Targeting Stereotyped B Cell Receptors from Chronic Lymphocytic Leukemia Patients with Synthetic Antigen Surrogates

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Chronic lymphocytic leukemia (CLL) is a disease in which a single B-cell clone proliferates relentlessly in peripheral lymphoid organs, bone marrow, and blood. DNA sequencing experiments have shown that about 30% of CLL patients have stereotyped antigen-specific B-cell receptors (BCRs) with a high level of sequence homology in the variable domains of the heavy and light chains. These include many of the most aggressive cases that have IG HV-unmutated BCRs whose sequences have not diverged significantly from the germ line. This suggests a personalized therapy strategy in which a toxin or immune effector function is delivered selectively to the pathogenic B-cells but not to healthy B-cells. To execute this strategy, serum-stable, drug-like compounds able to target the antigen-binding sites of most or all patients in a stereotyped subset are required. We demonstrate here the feasibility of this approach with the discovery of selective, high affinity ligands for CLL BCRs of the aggressive, stereotyped subset 7p that cross-react with the BCRs of several CLL patients in subset 7p, but not with BCRs from patients outside this subset.

Chronic lymphocytic leukemia (CLL) is the most common type of adult blood cancer leukemia with ~15,000 new cases reported every year in the United States (1). CLL involves the relentless expansion of a single B-cell clone, arguing that this occurs in response to a particular antigen, although the nature of these antigens is largely unknown (2). Many cases of CLL follow a relatively indolent course, whereas others are highly aggressive.

There have been impressive advances in the treatment of CLL. In addition to standard chemotherapy, anti-CD20 antibodies such as Rituximab, Ofatumumab, and Obinotuzumab are now used in combination or alone in standard clinical practice (1, 3). Inhibitors of Burton's tyrosine kinase, such as ibrutinib (4), which blunt B-cell activation, also have shown considerable promise. Nonetheless, all drugs currently on the market or in clinical trials are general immunosuppressants that fail to distinguish the pathogenic CLL cells from healthy B-cells. Ideally, drug targets would be identified that allow selective suppression or killing of the malignant CLL cells without interfering with the normal function of the humoral immune system (5).

One such target is the antigen-specific B-cell receptor (BCR) itself. Indeed, the pathogenic B-cells in a CLL patient are virtually the only cells in the body that express this particular receptor. Thus, if compounds highly selective for the receptor, with little off-target binding, could be discovered, it is possible that they could be employed chronically, unlike general B-cell killers. Although the antigen-binding pocket of an antibody is not generally considered a drug target, screening studies using phage-displayed peptide libraries have demonstrated the feasibility of obtaining selective ligands for the antigen-binding sites of some CLL BCRs (6–8). Moreover, we demonstrated recently that high affinity, non-peptidic, and serum-stable ligands for a CLL BCR can be discovered via combinatorial library screening (9). These studies support the feasibility of targeting CLL BCR for the development of highly selective drugs.

However, there are at least two major questions that have yet to be addressed with respect to drugging CLL BCRs. First, would a BCR-targeted strategy necessitate the development of a unique compound for each patient? This is obviously impractical. Large-scale DNA sequencing studies of the variable regions of BCRs from thousands of CLL patients has revealed that about one-third of CLL cases are “stereotyped,” meaning that the complementarity determining region 3 (HCDR3) of the pathogenic BCRs has significant homology to the CDR3s of CLL BCRs from other patients (10, 11). Indeed, patients exist whose entire IGHV-D-J amino acid sequences are identical, and some of these have remarkably identical IGHV/αL-β-Jx/A rearrangements. Thus, a critical question is whether a single compound could be identified that would cross-react with the BCRs of most or all of the patients falling into a particular stereotyped subset.

Second, the clinically most aggressive cases of CLL, where the need for new therapies is greatest, usually display so-called “IGHV-unmutated” (U-CLL) BCRs, defined as having a specific
Ig variable region gene (IGHV) sequence differing less than 2% from the germ line (2, 12). There is reason to believe that unmutated BCRs would be more challenging drug targets than BCRs that have undergone more extensive somatic mutation. This is because unmutated pockets are thought to be more flexible and dynamic (13, 14), which is consistent with reports that U-CLL BCRs exhibit significant polyreactivity (7, 15) and that previous attempts to identify peptide ligands for these receptors have only yielded low affinity molecules (7).

In this study, we probe these issues. We report the discovery of selective, high affinity synthetic ligands for the BCR of a patient from stereotyped subset 7P (10), which represents an aggressive, unmutated group of U-CLLs. These ligands were tested for binding to BCRs from other patients of the same stereotyped subset and BCRs from CLL patients outside of that subset. Gratifyingly, there is excellent cross-reactivity between the ligand and all of the 7P stereotype CLL BCRs, but not between the ligand and BCRs outside of that stereotyped subset. The CLL BCR ligands also do not bind detectably to healthy B-cells or T-cells. These data strongly support the idea that the development of BCR-targeted reagents for the treatment of most or all CLL patients of a given stereotyped subset is a tractable goal.

**Experimental Procedures**

Full details of all the experimental procedures not presented below are provided under supplemental "Materials and Methods."

**Design and Synthesis of the Combinatorial Library**—The one bead one compound combinatorial library was synthesized on solid phase (90 μm TentaGel beads, 0.28 mmol/g loading) following sub-monomer methods and split and pool techniques, resulting in each bead displaying many copies of a single compound. The strategy employed for high-throughput bead-based screening against soluble monoclonal antibodies is shown on the right. The library beads were pre-screened against human-IgG and goat anti-human IgG secondary antibody conjugated to a red quantum dot (QD). Any bead that displayed a red halo under a low power fluorescence microscope was removed from the library pool manually. The remainder of the beads was then incubated with recombinant CLL 014 BCR IgG. The hit beads were collected and the structure of the compounds were revealed using MALDI-TOF/TOF and LTQ-ETD mass spectrometry (supplemental Fig. S2).
ting red fluorescent (brightly fluorescent) light were collected manually using a micropipette. Beads were stripped of any bound proteins and the screening process mentioned above was repeated. From the final rounds of the selection process a total of 19 re-validated hit beads were identified. The individual hit beads were treated with 20 μl of 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 and LTQ-Orbitrap equipped with ETD (supplemental Figs. S2 and S3).

Biophysical Characterization of Initial Hit Compounds—Hit compounds were synthesized with a free cysteine molecule in the invariable linker (for fluorescein or biotin conjugation) and purified on HPLC using C18 reverse phase column (supplemental Figs. S4). A control compound with a similar branched backbone with random side chains was also synthesized and purified on HPLC (supplemental Fig. S4, bottom panel). Fluorescence polarization experiments were carried out using 50 nM of probe compound and serially diluted CLL 014 mAb in binding buffer (1× PBS, pH 7.4 containing 1% BSA) at room temperature for 45 min in the dark before recording fluorescence polarization using a Tecan Plate Reader (Infinite M100Pro). Biotinylated compound was immobilized on a streptavidin-coated 96-well ELISA plate and treated with increasing concentrations of CLL014 IgG followed by anti-human IgG1-HRP-conjugated secondary antibody. The plate was treated with TMB reagent and the binding data were recorded on a Tecan Plate Reader (Infinite M100Pro).

**FIGURE 2. Validation of binding of initial hit compounds.** A, fluorescence polarization assay to validate the binding of hit compounds against recombinant CLL 014 IgG. Serially diluted CLL 014 mAb was incubated with 10 nM fluorescein-tagged compounds/control in 1× PBST containing 1% BSA at room temperature for 45 min in the dark before recording fluorescence polarization using a Tecan Plate Reader (Infinite M100Pro). B, ELISA of five highest affinity compounds and a control compound with the same linker and branched backbone but random side chains (as shown in Fig. 2). Biotinylated compound was immobilized on a streptavidin-coated 96-well ELISA plate and treated with increasing concentrations of CLL014 IgG followed by anti-human IgG1-HRP-conjugated secondary antibody. The plate was treated with TMB reagent and the binding data were recorded on a Tecan Plate Reader (Infinite M100Pro).

**FIGURE 3. BLI assay for binding affinity measurements.** Two highest affinity compounds, KMS31 and KMS32 (biotin tagged), were immobilized on streptavidin sensors. The kinetic measurements were carried out by exposing sensors with serially diluted soluble CLL 014 IgG in binding buffer (1× PBS, pH 7.4 containing 1% BSA) in wells of a 96-well microtiter plate. The association and dissociation profiles of the compounds was monitored at 30 °C using an Octet RED96 system (Pall ForteBio). Binding curves were analyzed by global fitting of sensorgrams to a 1:1 binding model using data analysis software provided by Pall ForteBio.

**TABLE 1**

| Ligands  | ELISA $K_D$ | Octet $k_{on}$ | $k_{off}$ | $K_D$ |
|----------|-------------|----------------|----------|--------|
| $K_m$S31 | $67 \pm 13$ | $2.44 \times 10^4$ | $1.25 \times 10^{-3}$ | $51 \pm 11$ |
| $K_m$S32 | $167 \pm 39$ | $7.98 \times 10^3$ | $1.13 \times 10^{-3}$ | $141 \pm 27$ |
FIGURE 4. Binding selectivity of the highest affinity CLL 014 ligands. Biotin-tagged KMS30–32 or a control molecule not reactive with CLL 014 IgG was immobilized on streptavidin-coated ELISA plates and titrated with CLL 014, CLL 169, or CLL 068 monoclonal IgGs or a mixture of non-CLL human IgGs. None of the other antibodies represent the subset 7P to which the CLL 014 IgG belongs. The structures of the molecules are shown on the left and the binding curves on the right. Data analysis and curve fitting were performed with GraphPad Prism 6.0 using a nonlinear regression method. Error bars indicate the standard deviation of data obtained from three independent experiments.
Bio-layer Interferometry (BLI) Assay for Binding Affinity Measurements—Binding kinetics of various CLL IgGs with synthetic ligands were analyzed by BLI using an Octet RED96 system (Pall ForteBio). The highest affinity compounds, KMS31 and KMS32, were synthesized with biotin at the linker and immobilized on streptavidin sensors. The kinetic measurements were carried out by exposing sensors with serially diluted soluble CLL IgGs in binding buffer (1× PBS, pH 7.4, containing 1% BSA) in wells of a 96-well microtiter plate. The association and dissociation profiles of the compounds were monitored at 30°C. Binding curves were analyzed by global fitting of sensorgrams to a bivalent binding model using data analysis software provided by Pall ForteBio.

Binding of Multimeric Synthetic Antigen Surrogates with CLL Cells from Subset 7P Patients—Cryopreserved PBMCs from CLL patients from stereotyped subset 7P (CLL 014, CLL 529, CLL 675, and CLL 1297) and from other non-subset 7P patients were thawed and cultured in RPMI 1640 media supplemented with 10% FBS for 2 h at 37°C in 5% CO2. Re-suspended PBMCs in 1× PBS were carefully passed through a density gra-

FIGURE 5. Selective binding of dextran-conjugated multimeric ligands on HEK 293T cells expressing CLL smIgs. Flow cytometry analysis of dextran-conjugated multimeric ligand binding to cells displaying CLL 014 smIg and other non-selecting control smIgs as controls on HEK 293T cells. The V\textsubscript{H} expression vectors were re-engineered by introducing a transmembrane domain at the C terminus of the CH3 constant region to express CLL BCR as smIgG. HEK293T cells were transiently transfected with plg\textsubscript{γ}-transmembrane and lg\textsubscript{λ/x} plasmid pairs for CLL 014, CLL 068, CLL 169, and CLL 183 monoclonal IgG. The expression levels (bottom row) of the smIgs were determined by staining cells with anti-human Ig Fc-conjugated to allophycocyanin (anti-huIg Fc-APC) and analyzed on flow cytometry. The cells expressing smIgs were treated with bt-dext-KMS31 or bt-dext-KMS32 followed by staining with PE-conjugated streptavidin (sa-PE) before analysis by flow cytometry. Both of these multimeric ligands were selectively binding to cells only when they were expressing CLL 014 smIg. As shown, no significant staining was observed when cells were expressing non-selecting control CLL smIgs. bt-dex, biotinylated dextran.

FIGURE 6. V\textsubscript{H} and V\textsubscript{L} CDR3 sequence homology of BCRs in subset 7P. Protein sequence alignments and sequence logos of the variable heavy and light chain CDR3s of U-CLL IgG from stereotyped subset 7P.
dient using Ficoll-Paque Plus (from GE Healthcare) to remove dead cells. Approximately, 0.4 × 10^6 PBMCs from each CLL patient (CLL 014, CLL 529, CLL 675, CLL 1297, and CLL 3006) and from MEC1 cell line cells (DSMZ) were placed in each well of a 96-well microtiter plate for binding assays. The cells were pre-blocked with 3% BSA in 1× PBS containing 0.1% sodium azide and incubated with dextran-conjugated KMS31, KMS32, and control compounds (biotin-dextran, control compounds conjugated to dextran) in a 40 nm concentration for 40 min at 4 °C in binding buffer (1× PBS, pH 7.4, containing 1% BSA and 0.1% sodium azide). Following washing, cells were treated with SA-PE (to monitor binding), anti-human CD5-APC antibody, and anti-human CD19-FITC antibody (to identify CD5/CD19 CLL B-cell populations), and Po-Pro dye (for the staining of dead cells) for 30 min in the dark on ice. Selective staining of leukemic B-cells (CD5/CD19 populations) by multivalent compounds, dext-KMS31 and dext-KMS32, was detected by SA-PE signaling on flow cytometry (BD LSR II).

Results

Combinatorial Library Screening Provides Non-peptidic Ligands for an Unmutated, Stereotyped CLL BCR—We set as our target BCRs that belong to stereotype 7P, an example of U-CLL BCRs. After expressing the soluble form of the BCR from a patient in this sub-type (CLL 014) as an IgG in HEK 293T cells, the antibody was exposed to a one bead one compound library of molecules displayed on 90-μm TentaGel beads as a way to identify ligands for the antigen-specific binding site. The theoretical diversity of this library was just under one million compounds. As shown in Fig. 1, following a common linker, the variable region of the library contains two peptoid residues followed by a branch point derived from 4-azidoproline, from which two “arms” were constructed that contain units designed to impose conformational constraints (16, 17) (Fig. 1). The synthesis and characterization of this library are discussed in detail elsewhere.3

Screening was conducted largely as described previously (9). The library was first cleansed of beads that display ligands to the conserved regions of IgG antibodies or to antigen-binding sites of non-CLL antibodies. To do so, the beads were pre-blocked in 3% BSA and exposed to commercially available human total IgG, followed by anti-human IgG Fc antibody conjugated to red-emitting quantum dots. After thorough washing, the beads were examined under a fluorescent microscope and those that exhibited an obvious red halo were removed from the population manually using a micropipette (Fig. 1). The remainder of the library was then incubated with 100 nm soluble CLL 014 mAb. After another wash, the beads were incubated with labeled secondary antibody for visualization under the fluorescent microscope. A total of 83 brightly fluorescent beads were picked as putative hits.

Given the propensity of this procedure to yield false positives (19, 20), these beads were carried through another cycle of screening. First, the beads were washed with a denaturing buffer to remove any bound protein. After re-equilibration in a native buffer, the 83 beads were re-exposed to pooled human

3 M. Sarkar and T. Kodadek, unpublished data.
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KMS31

CLL014

CLL529

CLL675

CLL1297

KMS32

CLL014

CLL529

CLL675

CLL1297
TABLE 2

| Subset 7p IgG | K<sub>S31</sub> | K<sub>S32</sub> | K<sub>d</sub> Octet | K<sub>d</sub> ELISA |
|--------------|--------------|--------------|----------------|----------------|
| CLL014       | 67 ± 13      | 51 ± 11      | 167 ± 39       | 141 ± 37       |
| CLL529       | 331 ± 63     | 264 ± 57     | 391 ± 67       | 347 ± 59       |
| CLL675       | 239 ± 041    | 197 ± 36     | 237 ± 51       | 271 ± 33       |
| CLL1297      | 425 ± 73     | 340 ± 66     | 529 ± 90       | 489 ± 86       |

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constants were 2.44 × 10<sup>4</sup> M<sup>−1</sup> s<sup>−1</sup> and 1.25 × 10<sup>−3</sup> s<sup>−1</sup>, respectively. Under these conditions, the half-life of the complex was ~550 s.

To examine the selectivity of these three ligands, the immobilized biotinylated species were titrated with CLL 014 IgG, or IgGs derived from CLL BCRs outside of subset 7P (CLL 068 and CLL 169) as well as total human IgG using the ELISA format. Note that CLL 068 uses the same unmutated IGHV as the selecting mAb CLL 014. As shown in Fig. 4, KMS31 and KMS32 exhibited good selectivity for binding to CLL 014 as opposed to other human antibodies. KMS30, on the other hand, showed comparatively higher “off target” binding to the other human IgGs, so further characterization efforts were focused on KMS31 and KMS32.

To determine whether a similar level of selectivity is observed in a more native-like environment where the IgG is displayed on a cell surface, CLL 014, CLL 068, CLL 169, and CLL 183 IgGs were expressed on cells as surface immunoglobulins (smIg) by inserting a transmembrane anchoring domain at the C terminus of the heavy chain using methods described previously (9). HEK 293T cells were co-transfected with heavy and light chain expression vectors and the expression levels of surface membrane IgGs were determined by staining the cells with anti-human IgG Fc-specific antibody conjugated to allophycocyanin (anti-hulg-Fc-APC). Flow cytometry analysis confirmed expression of all 4 BCR IgGs of subset 7P on HEK 293T cells (supplemental Fig. S5).

To assess binding of KMS31 and KMS32 to these cells, the ligands were conjugated to a biotinylated dextran polymer that displays an average of 20–30 ligands per polymer chain (9, 22). They were then incubated with cells and binding was assayed by flow cytometry after staining with PE-conjugated streptavidin (Fig. 5). The dextran conjugates are employed in this type of experiment because the relatively rapid dissociation rate of the monomeric ligands (Fig. 3) preclude the use of flow cytometry for analysis of binding, as discussed previously (9). The multivalent dextran conjugates provide strong avidity-driven, long-lived binding. As shown in Fig. 5, both of the dextran-conjugated compounds, dext-KMS31 and dext-KMS32, stained the cells only when they were expressing CLL 014 smlg. Much lower levels of binding were observed for cells expressing the other CLL smIgs not belonging to subset 7P, including CLL mAb068 that uses the same IGHV1–69.

KMS31 and KMS32 Recognize Multiple BCRs within Subset 7P—With selective ligands for the CLL 014 BCR in hand, we turned to the critical question of whether these compounds would recognize other BCRs in subset 7P. Biotinylated KMS31 or KMS32 were immobilized on a neutravidin-coated 96-well ELISA plate or streptavidin sensors (for Octet) and titrated with increasing amounts of soluble monoclonal IgGs that represent...
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FIGURE 9. Flow cytometry analysis of ligand binding to cells displaying stereotyped CLL smIgs on HEK 293T cells. A, general scheme of the cell binding assay with dextran-conjugated multimeric ligands. CLL mAbs from CLL 014, CLL 529, CLL 675, and CLL 1297 and other control CLL IgG were transiently expressed on the HEK 293T cell surface. The cells were incubated with a biotinylated dextran polymer displaying 20–30 copies of the KMS31 or KMS32 and controls followed by staining with phycoerythrin-conjugated streptavidin (sa-PE). B, overlaid flow cytometry histograms showing the binding of dext-KMS31 on the cells expressing stereotyped smIgs from subset 7P and controls. C, overlaid flow cytometry histograms showing the binding of dext-KMS32 on cells expressing stereotyped smIgs from subset 7P and controls.
other BCRs from subset 7P (CLL 014, CLL 529, CLL 675, and CLL 1297; see Fig. 6 for the sequences of the CDR3s) or BCRs from CLL patients outside of subset 7P (CLL 169 and CLL 068). CLL 169 is a mutated, non-stereotyped CLL mAb, whereas CLL 068 is an unmutated CLL mAb from a different subset (subset 6) that nevertheless uses the same IGHSV1–69 (8). A mixture of commercially available human IgG was used as an additional control. As shown in Fig. 7, the Octet and ELISA data show clearly that KMS31 and KMS32 recognized the subset 7P CLL IgGs to a much greater extent than IgGs outside of this subset. Quantitatively, the $K_D$ values of the complexes between KMS31 and the other subset 7P IgGs are 4- to 7-fold higher (weaker binding) compared with the $K_D$ of the KMS31-CLL 014 IgG complex (Fig. 8 and Table 2). For KMS32, the range was 2- to 3.5-fold. Most importantly, no significant binding was observed for either compound against the other non-subset 7P control U-CLL or M-CLL Igs and against polyclonal human IgG.

Selective Binding of KMS31 and KMS32 to Primary CLL Cells from U-CLL “Subset 7P” Patients—CLL cells are known to express BCRs at lower density compared with healthy B-cells. Thus, whereas it is encouraging that the dextran conjugates of KMS31 and KMS32 bind to HEK 293T cells expressing subset 7P smIgs, it is important to analyze binding to bona fide patient-derived CLL B-cells. Frozen PBMCs from patients belonging to subset 7P (CLL 014, CLL 529, CLL 675, and CLL 1297) as well as CLL patients outside subset 7P were available in the collection at The Feinstein Institute for Medical Research. The frozen PBMCs were thawed and dead cells were removed by density gradient separation using Ficoll-Paque. The populations enriched for living cells were allowed to recover for 2 h before further manipulation. First, the isotype and expression levels of BCR Igs were confirmed by staining subset 7P and control cells with anti-human IgM (Fc5 fragment-specific) antibody (Fig. 10).

With expression of the BCR established, the cells were incubated with biotin-tagged dextran conjugates (KMS31, KMS32, or a control compound, not selected as a CLL 014 ligand, as shown in Figs. 4 and 11A). After washing, the cells were incubated with Po-Pro dye (to stain dead cells), as well as streptavidin-conjugated PE, anti-CD5-APC antibody, and anti-CD19-FITC antibody. CLL cells and T cells, but not healthy B-cells, express CD5 (23), whereas only B-cells (CLL and normal cells), but not T cells, express CD19, allowing these three cell types to be distinguished by these two markers. Double positive CD5+/CD19+ CLL cells (Fig. 11B, quadrant...
2 (Q2)) were gated and monitored for staining by the synthetic molecule-dextran-streptavidin-conjugated PE by flow cytometry. Similarly, other populations of the blood lymphocytes (CD5+/CD19+ T cells, CD5−/CD19+, and CD5−/CD19− cells; Q1 and Q3/Q4, respectively, in Fig. 11B) in PBMCs from the same CLL patient were also monitored for...
In this paper, we explore a novel strategy of this sort for the development of therapeutics for CLL. As mentioned above, CLL involves the accumulation of a single antigen-specific B-cell clone, arguing for antigen stimulation. In theory because of the tremendous diversity in the structure of antigen-binding sites on Ig that recombination, N-addition, and somatic mutation impart, no other cell in the body displays this receptor, making these pathogenic BCRs attractive targets for therapy.

Moreover, most CLL B-cells are frozen at the smlg stage of B-cell development and therefore do not differentiate into plasma cells. Hence, the blood of CLL patients does not contain high levels of antibodies corresponding to the antigen-specific BCR displayed on the CLL cells. Therefore, interception of the “drug” by secreted Ig before it reaches the target cell is much less likely than for other B-cell malignancies for which the malignant cell is at a later stage of B-cell maturation and the levels of soluble Ig are likely higher, e.g. Hairy Cell Leukemia and non-Hodgkins lymphoma.

However, many hurdles must be surmounted to execute this strategy. First, the antigen sites of BCRs are not generally considered druggable targets. There are a few reports of the identification of peptide ligands for CLL BCRs (6–8) but, prior to our work (9), no examples of non-biological, proteolytically stable CLL BCR ligands. Although, the peptide ligands against CLL 014 U-CLL BCR identified by Chiorazzi and co-workers (15) were selective, they bound with low affinity. Indeed the binding was too weak to measure a binding constant, and highlight the major improvement realized with the ligands reported here. Moreover, the BCRs from highly aggressive U-CLL patients are highly polyreactive (15), which is thought to be a reflection of a “looser” and more dynamic antigen-binding pocket (13, 14) because these receptors have not undergone somatic hypermutation. This could make them more difficult targets for the development of highly selective and potent drugs (7). In addition, there is the question of whether or not BCR-targeted drugs would be absolutely patient-specific. However, sequencing studies have revealed the existence of several CLL stereotyped subsets that account for about one-third of all CLL cases (10, 25). The high level of sequence homology between the BCRs of patients within one of these stereotyped subsets suggests that a compound isolated against a given stereotyped BCR might exhibit considerable binding affinity for other Igs in the same subset.

The data reported here show that relatively high affinity, non-peptidic, serum-stable (Fig. 12) ligands for a stereotyped U-CLL BCR can indeed be discovered through combinatorial library screening. We identified several molecules from a library of almost one million bead-displayed targets that bound a soluble, IgG version of a CLL BCR from stereotyped subset 7P
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(8). Two of these, KMS31 and KMS32, proved to have high affinity and selectivity for the CLL 014 IgG. Thorough in vitro characterization of the binding properties of these two molecules showed that they did indeed bind well to three other CLL IgGs from patients in subset 7P, with only a modest 2- to 7-fold reduction in affinity, and with little or no binding to IgGs outside of this subset. Experiments using dextran conjugates of these molecules and HEK 293T cells expressing CLL smlIgGs provided similar results. Most importantly, as shown in Fig. 11, the KMS31- and KMS32-dextran targets stained patient-derived primary CLL cells if and only if they displayed a stereotyped subset 7P BCR. These data provide strong support that it should indeed be feasible to develop BCR-targeted drugs applicable to the treatment of most or all CLL patients in a given stereotyped subset.

KMS31 and KMS32 are primary screening hits from a completely unbiased combinatorial library (Fig. 1). Thus it will be important, and should be quite feasible, to affinity mature them into much higher potency BCR-targeting agents. We have shown recently that this kind of hit improvement can be achieved through the construction of "derivative libraries" that explore chemical space around the structure of the primary hit in hopes of optimizing the fit of the molecule in the protein pocket (18). Another important step will be to tether KMS31, KMS32, or improved derivatives to some type of toxic moiety or immunological effector function to kill the CLL target cells. This work is underway.

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