α -MEM medium with 10% FBS and OCI-Ly1, OCI-Ly3, and OCI-Ly10 were grown in IMDM medium with 20% FBS and 50uM 2-mercaptoethanol. HEK 293T/17 cells were grown in DMEM with 10% FBS. All medium and supplements were from Invitrogen (Carlsbad, CA), whereas FBS was from Sigma (St. Louis, MO).

Supporting Information. Preparation and additional characterization for final compounds; NMR conformational analysis for **8** and **11**; effect of **31** in *in vitro* radioligand binding enzyme and functional assays; kinase selectivity data for **11** and **31**; crystallization and structure determination for **1**, **2**, **7**, **11**, with BCL6 and **11** with CK2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession codes. Atomic coordinates for the crystal structures of BCL6 with compounds **1**, **2**, **7** and **11**, and CK2 with compound **11** can be accessed using PDB codes 5N20, 5N1X, 5N21 5N1Z and 5N1V respectively. Authors will release the atomic coordinates and experimental data upon article publication.

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Notes

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Abbreviations Used

BCL6, B-cell lymphoma 6; BCOR, BCL6 corepressor; BTB, Broad-Complex, Tramtrack and Bric a brac; DIPEA, *N,N*-diisopropylethylamine; DLBCL, diffuse large B-cell lymphomas; EtOAc, ethyl acetate; FRET, fluorescence resonance energy transfer; GC germinal center; LE, ligand efficiency; LLE, ligand lipophilicity efficiency; MM, multiple myeloma; NCOR, nuclear receptor corepressor; PPI, protein-protein interaction; SBDD, structure based drug design; SEM, standard error of the mean; SMRT, silencing mediator for retinoid or thyroid-hormone receptors; SPR, surface plasmon resonance.

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Table of Contents Graphic

Fragment hit
 $K_d = 689 \mu\text{M}$

11
 $K_d = 0.0065 \mu\text{M}$