2014

Recognition of antigen-specific B-cell receptors from chronic lymphocytic leukemia patients by synthetic antigen surrogates

M. Sarkar

Y. Liu
Zucker School of Medicine at Hofstra/Northwell

J. Morimoto

H. Peng

C. Aquino

See next page for additional authors

Authors
M. Sarkar, Y. Liu, J. Morimoto, H. Peng, C. Aquino, C. Rader, N. Chiorazzi, and T. Kodadek

Published in final edited form as:
Chem Biol. 2014 December 18; 21(12): 1670–1679. doi:10.1016/j.chembiol.2014.10.010.

Recognition of Antigen-Specific B Cell Receptors From Chronic Lymphocytic Leukemia Patients By Synthetic Antigen Surrogates

Mohosin Sarkar[1], **Yun Liu**[4], **Jumpei Morimoto**[1], **Haiyong Peng**[2], **Claudio Aquino**[1], **Christoph Rader**[2,3], **Nicholas Chiorazzi**[4], and **Thomas Kodadek**[1,2]

Thomas Kodadek: kodadek@scripps.edu

[1]Department of Chemistry, The Scripps Research Institute, Jupiter, FL 33458

[2]Department of Cancer Biology, The Scripps Research Institute, Jupiter, FL 33458

[3]Department of Molecular Therapeutics, The Scripps Research Institute, Jupiter, FL 33458

[4]The Karches Center for Chronic Lymphocytic Leukemia Research, Hofstra North Shore-LIJ School of Medicine, The Feinstein Institute for Medical Research, Manhasset, NY

Abstract

In patients with chronic lymphocytic leukemia (CLL), a single neoplastic antigen-specific B cell accumulates and overgrows other B cells, leading to immune deficiency. CLL is often treated with drugs that ablate all B cells, leading to further weakening of humoral immunity, and a more focused therapeutic strategy capable of targeting only the pathogenic B cells would represent a significant advance. One approach to this would be to develop synthetic surrogates of the CLL antigens allowing differentiation of the CLL cells and healthy B cells in a patient. Here, we describe discovery of non-peptidic molecules capable of targeting antigen-specific B cell receptors with good affinity and selectivity using a combinatorial library screen. We demonstrate that our hit compounds act as synthetic antigen surrogates and recognize CLL cells and not healthy B cells. Additionally, we argue that the technology we developed can be used for discovery of other classes of antigen surrogates.

Keywords

Chronic Lymphocytic Leukemia; Combinatorial Chemistry; B Cell Receptor; Antigen Surrogate; Antibody

Chronic lymphocytic leukemia (CLL) is a cancer of B lymphocytes that occurs via the relentless accumulation of a single antigen-specific B cell clone (Chiorazzi et al., 2005). The

Correspondence to: Thomas Kodadek, kodadek@scripps.edu.

nature of the antigens that drive these events in CLL patients are generally not known (Catera et al., 2008; Chu et al., 2010). Like many diseases of the immune system, CLL is often treated with immunosuppressive compounds (Porter et al., 2011). For example, monoclonal antibodies (mAbs) such as rituximab that eliminate all $CD20^+$ B cells are employed commonly in combination with systemic chemotherapy (Jaglowski et al., 2010). There is also great interest in targeting molecules involved in, or associated with, B cell activation, such as Bruton's tyrosine kinase (Byrd et al., 2013). However, while these B cell-targeted drugs are a great improvement over standard cytotoxic chemotherapy for CLL and other B cell malignancies, they fail to discriminate between healthy and pathogenic B cells and thus leave the patient without a functional humoral immune system. Therapeutics selective for the pathogenic B cells would be of considerable interest. An intriguing target for such a strategy would be the antigen-specific surface membrane immunoglobulin (smIg) component of the B Cell Receptor (BCR) of the pathogenic lymphocytes.

Indeed, Levy and co-workers have shown that vaccination of CLL patients with their own Ig can induce useful anti-idiotype antibodies and T cells that selectively target the pathogenic B cell clones and recruit effector functions to them, resulting in their elimination (Meeker et al., 1985). This strategy that has been applied to other B-cell malignancies as well (Timmerman et al., 2009). Excellent clinical efficacy has been observed in several cases, providing compelling evidence for the feasibility of a strategy that selectively targets only the pathogenic cells. However, there has been little progress on the development of drug candidates that are selective for pathogenic B cells. In this vein, one would require a highly selective ligand capable of binding to the antigen-binding site of the leukemic Ig that could be tailored to carry a toxic cargo. While BCR-targeting antibody-drug conjugates (ADCs) could be imagined as potential therapeutics, similar to the CD30-targeting ADC that is approved for Hodgkin lymphoma (Bander et al., 2012), their production is technically challenging (Adem et al., 2014) and antibodies are able to carry only a small amount of drug payload, necessitating the use of only a few extremely toxic compounds (Anderl et al., 2013). Easily manipulable synthetic ligands for leukemic Igs would have several advantages in this regard. To the best of our knowledge however, the only report of synthetic ligands that recognize an antigen-specific CLL Ig are 12-residue peptides isolated via phage display (Seiler et al., 2009). Simple linear peptides are poor candidates for this application since they have limited stability in serum. More drug-like molecules with this type of binding selectivity are unknown.

Here we report a potentially general approach to screen synthetic one bead one compound (OBOC) libraries to identify leukemic Ig-binding “antigen surrogates”. In this study, a library of molecules called COPAs (chiral oligomers of pentenoic acids) (Aquino et al., 2011) (Fig. 1) was mined for such ligands. These polyketide-inspired molecules are calculated to adopt discreet conformations determined by the absolute chirality in each monomeric unit (Aquino et al., 2011). Thus, oligomers of these units are predicted to be much “stiffer” than relatively “floppy” small peptides and peptoids and are expected to be a superior source of protein ligands. In line with this expectation, previous work (Aquino et al., 2011) has shown that libraries of COPAs are capable of providing ligands to protein targets for which no good binders could be mined from a comparable library of peptoids. Indeed, COPA ligands for antigen-specific CLL Igs are described in this study that bind

selectively with sub-micromolar affinities to a soluble, recombinant CLL patient-derived monoclonal Ig. When displayed on a multimeric scaffold, these ligands distinguish between cells that express different antigen-specific Igs. This work represents the critical first step in the development of non-peptidic, drug-like molecules capable of distinguishing pathogenic from healthy lymphocytes.

Results

A screen for synthetic “antigen surrogates”

We hypothesized that one could mine COPA libraries for high affinity and selectivity ligands that would recognize the antigen-binding site of CLL patient-derived smIgs by first denuding the library of ligands to conserved regions of IgGs and then screening the remainder of the library against one or a few antigen-specific targets. To test this idea, a library of COPAs with the general structure shown in Fig. 1 was constructed. The library, which has a theoretical diversity of 1.28 million compounds, was created using 2.5 g of 160 μm TentaGel beads (≈ 1.3 million beads) using split and pool solid-phase synthesis (Aquino et al., 2011). The beads were suspended in buffer containing a high concentration of BSA, and then exposed to a mixture of human IgG antibodies obtained from individuals that did not suffer from CLL (Fig. 2). After washing, the beads were exposed to a red quantum dot-conjugated anti-human IgG secondary antibody. The beads that bound significant levels of the human IgG antibodies, or bound directly to the secondary antibody preparation, acquired an intense red fluorescence. These beads were removed from the population manually using a pipette under a low power fluorescence microscope.

We assumed that the remainder of the beads should be largely devoid of ligands for conserved regions of human IgGs. Thus, these beads were incubated with three soluble recombinant CLL monoclonal Igs, expressed and purified from HEK 293 T cells (called CLL 068, 169 and 183, respectively (100 nM each)). Again, subsequent incubation with the fluorescently labeled secondary antibody was employed to “light up” the “hits”. A total of one hundred ten beads fluoresced bright red and were collected.

Since the re-synthesis and analysis of screening hits is the most time-consuming portion of this type of discovery experiment, we chose to repeat the experiment on these 110 putative hits. Therefore, antibodies were stripped from the beads using a denaturing buffer. After re-equilibration, the beads were again exposed to control human IgG followed by the labeled secondary antibody. Beads that emitted any observable fluorescence were eliminated from the population. Those that did not were then exposed to each of the three CLL Igs, one at a time, followed by labeled secondary antibody. At each step the bright beads were harvested. This resulted in 18 hits against CLL 169 and 13 hits against CLL 183 mAb. None of the beads bound significant levels of CLL 068, indicating that there were no high affinity ligands for the antigen-binding site of this Ig in the COPA library employed.

The beads displaying putative CLL 169 and 183 ligands were placed in individual wells of a 96-well plate and treated with CNBr to release the compounds into solution via cleavage of the methionine residue in the conserved linker (Fig. 1). The molecules were analyzed by a combination of MALDI TOF-TOF and ESI-ETD mass spectrometry (Sarkar et al., 2013).

From these data the structures of 14 of the CLL 169 hits and 9 of the CLL 183 hits could be elucidated unambiguously (Supp. Fig. S1). All of these compounds were re-synthesized and purified by HPLC. In each case, a C-terminal cysteine was included to facilitate site-specific labeling or surface immobilization of the COPA molecule (Supp Fig 2).

Biophysical analysis of COPA-antibody binding

To evaluate the affinities of these small molecules for their target, they were labeled with fluorescein and titrated with recombinant, soluble Ig. Binding was monitored by fluorescence polarization (FP) spectroscopy. Six of the 14 putative CLL 169 compounds showed clear binding in this experiment, with the best ligand, KMS5, exhibiting a K_D of approximately 500 nM. (Supp. Fig. S3 a). Three out of 9 putative CLL 183 ligands were validated in this assay format exhibited K_D values ranging from 0.6 μM to 3 μM (Supp. Fig. S3 b). Similar results were obtained when the compounds were biotinylated, immobilized on streptavidin-coated ELISA plates, and titrated with increasing amounts of soluble Ig (Fig. 3a and Supp. Fig. S3 c). The derived K_D values were lower, presumably due to avidity effects.

The fact that the majority of the hits proved to be poor ligands in solution or on ELISA plates was not surprising. Work in our laboratory on small molecule-antibody interactions has shown that TentaGel beads are heterogeneous and that a small fraction of the bead population displays the compound at exceedingly high density, which probably allows very weak ligands that happen to be displayed at such densities on these rare beads to stably capture an antibody on the surface due to “kinetic trapping” effects (Doran et al., 2014).

Analysis of binding selectivity

To address the critical question of whether these compounds target the antigen-binding site of the CLL-specific smIg, the three that exhibited the highest affinity for CLL 169 (KMS5, KMS9 and KMS11) were immobilized on an ELISA plate and titrated with various soluble Igs (CLL 014, CLL 068 and CLL 183) as well as human serum IgG. Two of the compounds, KMS9 and KMS11, had modest selectivity for CLL 169 (Supp. Figs. S3 d and e), whereas binding of KMS5 to CLL 169 was highly selective (Fig. 3c). Moreover, a scrambled version of KMS5 bound to CLL 169 with a much lower affinity (Fig. 3). These data argue that KMS5 recognized the antigen-binding site of the CLL 169 Ig selectively.

To investigate this point further, a competition binding experiment was carried out against a 12-residue peptide, called 169-8, shown previously to bind the antigen-binding site of CLL 169 (Seiler et al., 2009). As shown in Fig. 4a, soluble COPA KMS5 competed binding of the CLL 169 IgG to immobilized KMS5, as expected. The same result was obtained when soluble peptide 169-8 was added to the CLL Ig, but a control soluble peptide had no effect. These data argue that KMS5 and peptide 169-8 compete for the same binding site on CLL 169 monoclonal Ig.

The competition data in Fig. 4a also suggest that the affinity of the COPA and the peptide are similar. To validate this, ELISA experiments were conducted in which each compound, or a control COPA compound or peptide, was immobilized on the ELISA plate and titrated with CLL 169 IgG. As shown in Fig. 4b, this experimental format confirmed that the affinities of the peptide and COPA for CLL 169 are similar, with the peptide binding

approximately 2-fold more tightly (K_D = 49 ± 11 nM for the peptide-IgG complex, K_D = 90 ± 20 nM for the COPA-IgG complex). While the affinities are similar, the synthetic COPA compound is resistant to degradation by human serum proteases (Supp. Fig. 4) and thus would be preferable for selective drug delivery to CLL cells.

Selective recognition of cells expressing CLL-specific smIgs

With respect to the ultimate goal of delivering toxic cargo to CLL B cells, a critical issue is whether the COPA ligands can recognize the smIgs for which they were selected. Ig heavy chain secreted-expression plasmids (pIg-gamma) were re-engineered by introducing an IgG1 transmembrane (TM) domain at the C-terminus (Supp. Figs. S5 a and b). HEK 293 T cells were transfected with these expression vectors and the expression levels of surface membrane IgGs was determined by staining the cells with anti-human Ig Fc specific antibody conjugated to allophycocyanin (anti-huIgFc-APC) and analyzing by flow cytometry (Fig. 5). As expected from a transient transfection, the population has a distribution of smIgG expression levels, reflected in the width of the peak in the flow cytometry plots.

Initial experiments, in which biotin-tagged KMS5 or biotin-tagged 169-8 peptide were incubated with cells displaying CLL 169 or other BCRs were unsuccessful. Only a modest amount of non-selective binding was observed (Supp. Figs. S5 c and d). We hypothesized that this is due to the modest affinity of these artificial CLL 169 ligands. Therefore, we turned to the multimerization of the peptide and COPA ligands in the hope that avidity effects would provide a more favorable result. Dextran was chosen as the backbone for these multimeric compounds given its excellent solubility and biocompatibility. An estimated average of 31 equivalents of KMS5 or peptide 169-8 was conjugated to biotinylated dextran using the chemistry depicted in Supp. Fig. S6. To investigate if these multivalent conjugates indeed increase the binding affinity for CLL 169 Ig, we carried out competition ELISAs using KMS5 and dextran conjugated KMS5 (Fig. 4a), and 169-8 peptide and dextran conjugated 169-8 peptide (Supp. Fig. 3f). Both of these experiments showed that multimerization increased the affinity of the compound for CLL 169 Ig by more than 10-fold (Supp. Table S1). These polymers were then incubated with cells expressing the smIg form of CLL 169, one of three alternative CLL smIgs, or cells lacking any smIg. Binding was followed by staining with PE-conjugated streptavidin and flow cytometry.

As shown in Fig. 5, KMS5-Dextran showed significant binding to the cells expressing CLL 169, exhibiting a plot similar to that observed when the CLL 169-displaying cells were incubated with the anti-huIg Fc-APC antibody. In contrast, only a small amount of binding of KMS5 to cells expressing any of the other CLL IgGs, or to the control cells, was observed. A similar result was obtained with the 169-8 peptide-dextran conjugate, again showing the similar behavior of the peptide and much smaller COPA ligand.

Selective binding of multivalent ligands with primary CLL cells from patients

Given these promising results using smIg-expressing cells, we evaluated the extent that KMS5 selectively recognized the patient-derived primary CLL B cells. Frozen peripheral blood mononuclear cells (PBMCs) containing the CLL 169 cells were thawed and allowed

to recover for 2 hours. To enrich for living B cells, the PBMCs were subjected to a Ficoll gradient and then a commercial kit (from Milteneyi Biotec) was employed to enrich B cells in the population by selectively depleting non-B cells. These cells were then incubated with biotin-tagged dextran-KMS5, the equivalent construct displaying peptide 169-8, or biotin-dextran conjugates displaying a control COPA or peptide. After washing, the cells were then incubated with streptavidin-conjugated PE (to monitor binding of the dextran conjugates to the cells), anti-CD5-APC antibody and anti-CD19-FITC antibody (to identify the $CD5^+$/$CD19^+$ CLL B cell population), and Po-Pro dye (to stain dead cells). Four-color flow cytometry was carried out to focus the analysis on binding of the dextran conjugates to living CLL B cells (Fig. 6a). As shown in Fig. 6b, the biotinylated dextran conjugates displaying the COPA KMS5 and the peptide 169-8 associated with the CLL 169 B cells (Q2, $CD5^+$/$CD19^+$), whereas biotinylated dextran conjugates displaying a control COPA or peptide did not. On the other hand, the biotinylated dextran conjugates did not exhibit any significant affinity for the small population of normal (non-CLL) B cells (Q3, $CD5^-$/$CD19^+$) from the same patient (Fig 6c). Furthermore, when the KMS5 and 169-8 conjugates were incubated with B cells isolated from a different CLL patient, no significant binding was observed (Fig. 6d). In this case, the level of signal in the PE channel observed when the KMS5 or 169-8 dextran-biotin conjugates were incubated with the cells was essentially identical to that observed when the cells were mixed with the control conjugates. Furthermore these, in turn, were similar to that observed when no dextran-biotin conjugate at all was used. The KMS5 and 169-8 conjugates also did not bind to the T-cell population (Q1, $CD5^+$/$CD19^-$) of CLL 169 PBMCs nor to the CLL cell line MEC1 (Supp. Fig. S7). The CLL 169 and control CLL B cells had similar levels of smIg, as determined by staining with anti-Human IgM (Fc5μ fragment specific) antibody as shown in Supp. Fig. 7d. Therefore, these data show that the KSM5-dextran conjugate (as well as the peptide 169-8-dextran conjugate) is a highly selective ligand for native B cells displaying the CLL 169 BCR. Notably, the binding is independent of the isotype of the BCR (IgG on HEK 293 T cells and IgM on CLL B cells), further corroborating selective targeting of the antigen-binding site.

Discussion

This study focused on determining the feasibility of screening combinatorial libraries of unnatural, non-peptidic compounds for ligands to antigen-specific B cell receptors. Such molecules could be used as “delivery agents” to bring toxic cargo or attract effector functions selectively to specific Ig-bearing cells, providing an attractive strategy to attack pathogenic B cells while sparing healthy B cells. High affinity ligands for a CLL Ig could also potentially be employed in a diagnostic mode, for example in a flow cytometry-based assay to monitor the return of the pathogenic B cell clone following initial chemotherapy. While BCR-binding (anti-idiotypic) antibodies and peptides have been described previously, to the best of our knowledge, there are no reports of synthetic, unnatural molecules capable of distinguishing B cells by their antigen specificity.

A library of COPA molecules (Fig. 1) was chosen as the starting point. These compounds are insensitive to serum peptidases that would rapidly degrade simple peptides (Supp. Fig. S4). The OBOC COPA library was first denuded of ligands for conserved regions of human IgG molecules by screening it against a mixture of antibodies from healthy controls and

removing binders with this step (Fig. 2). The remainder of the library was then screened against the recombinant, soluble version of CLL Igs. These hits were then stripped and re-subjected to the screening conditions again to ensure that they were not artifacts. The structures of the compounds that bound one of the CLL Igs employed in this study were then deduced by mass spectrometry after release from the beads.

Several of the molecules identified in the screen proved to be good ligands for a particular CLL smIg, as evidenced by binding data from ELISA and fluorescence polarization experiments (Fig. 3 and Supp. Figs. S3). KMS5, the best ligand for the CLL 169 Ig was characterized in some detail. It bound to the antigen-binding site of the soluble version of this Ig with an affinity of approximately 500 nM in solution (Supp. Fig. 2a) and 90 nM when immobilized on an ELISA plate, the difference presumably reflecting avidity effects in the latter case. This affinity was similar to that displayed by a 12-residue peptide ligand for CLL169 isolated previously by phage display (Seiler et al., 2009) and the two compounds competed for the antibody, indicating recognition of the antigen-binding site.

Finally, while neither the biotin-labeled COPA KMS5 or peptide 169-8 could be seen to bind detectably to cells expressing the CLL 169-specific Ig, probably due to the kinetic instability of the complexes, selective binding was observed when either molecule was displayed on a biotinylated dextran polymer (Fig. 5), again likely due to avidity effects. We have found that ligands displayed on dextran can engage both arms of antibodies (Morimoto, et al., 2014) and there is the further opportunity for binding of a single dextran conjugate to multiple Igs on the cell surface. In experiments using HEK 293 T cells expressing membrane-anchored IgG, flow cytometry plots showed a clear shift in the fluorescence of the CLL 169-expressing cells, but not cells expressing CLL 014, CLL 068, CLL 183 or no BCR (Fig. 5). For the CLL 169-expressing cells, the gain in fluorescence was similar when the cells were incubated with the peptide-dextran complex, the COPA-dextran complex or an anti-Fc antibody, indicating highly efficient binding. Similar results were observed using native B cells isolated form the CLL 169 patient and not from CLL cells from a different patient whose smIg was structurally distinct. Highly selective binding to the former was observed (Fig. 6).

In summary, we have developed a potentially general protocol for the isolation of non-peptidic ligands for antigen-specific BCRs. The molecules that result from this screen have modest affinity, but high selectivity, for the antigen-binding site of the smIg of the primary leukemic cells of the original CLL patient. By oligomerizing these molecules through conjugation to dextran, high affinity is also achieved via avidity effects. We, and others, have described the synthesis of a variety of different OBOC libraries containing unnatural molecules (Alluri et al., 2003; Gao and Kodadek, 2013; Kritzer et al., 2005; Peng et al., 2006; Suwal and Kodadek, 2013; Wu et al., 2014), all of which could be applied to this screening protocol, thus allowing many different chemotypes to be explored as antigen surrogates. Future work will focus on the isolation of many such molecules, optimization of the initial hits to higher affinity leads and the creation of chimeric molecules capable of delivering toxic cargo to B cells expressing the target receptor.

Significance

The ability to manipulate antigen-specific immune responses would have a significant impact in several areas of biology and medicine. One such example is chronic lymphocytic leukemia, where the antigen-specific B cell receptor is an attractive drug target. Here we describe the first examples of high affinity and selectivity non-peptide ligands capable of engaging antigen-specific CLL B cells. The development of this technology opens the door to the development of chimeric “antigen-surrogate”-toxin conjugates capable of killing only pathogenic CLL cells without harming the normal function of the humoral immune system.

Materials and Methods

Experimental procedures

Design and synthesis of COPA library—The library was synthesized on 160 μm TentaGel MB NH_2 resin (0.29 mmol/g loading, 520,000 beads/g, from Rapp Polymere, Germany). Total theoretical diversity of the library was 1.28 million and 2.5 g (2.5×520000 beads) of resin was used to cover at least one copy of each library member. The TentaGel beads were derivatized with a peptoid linker as shown in Fig 1. A four-mer COPA library was synthesized following the linker using solid-phase sub-monomer methods and split-and-pool techniques from (*R*) and (*S*) stereoisomers of 5-chloropentenoic acids and commercially available 20 different primary amines. The pentenoic acid sub-monomers (as depicted in Fig. 1) were synthesized following the protocols described in our previous paper (Aquino et al, Nature Chemistry 2011). To correlate differences in the mass of fragment ions with absolute stereochemistry the *S*-isomer was synthesized with an ethyl group at the C4 position. The synthesis of COPA oligomers was described in details by Aquino et al (Nature Chemistry 2011). The synthesis of COPA oligomers was described in details by Aquino et al. (Aquino et al., 2011). To determine the quality of the library, 24 random beads were isolated from the library and treated individually with 20 μL of CNBr solution to release the compounds. The parent ion mass of the compounds was determined by MALDI-TOF and the sequence of the unknown COPA tetramer was determined by multiple sequence specific product ions obtained either from $[M+2H]^{2+}$ or $[M+3H]^{3+}$ or from both precursor ions by ESI Linear Ion Trap Mass Spectrometer (LTQ-ETD, ThermoFinnigan). The method of structure elucidation of COPA oligomers by MALDI and LTQ-ETD mass spectrometry was described in details by Sarkar et al. (Sarkar et al., J. Am. Soc. Mass Spectrom., 2013). From LTQ-ETD fragmentation patterns, the sequence of the COPA oligomers was decoded unequivocally for 22 out of 24 (91%) random beads. Representative spectra of MALDI-MS and ETD fragmentation patterns from a random hit bead are shown in Supp. Fig. S1.

Expression and purification of CLL BCR IgGs—CLL monoclonal antibodies (CLL 014, CLL 068, CLL 169 and CLL 183) were expressed as IgG isotype from IgG-lambda or IgG-kappa (light chain) and IgG-gamma (heavy chain) expression plasmids obtained from professor Nicholas Chiorazzi Laboratory (Feinstein Research Institute, NY). The cloning, expression, and purification of mAbs were performed as reported by Seiler et al. (Seiler et al., 2009)._Heavy and light chain variable gene segments from CLL clones were amplified and sub-cloned into IgG heavy chain and IgG light chain expression plasmids to express the

CLL antibodies as IgG isotype. Human embryonic kidney 293 T (HEK 293T) cells were transiently co-transfected with heavy and light chain plasmids and grown for 5 days in serum free media supplemented with nutridoma-SP (from Roche Applied Science). CLL antibodies were purified from media on a Protein G Plus agarose column (from Thermo Scientific), eluted in 1×PBS and stored in 1% BSA at −20 °C before use.

In order to display full length IgG1 on cell surface, the heavy chain trans-membrane (TM) domain of human IgG1 was added to the C terminus of human CH3 domain in expression vector pIg-gamma. Primers used for TM cloning are shown in supporting information (Fig S4). The Surface membrane immunoglobulins (smIg) were expressed on transiently co-transfected HEK 293 T cells and the expression level (CLL 014, CLL 068, CLL 169 and CLL 183) was determined by staining cells with Goat anti-human Fc-IgG conjugated to allophycocyanine (Fc-APC, from Jackson ImmunoResearch Laboratory Inc) and analyzing by fluorescence activated cell sorting (BD FACSCanto II).

High-throughput bead-based screening of COPA library—The TentaGel beads displaying COPA library were pre-blocked with 1×TBST StartingBlock (Tris-buffered saline, pH 7.5, 0.05% Tween 20) containing 1% BSA before incubation with a 1:500 dilution of goat $F(ab')_2$-anti-human IgG Qdot 655 (Life Technologies) for 45 min at RT in 1×TBST. The beads were visualized under a fluorescence microscope (Olympus BX-51) equipped with a 10×DAPI filter. Any beads emitting red light were collected manually by a micropipette and discarded. The process was repeated by incubating with 100 nM of pooled human IgG (from Sigma-Aldrich) following goat $F(ab')_2$-anti-human IgG-Qdot. The denuded COPA library beads were incubated with 300 nM of CLL BCR antibodies (100 nM each of CLL 068, CLL 169 and CLL 183) in binding buffer for 45 min at RT followed by incubation with a 1:500 dilution of goat $F(ab')_2$-anti-human IgG Qdot for 45 min at RT. Individual beads emitting red fluorescent light were collected manually using a micropipette. A total of 110 beads (brightly fluorescent) were collected, washed with 1×TBST, treated with 1% SDS at 90 °C for 10 min to remove bound proteins, Qdot conjugated antibodies and any non-specific proteins bound to the beads. In the final rounds of selection process initial hit beads were incubated serially with CLL 068, CLL 169 and then CLL 183 to separate the individual hits. A total of 18 hit beads against CLL 169 mAb and 14 hit beads against CLL 183 were identified.

The hit beads were treated with 20 **μ**L CNBr solution overnight to release the compounds and the sequence of the compounds were elucidated by MS and tandem MS/MS using 4800Plus MALDI-TOF Analyzer (AB SCIEX) LTQ-Orbitrap XL (Thermo Scientific), San Jose, CA, USA) equipped with ETD at a resolving power of 60,000 at m/z 400. The structures of the hit compounds were elucidated by careful analysis of the product ions.

Biophysical characterization of initial hit compounds—Putative hit compounds (14 for CLL 169 and 9 for CLL 183) were resynthesized with a fluorescein or biotin group at the linker position and purified on HPLC using C18 reverse phase column. For the fluorescence polarization experiment, fluorescein-COPA compounds (50 nM) were added to the serially diluted CLL BCR mAbs in binding buffer (50 mM Tris, 100 mM NaCl, 0.05% Tween 20, pH 7.6) in a 384-well microtiter plate and incubated in the dark at room temperature until

the binding reached to the equilibrium. Fluorescence polarization (mP units) was measured on an EnVision Multilabel Reader from Perkin Elmer. For ELISA, biotin-tagged compounds and peptide were immobilized on a streptavidin or neutravidin coated 96-well microtiter plate and titrated with serially diluted CLL mAbs for 45 min at RT, followed by a 1:5000 (1-Step Ultra TMB ELISA substrate) or 1:20,000 (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific) dilution of IgG1 conjugated HRP (Southern Biotech) for 30 min at RT. The plates were treated with 1-Step Ultra TMB ELISA substrate (3,3′, 5,5′ - tetramentyl benzidine) or West Pico chemiluminescent substrate (Thermo Scientific) according to the supplier's protocols and the data were recorded on an EnVision Multilabel Reader at 450 nm or on a Tecan Microplate Redaer (Infinite 200Pro) using blue light. For competitive ELISA, KMS5 (or 169-8) peptide was immobilized on to a neutravidin coated 96-well microtiter plate and incubated with 120 nM of CLL 169 mAb for equilibrium binding. The multiwell plate was then treated with serial dilution of KMS5 (self competition) or 169-8 peptide or control (random side chains) for 45 min at RT followed by anti-IgG1-HRP antibody (1:20,000) for 30 min at RT. SuperSignal West Pico Chemiluminescent substrate was used for colorimetric development and the luminescence was recorded on a Tecan Microplate Reader (Infinite 200Pro)

Serum stability test of COPA compounds—Standard solutions of COPA compounds (1.0 mM) were prepared in DMSO. KMS5 (4 μL) was added to a mixture of H2O (96 μL) and human serum (100 μL; Sigma Aldrich), and incubated at 37°C. A 20 μL of plasma was aliquoted from the incubation solution at 0, 24, 48, 72, 96, 120 and 144 h time points. A 1 μL of 0.2 mM internal standard COPA compound was added to the mixture and immediately applied to C18-spin column (Thermo Scientific). The COPA compounds were purified following supplier's protocol and eluted with a 40 μL of an elution solution (80% acetonitrile, 20% H_2O). Resulting solution was diluted with 0.1% formic acid and analyzed by LC-MS ((LC/MS 1100 Series equipped with SBC18 column from Agilent)) with a linear gradient of (5% acetonitrile, 0.05% formic acid/aqueous solution) to (80% acetonitrile, 0.05% formic acid/aqueous solution).

Peptide synthesis—169-8 peptide (CGSSG-DNYAAALAQRAR, grey-linker) was synthesized following standard solid-phase peptide synthesis (SPPS) protocol using HOBt (Hydroxybenzotriazole), HBTU (2-(1H-Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium Hexafluorophosphate), DIPEA (diisopropylethyl amine) and Fmoc-amino acid in DMF. Biotin-maleimide (from Sigma) or Fluorescein-5-maleimide (from Pierce) was conjugated to the peptide by Michael reaction through the free thiol group on Cys. Peptides were purified on HPLC using C18 reverse phase column, lyophilized and stored at −20 °C before use. Control peptide (CGSSG-FLAQKLWSALEY) was custom prepared from Sigma and the biotin was conjugated to it by Michael reaction using biotin-maleimide (from Sigma).

Multivalent COPA and peptide ligand synthesis on dextran polymer—Amino-dextran was synthesized as previously reported (Nakamura et al., 2010). The synthesis and chemical characterization of dextran conjugates were described in details in our previous paper (Morimoto et. al., Bioconjugate Chem. 2014). In short, dextran (500 mg, average molecular weight 35,000–45,000, from *Leuconostoc mesenteroids*, Sigma) was dissolved in

50 mL of anhydrous DMF and treated with 126 mg of *N*,*N*′-carbonyldiimidazole and 250 μL of ethylenediamine. Aminodextran was treated with biotin-NHS ester in DMSO at 4 °C for overnight to conjugate biotin. COPA or peptide ligands were conjugated to the biotinylated-dextran polymer through *N*-(α-Maleimidoacetoxy) succinimide ester (AMAS, from Thermo scientific) in DMSO for two hours in the dark. The unreacted reactants were removed by filtering through Amicon Ultra 4 mL centrifugal filter unit (10,000 NMWL, from Millipore). The ligand-dextran conjugate in the filter unit was recovered in 1×PBS according to the manufacturer's protocol. The concentration of biotin in the solution was quantitatively determined using the Fluorescence Biotin Quantitation Kit (Thermo Scientific). The procedure to estimate the number of ligands conjugated to dextran polymer is described in details in Supplemental experimental procedures.

Cell binding assay by flow cytometry—HEK 293 T cells grown to 70 % confluency 1 day after passage were transiently co-transfected with IgG light chain (pIg-lambda or-kappa) and heavy chain (pIg-gamma-TM) plasmids using 293-fectin transfection reagents (from Life Technologies) following the manufacturer's instructions. Cells were grown for 48 to 72 h at 37 °C in 5% CO_2, collected from the flask, aliquoted ($\sim 0.5\times10^6$ cells) in each well of a microtiter 96-well plate and pre-blocked with 2% BSA in 1×PBS containing 0.1% sodium azide. The cells were incubated with biotin-tagged COPA compounds (50 nM) or 169-8 peptide (20 nM) or dextran conjugated multivalent ligands for 30 min at 4 °C in binding buffer (1×PBS, pH 7.4 containing 1% BSA and 0.1% sodium azide) followed by treating with 1:40 dilution of Streptavidin-Phycoerythrin (saPE, from BD Bioscience) and Fc-APC for 30 min on ice. The cell surface expression of smIg (CLL 014, CLL 068, CLL 169 and CLL 183) was detected as APC signal and the binding was detected as PE signal in flow cytometry (BD FACSCanto II).

Binding assay of multivalent ligand-dextran conjugates with primary CLL Cells from patient—Cryopreserved PBMCs from CLL patient 169 (Jaglowski et al., 2010) were thawed and recovered in AIM V media for 2 h at 37 °C in 5% CO_2. Re-suspended PBMCs in 1×PBS were carefully passed through a density gradient using Ficoll-Paque Plus (from GE Healthcare) to remove the dead cells. B cells were further purified using a B cell purification kit (from Miltenyi Biotec) following the supplier's protocols. Four-color flow cytometry-based analysis was carried out using streptavidin conjugated PE (to determine binding affinity), anti-human CD5-APC antibody and anti-human CD19-FITC antibody (to identify $CD5^+/CD19^+$ CLL B cell populations, from BD biosciences), and Po-Pro dye (for the staining of dead cells, from Life Technologies). About 0.4×10^6 cells were aliquoted into 96-well microtiter plate and incubated with 1×PBS containing 2% BSA and 0.1% sodium azide for blocking followed by binding with biotin conjugated multivalent COPA, peptide ligand, control COPA compound and control peptide in binding buffer for 30 min on ice. The cells were stained with streptavidin conjugated PE, anti-CD5-APC antibody, anti-CD19-FITC antibody, and Po-Pro dye for 30 min on ice and analyzed on BD FACSCantoII for recognition to multivalent antigen surrogates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the NIH (NHLBI Proteomics Center contract (No1-HV-00242) to T.K and RO-1 CA CA81554 to N.C). J.M. was supported by the JSPS Postdoctoral Fellowship for Research Abraod program.

References

Adem YT, Schwarz KA, Duenas E, Patapoff TW, Galush WJ, Esue O. Auristatin antibody drug conjugate physical instability and the role of drug payload. Bioconjug Chem. 2014; 25:656–664. [PubMed: 24559399]

Alluri PG, Reddy MM, Bachhawat-Sikder K, Olivos HJ, Kodadek T. Isolation of protein ligands from large peptoid libraries. Journal of the American Chemical Society. 2003; 125:13995–14004. [PubMed: 14611236]

Anderl J, Faulstich H, Hechler T, Kulke M. Antibody-drug conjugate payloads. Methods Mol Biol. 2013; 1045:51–70. [PubMed: 23913141]

Aquino C, Sarkar M, CHalmers MJ, Mendes K, Kodadek T, Micalizio G. A biomimetic polyketide-inspired approach to small molecule ligand discovery. Nature Chem. 2011; 4:99–104. [PubMed: 22270625]

Bander NH, Czuczman MS, Younes A. Antibody-drug conjugate technology development for hematologic disorders. Clin Adv Hematol Oncol. 2012; 10:1–16. [PubMed: 23072775]

Byrd JC, Furman RR, Coutre SE, Flinn IW, Burger JA, Blum KA, Grant B, Sharman JP, Coleman M, Wierda WG, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. The New England journal of medicine. 2013; 369:32–42. [PubMed: 23782158]

Catera R, Silverman GJ, Hatzi K, Seiler T, Didier S, Zhang L, Herve M, Meffre E, Oscier DG, Vlassara H, et al. Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. Mol Med. 2008; 14:665–674. [PubMed: 19009014]

Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. The New England journal of medicine. 2005; 352:804–815. [PubMed: 15728813]

Chu CC, Catera R, Zhang L, Didier S, Agagnina BM, Damle RN, Kaufman MS, Kolitz JE, Allen SL, Rai KR, Chiorazzi N. Many chronic lymphocytic leukemia antibodies recognize apoptotic cells with exposed nonmuscle myosin heavy chain IIA: implications for patient outcome and cell of origin. Blood. 2010; 115:3907–3915. [PubMed: 20110421]

Doran TM, Gao Y, Mendes K, Dean S, Simanski S, Kodadek T. The utility of redundant combinatorial libraries in distinguishing high and low quality screening hits. ACS Comb Sci. 2014; 16:259–270. [PubMed: 24749624]

Gao Y, Kodadek T. Synthesis and screening of stereochemically diverse combinatorial libraries of peptide tertiary amides. Chem & Biol. 2013; 20:360–369. [PubMed: 23521794]

Jaglowski SM, Alinari L, Lapalombella R, Muthusamy N, Byrd JC. The clinical application of monoclonal antibodies in chronic lymphocytic leukemia. Blood. 2010; 116:3705–3714. [PubMed: 20610811]

Kritzer JA, Luedtke NW, Harker EA, Schepartz A. A rapid library screen for tailoring beta-peptide structure and function. Journal of the American Chemical Society. 2005; 127:14584–14585. [PubMed: 16231906]

Meeker TC, Lowder J, Maloney DG, Miller RA, Thielemans K, Warnke R, Levy R. A clinical trial of anti-idiotype therapy for B cell malignancy. Blood. 1985; 65:1349–1363. [PubMed: 3888313]

Morimoto J, Sarkar M, Kenrick S, Kodadek T. Dextran as a generally applicab;e multivalent scaffold for improving immunoglobulin-binding affinities of peptide and peptidomimetic ligands. Bioconj Chem. 2014; 25:1479–1491.

Nakamura J, Nakajima N, Matsumura K, Hyon SH. Water-soluble taxol conjugates with dextran and targets tumor cells by folic acid immobilization. Anticancer research. 2010; 30:903–909. [PubMed: 20393013]

Peng L, Liu R, Marik J, Wang X, Takada Y, Lam KS. Combinatorial chemistry identifies high-affinity peptidomimetics against alpha4beta1 integrin for in vivo tumor imaging. Nature Chem Biol. 2006; 2:381–389. [PubMed: 16767086]

Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. The New England journal of medicine. 2011; 365:725–733. [PubMed: 21830940]

Sarkar M, Pascal BD, Steckler C, Micalizio GC, Kodadek T, Chalmers MJ. Decoding split and pool combinatorial libraries with electron transfer dissociation tandem mass spectrometry. J Amer Soc Mass Spec. 2013; 24:1026–1036.

Seiler T, Woelfle M, Yancopoulos S, Catera R, Li W, Hatzi K, Moreno C, Torres M, Paul S, Dohner H, et al. Characterization of structurally defined epitopes recognized by monoclonal antibodies produced by chronic lymphocytic leukemia B cells. Blood. 2009; 114:3615–3624. [PubMed: 19690339]

Suwal S, Kodadek T. Synthesis of libraries of peptidomimetic compounds containing a 2-oxopiperazine unit in the main chain. Org Biomol Chem. 2013; 11:2088–2092. [PubMed: 23440085]

Timmerman JM, Vose JM, Czerwinski DK, Weng WK, Ingolia D, Mayo M, Denney DW, Levy R. Tumor-specific recombinant idiotype immunisation after chemotherapy as initial treatment for follicular non-Hodgkin lymphoma. Leuk Lymphoma. 2009; 50:37–46. [PubMed: 19125383]

Wu, H.; Li, Y.; Bai, G.; Niu, Y.; Qiao, Q.; Tipton, JD.; Cao, C.; Cai, J. Chemical communications. Vol. 50. Cambridge; England: 2014. gamma-AApeptide-based small-molecule ligands that inhibit Abeta aggregation; p. 5206-5208.

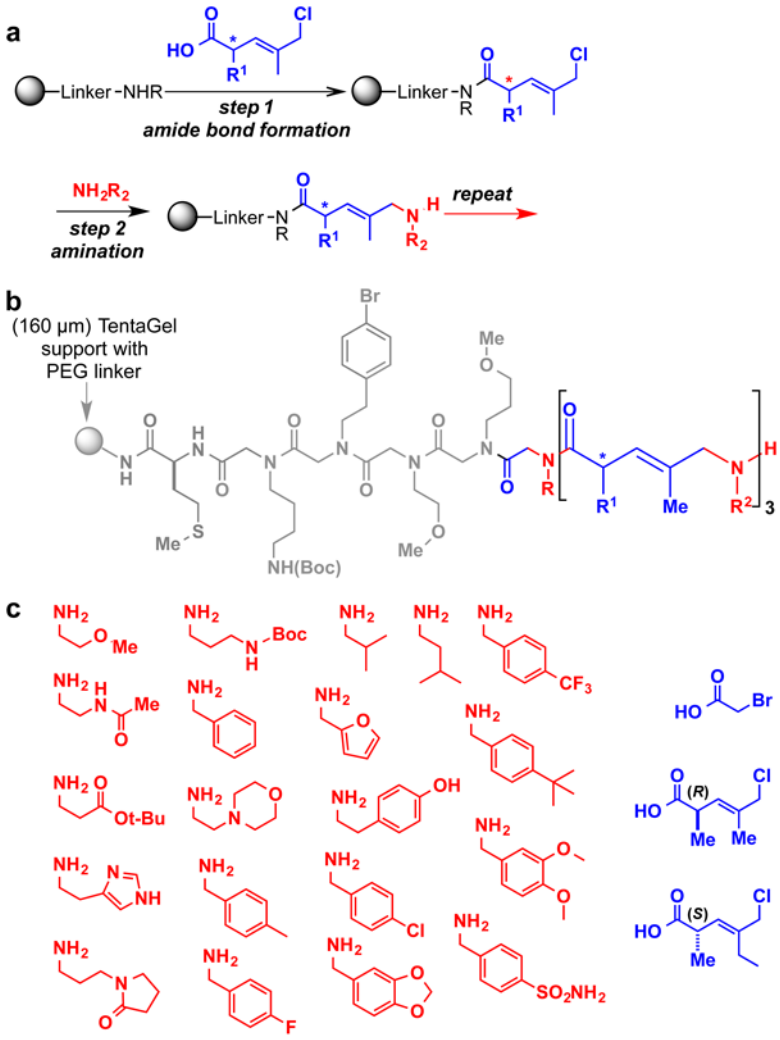

Figure 1. Design and solid-phase synthesis of a one bead one compound library of chiral oligomers of pentenoic acids (COPAs)

a. General synthetic scheme for the solid-phase synthesis of COPAs. b. General structure of the library used in this study comprised of a peptoid unit in the first position followed by three COPA units. The part of the structure shown in grey is an invariant linker present in all of the molecules. The library was constructed by split and pool synthesis on 160 μm TentaGel beads (0.29 mmol/g loading). C. Structures of the acid and amine sub-monomers employed in the library synthesis. By using 20 amines at each of the four variable positions and either (*R*)-2-methyl-5-chloro-3-pentenoic acid or (*S*)-2-methyl-4-ethyl-5-chloro-3-pentenoic acid at positions 2-4, the total theoretical diversity of the COPA library was 1.28 million compounds. 2. g of resin (≈ 1.3 million beads) was used to create the library.

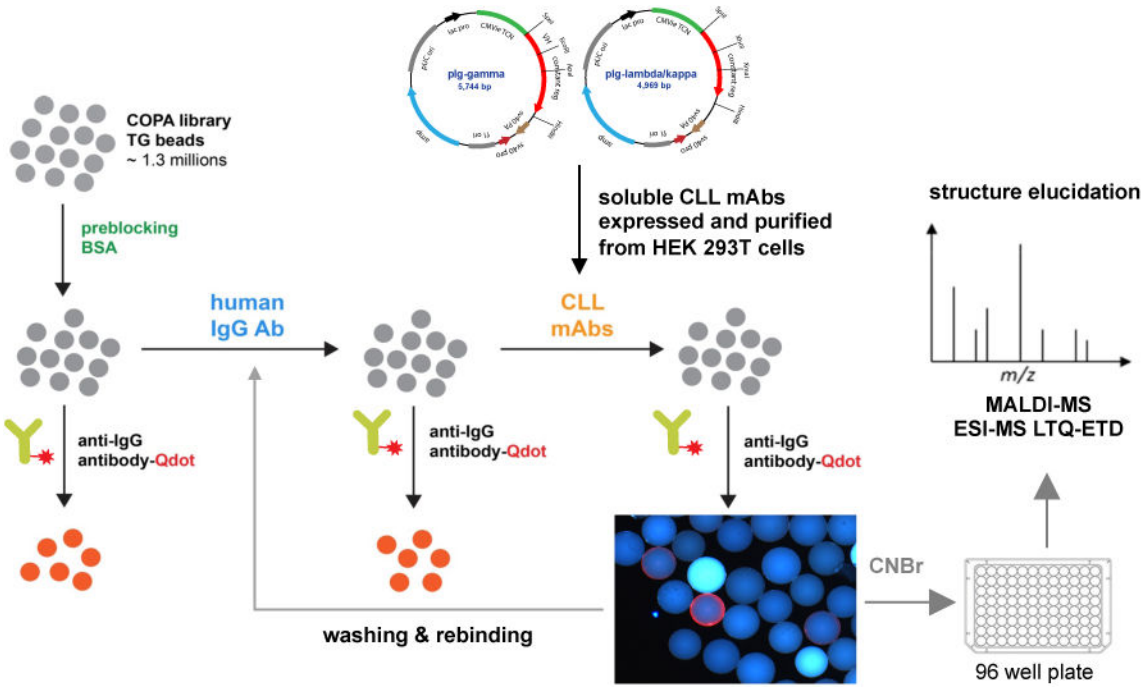

Figure 2. Schematic of the high-throughput bead-based screening strategy

The combinatorial library shown in Fig. 1 (≈ 1.2 million beads) was first incubated with goat anti-human IgG secondary antibody conjugated to a red quantum dot (QD) and beads that bound the secondary antibody, as judged by the presence of a red halo when observed under a low power fluorescence microscope, were removed using a pipette. The remainder of the beads were then incubated with a mixture of human IgGs obtained from individuals who did not have CLL, followed, after washing, by the quantum dot-conjugated secondary antibody. Again, fluorescent beads were removed from the population. The denuded library was then screened against a mixture of the CLL monoclonal Igs, CLL 068, 169 and 183 (100 nM each), followed by the QD-conjugated secondary antibody. The fluorescent beads were pooled and stripped of protein. They were again exposed to control human IgGs and QD-conjugated secondary and beads with any noticeable fluorescence were removed. Finally, the remainder of the beads were exposed to the three CLL antibodies, one at a time, followed by QD-conjugated secondary antibody. The fluorescent beads were collected and placed in the wells of a microtiter plate (one bead per well). The compounds were released from the bead using CNBr and the structures were determined using a combination of MALDI and LTQ-ETD Mass spectrometry. See also Supp. Fig. S1.

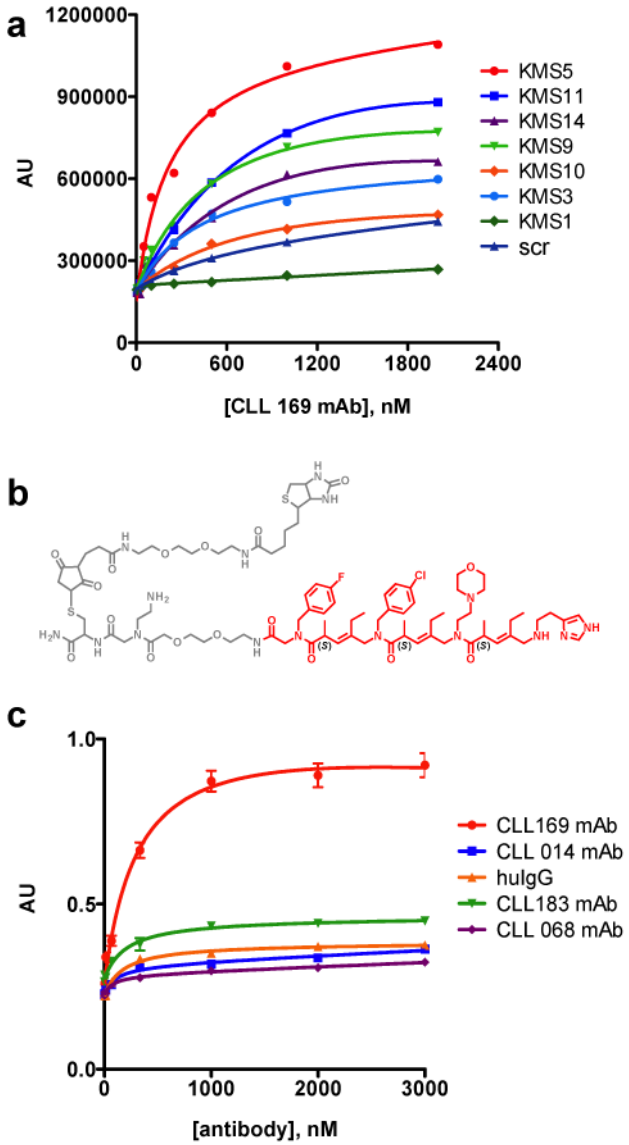

Figure 3. Binding properties of the library screening hits

a. Biotinylated derivatives of six of the COPA screening hits found to bind to CLL 169 in solution (Supp. Fig. S3) were immobilized on streptavidin-coated ELISA plates and titrated with the CLL 169 monoclonal Ig, followed, after washing, by mouse-anti human IgG1-HRP. The K_D of the highest affinity complex (KMS5) was 90 ± 20 nM **(b)** Chemical structure of the biotinylated derivative of KMS5. **(c)** Selectivity of KMS5 for CLL 169. Immobilized KMS5 was titrated with the indicated antibodies, followed by the HRP-conjugated secondary antibody. Error bars indicate standard deviation (SD) of data obtained from three independent experiments. See also Supp. Fig. S3.

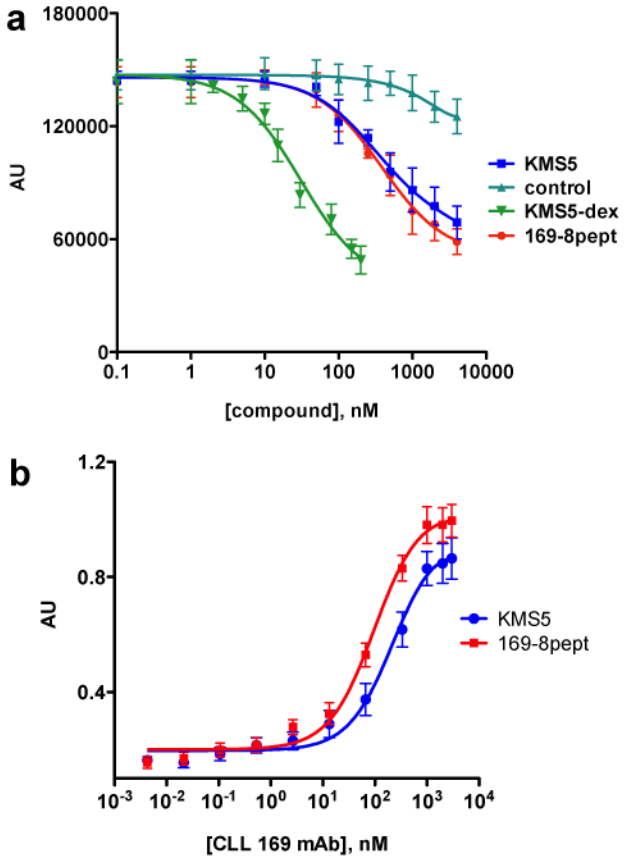

Figure 4. Comparison of the COPA and peptide ligands for CLL 169

(a) Competition binding assays in which the CLL 169 Ig-containing solution (120 nM) was blocked with the indicated concentration of soluble KMS5, KMS5-dextran, 169-8 peptide, or a control peptide, then added to an ELISA plate displaying immobilized KMS5. After washing the level of bound antibody was determined using mouse anti-human IgG1 antibody conjugated to HRP. **(b)** Biotinylated derivatives of COPA KMS5 or peptide 169-8 (DNYAAALAQRAR) were immobilized on streptavidin-coated ELISA plates and titrated with CLL 169 followed, after washing, by mouse-anti human IgG1-HRP. Data analysis and curve fitting were performed with GraphPad Prism 5.0 (GraphPad Software) using a nonlinear regression method. Error bars indicate standard deviation (SD) of data obtained from three independent experiments.

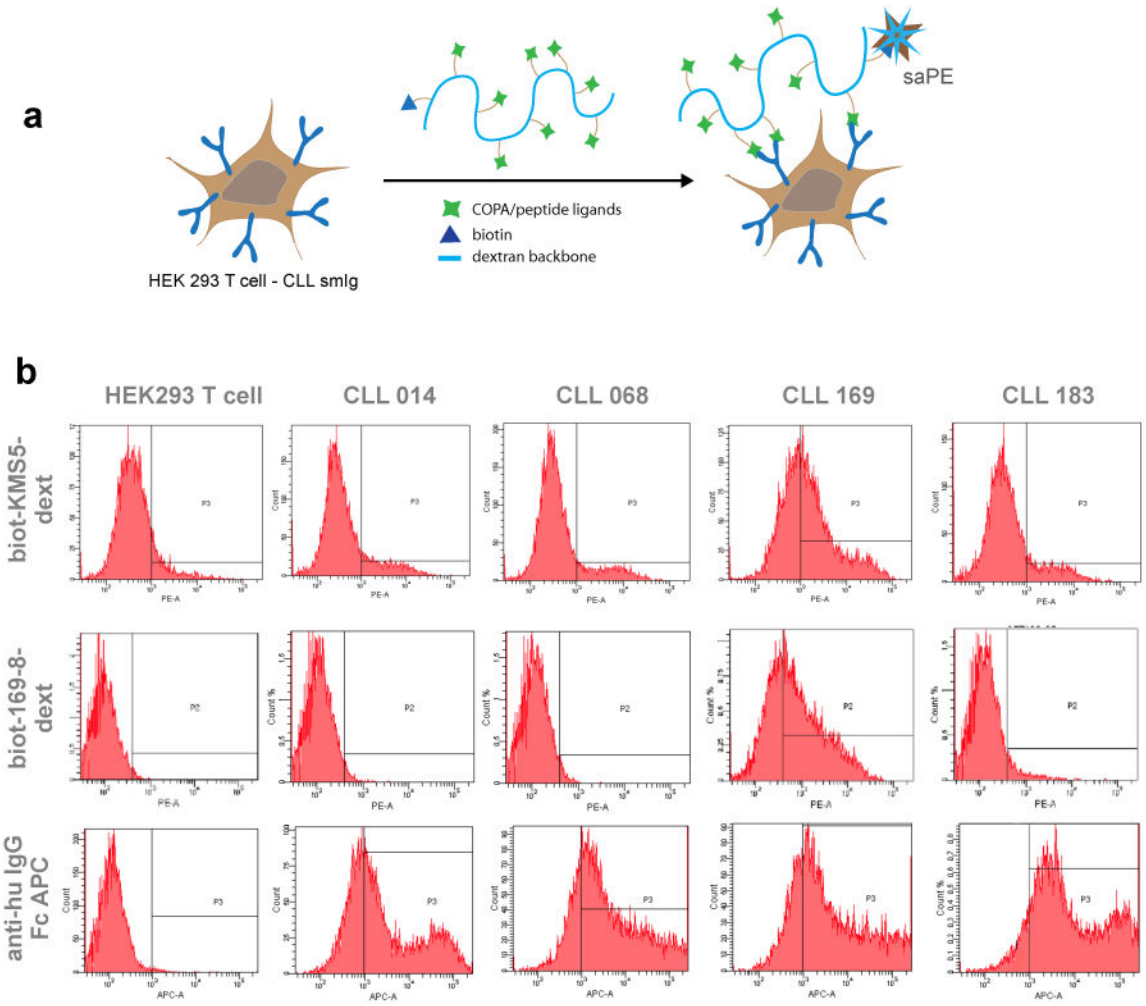

Figure 5. Flow cytometry-based analysis of ligand binding to cells displaying different CLL BCRs

(a) Schematic depiction of the assay. The pIg-gamma expression vectors were re-engineered by introducing a transmembrane (TM) domain at the C-terminus of the CH3 constant region to express CLL BCR as surface membrane immunoglobulin (smIg). HEK 293 T cells were transiently transfected with pIg gamma-TM and Ig lambda/kappa plasmid pairs for CLL 014, CLL 068, CLL 169 and CLL 183 monoclonal Igs and the expression levels of these smIgs were determined by staining the cells with anti-human Ig Fc conjugated to allophycocyanin (anti-huIgFc-APC) flowed by analysis using flow cytometry. The plots indicate that all four of the smIgGs were expressed (bottom row, panel b). The cells were then mixed with a biotinylated dextran polymer displaying 20-30 copies of the COPA KMS5 or peptide 169-8. See also Supp. Fig. S5. **(b)** Flow cytometry plots obtained by treating the cells expressing the indicated smIg with the selecting antibody or dextran polymer, followed by PE-conjugated streptavidin (SA-PE). See also Supp. Fig. S7.

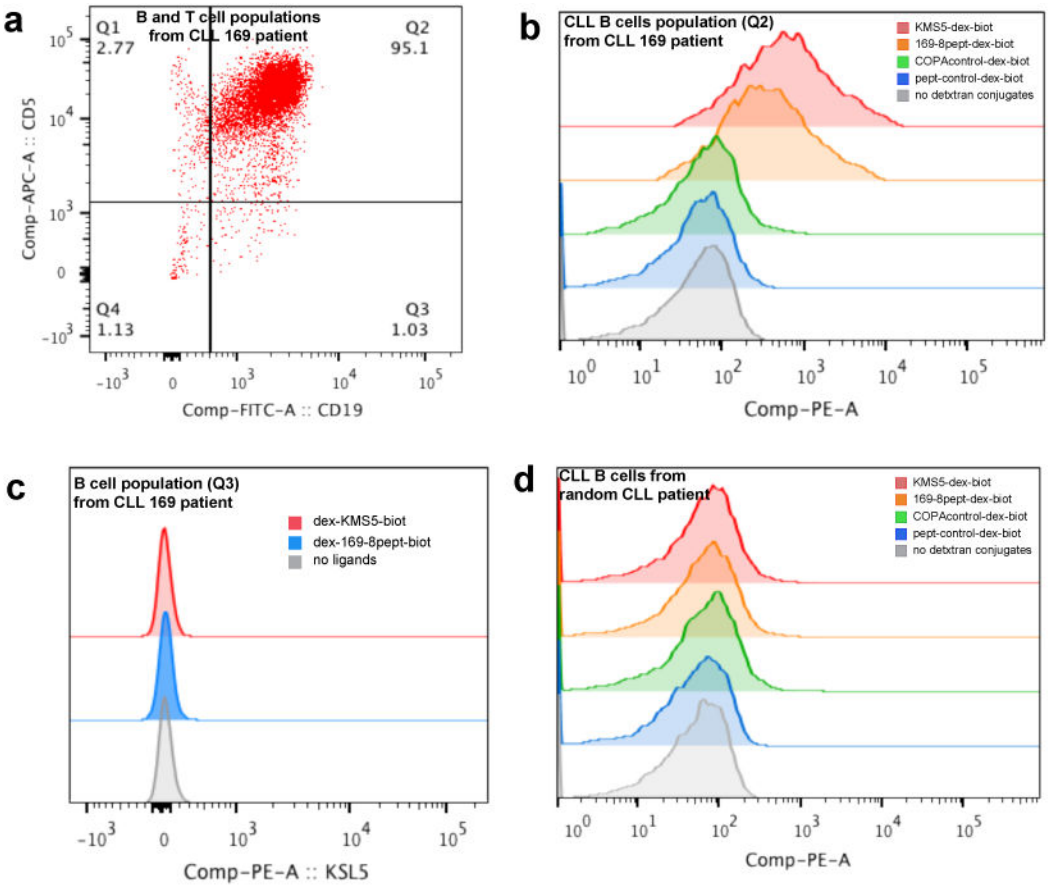

Figure 6. Flow cytometry-based analysis of ligand binding to primary B cells from patients

Purified CLL 169 B cells from PBMCs were incubated with multivalent compound (KMS5-dex-biot), peptide (169-8pept-dex-biot) or controls (COPA compound and peptide that were not selected as CLL 169 ligands) followed by staining and flow cytometry analysis. The stains used were phycoerythrin (PE)-conjugated streptavidin (SA-PE), anti-human CD5-APC antibody, anti-human CD19-FITC antibody, and Po-Pro dye, which dyes dead cells. **(a)** Leukemic B cell populations ($CD5^+/CD19^+/Po\text{-}Pro^-$) and regular B cell populations ($CD5^-/CD19^+/Po\text{-}Pro^-$) from CLL 169 patient are shown in Q2 and Q3 quadrants, respectively, on the dot plot. **(b)** Observed level of SA-PE staining of $CD5^+/CD19^+/Po\text{-}Pro^-$ CLL 169 B cells after incubation with the dextran conjugates indicated. (**c**) Observed level of SA-PE staining of regular B cell populations ($CD5^-/CD19^+/Po\text{-}Pro^-$) after incubation with the dextran conjugates indicated. **(d)** Observed level of SA-PE staining of $CD5^+/CD19^+/Po\text{-}Pro^-$CLL B cells obtained from a different patient (not CLL 169) after incubation with the dextran conjugates indicated (See also Supp. Fig. S7).