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14. ABSTRACT The proposed research set to: 1)create and characterize CD22-binding peptides that initiate signal transduction and apoptosis in NHL., 2) optimize CD22-mediated signal transduction and lymphomacidal properties of ligand blocking anti-CD22 mAbs and peptides with CD22-specific phosphatase inhibition and 3) correlate mAb-mediatedand anti-CD22 peptide-mediated in vivo physiologic changes, efficacy, and tumor targeting using advanced iPET and FDG-PET imaging technology. Since funding we have identified five peptides that are based on CDR's of anti-CD22 mAbs. Only the sequence derived from heavy chain CDR2 (Peptide 5) demonstrated significant B-cell binding. Peptide5 bound to both malignant and primary B-cells with very little T-cell binding. The affinity had a Km of 5x10-6M. Peptide 5 mediated killing of several NHL cell lines to a degree similar to that of the parent mAb (HB22.7). Peptide 5's loop structure was shown to be crucial for B-cell binding and ligand blocking. Mutational analysis revealed that most amino acids were critical for B cell binding. Using a CD22 transfected COS cell line, we demonstrated CD22-specific binding and CD22 ligand blocking to a degree similar to HB22.7. Finally Peptide 5 was used as a vehicle to deliver a pro-apoptotic peptide into NHL cells. Peptide 5 was fused to a BH3 death domain-containing peptide which demonstrated more effective NHL cell killing than the parent peptide.					
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Introduction

CD22 is a B-lymphocyte-specific glycoprotein that functions as an adhesion molecule capable of binding multiple hematopoietic cell types; it also transduces signals to the cell interior. Our studies have begun to dissect the CD22 signaling cascade at the biochemical level. We identified anti-CD22 monoclonal antibodies (mAbs) that bind the two NH₂-terminal immunoglobulin domains of CD22 and specifically block the interaction of CD22 with its ligand. These “blocking” mAbs induce proliferation of primary B-cells, but apoptotic responses in neoplastic B-cells. Preliminary data show that CD22 ligand blocking mAbs that effectively crosslink CD22 have *distinct* functional properties and facilitate assembly of an effector protein complex. These anti-CD22 mAbs (like HB22.7) are unique and functionally distinguishable from other anti-B-cell, and even other anti-CD22 mAb. Therefore, HB22.7 has the potential to become an exciting, new treatment for non-Hodgkin’s lymphoma (NHL). The NCI approved, funded, and recently completed humanization of the HB22.7, blocking, anti-CD22 mAb through the Rapid Access Intervention Drug (RAID) Program. Humanization of HB22.7 may permit recruitment of immune mechanisms such as antibody and complement dependent cellular cytotoxicity. We hypothesize that enhancing the intrinsic pro-apoptotic properties of HB22.7 by humanization will translate into even better clinical efficacy. Humanized HB22.7 (hHB22.7) could become a new therapy for patients with CD22-positive NHL, much as rituximab (Rituxan) is an option for patients with CD20-positive NHL. However, before the NCI RAID program will produce hHB22.7 for clinical trials, validation of the safety, biodistribution, and pre-clinical efficacy is necessary. Based on these hypotheses our Specific Aims are:

Aim I is to *identify and characterize CD22-binding peptides that initiate signal transduction and results in apoptosis. CD22 binding and internalization will be optimized to enhance the highly specific and effective lymphomacidal properties demonstrated by the parent mAbs.*

Hypothesis: Peptides derived from the highly conserved CDRs of anti-CD22 ligand blocking mAbs can bind CD22 and will be effective treatment for NHL.

Rationale: MAb that target cell surface receptors are proving to be powerful tools for modulation of cellular function. However, mAb have limitations: need for costly humanization, expense of production and purification, and potentially suboptimal penetration into larger tumors. Peptides, in contrast, lend themselves to easy and cost-effective production and purification. The ability to manipulate the sequence of peptides (which we have already demonstrated) has the potential to further enhance their efficacy. In addition given the specific nature of their targeting and internalization, the peptides can be used as vehicles for delivery of cytotoxic drugs, signaling modulators, or apoptosis inducers.

The goals of Aim I are:

1. To design and synthesize peptides derived from the highly conserved CDRs of anti-CD22 ligand blocking mAbs and characterize their binding *in vitro* to B-cell NHL lines and normal tonsillar B-cells.
2. The physiologic effects of high affinity peptides: initiation of signal transduction, and effects on cell growth and apoptosis, will be studied.
3. High affinity binding peptides will be further characterized by N and C-terminal deletion analysis and alanine walk analysis to identify the crucial amino acids for molecular

recognition. Mutational analysis will be done to identify more peptides with enhanced affinity.

4. Promising peptides that initiate signal transduction and mediate apoptosis will be further assessed *in vivo* for their lymphomacidal properties using a nude mouse xenograft model.

Aim II is to optimize CD22-mediated signal transduction and the lymphomacidal properties of the ligand blocking anti-CD22 mAbs and peptides with CD22-specific phosphatase inhibition.

Hypothesis: Phosphatase inhibition will specifically augment the lymphomacidal properties of the anti-CD22 blocking mAbs and CD22-targeting peptides.

Rationale: Our lab and others have spent years elucidating the details of CD22-mediated signal transduction. It was ascertained that the tyrosine phosphatase SHP-1 (aka PTP-1C) preferentially associates with the cytoplasmic tail of CD22 and down modulates CD22-mediated and BCR-mediated signals. The other B-cell-specific receptors (CD19, CD20, and the BCR) do not have appreciable amounts of SHP-1 or other known tyrosine phosphatases physically associated with them. Therefore the SHP-1/CD22 association is specific. We have demonstrated that phosphatase inhibition (PI) significantly enhances CD22-mediated signals, apoptosis, and lymphomacidal effects (figures 12-14).

Goals for Aim II are:

1. To analyze CD22-mediated signal transduction and apoptosis manipulated by tyrosine phosphatase inhibition *in vitro*.
2. To assess the efficacy of combining phosphatase inhibitor(s) with the anti-CD22 ligand blocking mAb and peptides in human NHL xenograft models.

Aim III: to correlate mAb-mediated and anti-CD22 peptide-mediated *in vivo* physiologic changes, efficacy, and tumor targeting using advanced iPET and FDG-PET imaging technology. The influence of phosphatase inhibitors will also be evaluated.

Hypothesis: iPET scanning will allow for serial noninvasive monitoring of targeting and all for correlation of targeting with response and efficacy.

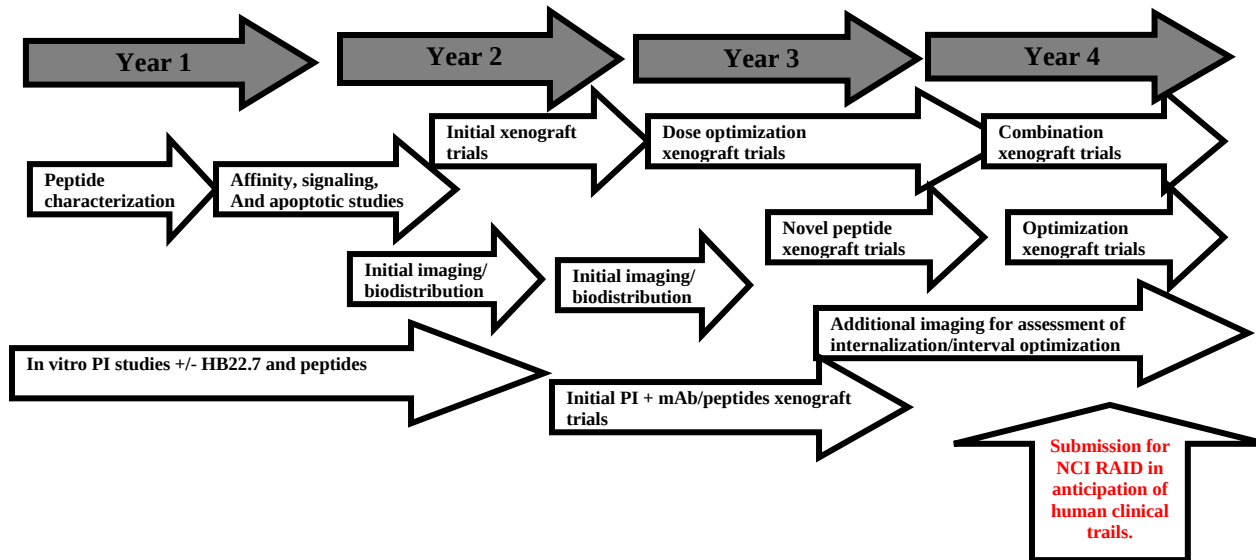
Rationale: A better understanding of CD22 targeting and the resultant physiologic effects will facilitate translation of peptides and phosphatase inhibitors from a research endeavor to exciting new drugs for patients.

The goals for Aim 3 are:

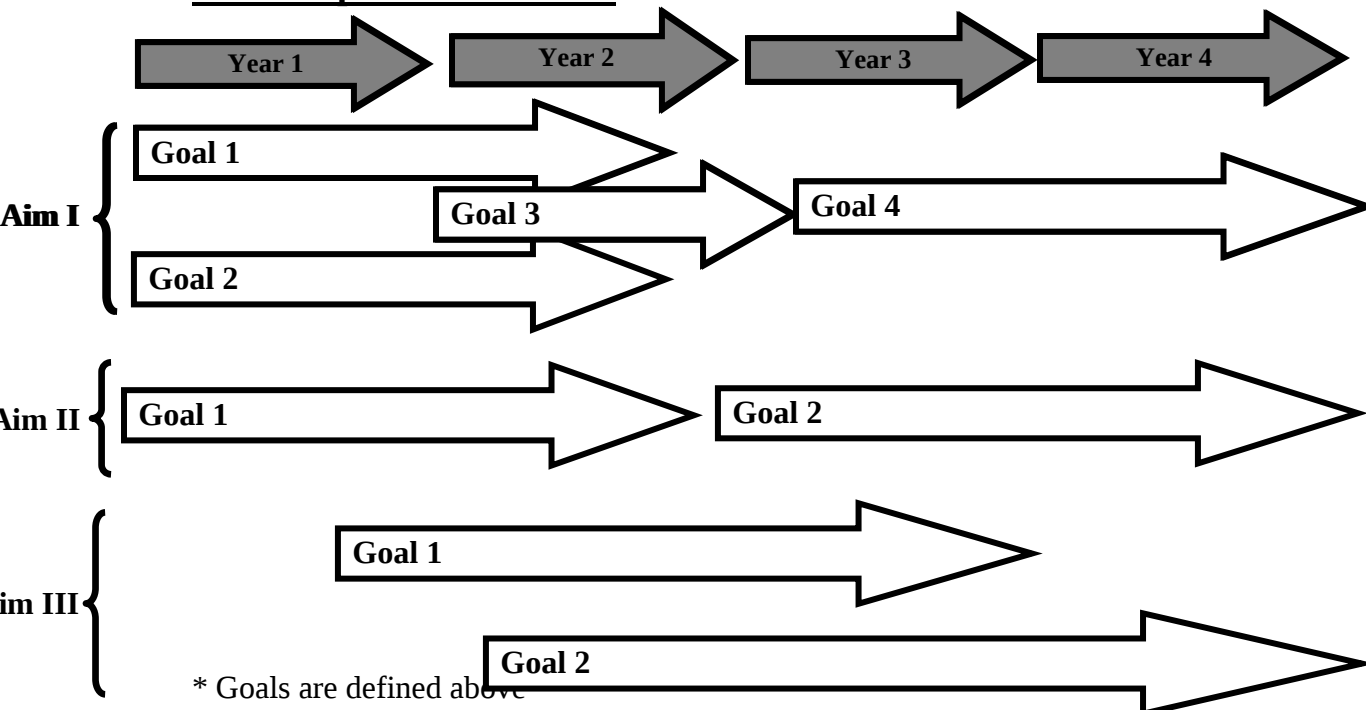
1. To assess *in vivo* tumor metabolism by: FDG-PET imaging (which shows tumor metabolic activity), and iPET imaging (a highly sensitive method to assess *in vivo* tumor-targeting). IPET with peptides will either employ ^{64}Cu -DOTA-peptide or ^{18}F -peptide depending on the amino acid sequence of the peptide then under study. Small animal PET imaging is available at only a few institutions: the Bio-imaging Center at UCD is one of them. IPET can be highly useful for understanding the “real time” *in vivo* consequences of treatment. Radiolabeling of tumor targeting peptides with radionuclides appropriate for PET is going to be done, however, the precise labeling techniques can only be described after the amino acid sequence of the peptide chosen for study is determined in Aim I.

2. To serially confirm and correlate the imaging data with the clinical effect (response rate) and *in vitro* physiologic effects (signaling, apoptosis) by using fine needle aspirates (FNA) and flow cytometry (FACS).

Timeline



Goal*-dependent Timeline



Annual Report Summary/Key Research Accomplishments

Since initiation of funding in 2007 we have made substantial progress in achieving goals 1,2, and 3 of Aim I as predicted by the timeline described above in the statement of work. Much of this

work has recently been accepted for publication in the *International Journal of Peptide Research* (appendix 1).

In this report, we demonstrate that CDR-based peptides derived from the anti-CD22 ligand blocking mAb are capable of binding CD22 with resultant lymphomacidal activity. Previously described combinatorial chemistry techniques were used to effectively present and screen CDR based peptides in primary B and T-cells, and B-cell NHL cell lines. Peptide 5 a peptide that contains the sequence of CDR2 of the anti-CD22 mAb HB22.7 was extensively studied due to its superior binding to Karpas 422 cells (B-cell NHL), and normal primary B-cells when compared to the four other synthesized CDR-based peptides, (appendix 1, figure 2). Binding studies revealed Peptide 5 to be relatively B-cell specific with only minimal T-cell binding (appendix 1, figure 3). Pre-incubation of B cells with HB22.7 abrogated Peptide 5-mediated binding which is consistent with the hypothesis that Peptide 5 binds to the same CD22 epitope as one of the parent mAbs, HB22.7. Structural examination revealed that the Peptide 5 loop structure and that all 21 amino acids of Peptide 5 appears to be required to achieve cellular specificity and binding to CD22. Cysteine residues were added at both ends of the peptide for cyclization to mimic the CDR structure. Loop reduction with DTT disrupts the disulfide bonds necessary for binding to CD22, (appendix 1, figure 4). Consequently, the three dimensional structure of Peptide 5 appears crucial for B-cell binding. Next the alanine walk mutational analysis and the N- and C-terminal deletion analysis demonstrated that all but two amino acids were critical for CD22 binding (appendix 1, figure 5). The non-blocking CD22 mAb (HB22.27) and blocking CD22 mAb (HB22.7) differ dramatically in the percent inhibition of ligand binding; they have been previously shown to bind different regions of CD22. Next a formal analysis of CD22 ligand blocking was done to verify that Peptide 5 binds to domains 1 and 2 of CD22 and blocks CD22 ligand binding. When compared to HB22.7 and HB22.27, Peptide 5 has intermediate blocking activity, whereas Peptide 1 demonstrated very little CD22 ligand blocking activity (appendix 1, figure 6). This supports the hypothesis that Peptide 5 binds CD22 domains 1 and 2 and at least partially blocks CD22 ligand binding. The small size of Peptide 5 and the fact that HB22.7 contains 12 CD22-binding CDRs may account for the inferior blocking capability of Peptide 5.

The CD22-binding affinity of Peptide 5 was assessed using a flow-based Scatchard analysis which demonstrated a K_d of 5×10^{-6} M (appendix 1, figure 7). While this is considerably lower than what has been measured for HB22.7 (10^{-9} M), it is consistent with the affinity of other CDR-mimetic peptides. The difference can be, in part accounted for by the increased number of CDRs within the parent blocking mAbs. Studies utilizing focused peptidomimetic libraries are currently being used to improve the affinity of Peptide 5.

Based on previous data with HB22.7, we hypothesized that CD22 ligand blocking is required for CD22-mediated lymphomacidal activity. Our studies reveal that Peptide 5 has similar lymphomacidal effects when compared to HB22.7 despite some difference in its ability to block CD22 ligand binding, (appendix 1, figure 8). One of the advantages of peptide-based therapeutics is that they are easily manipulated to modify affinity and specificity. In addition, they can be used as vehicles to carry cytotoxic payload. CD22 is a unique therapeutic target as it is B-cell specific, found on the majority of B-cell NHL, and is internalized once bound.

While not originally proposed in the current proposal, based on the unique targeting, internalization, and pro-apoptotic potential of this peptide we decided to explore it's use as a carrier vehicle. We harnessed the death-promoting alpha helical properties of the BH3 domain of BAD by fusing it to Peptide 5 which will promote B cell internalization. Previous studies have used this approach by fusing the BH3 domain to the internalizing antennapedia (ANT) domain.

This study demonstrated Bcl-2 independent pro-apoptotic effects; however the ANT domain is not tissue specific. Treatment of Ramos NHL cells with Peptide 5-BAD resulted in dose responsive lymphomacidal activity that was more effective than the parent mAb, HB22.7, or Peptide 5 alone (appendix 1, figure 9). Studies that specifically examine the mechanism by which Peptide 5-BAD mediates its lymphomacidal activity are ongoing.

In terms of Aim 2 those studies are just getting underway. Initial signaling studies revealed that similar to the parent mAb HB22.7, Peptide 5 also activates the p38 and SAPK signaling pathway figure 1 (below). While these studies need to be further verified they suggest that the peptides initiate the same signaling pathway as the parent mAb and this sets the stage for manipulation as described in Aim 1.

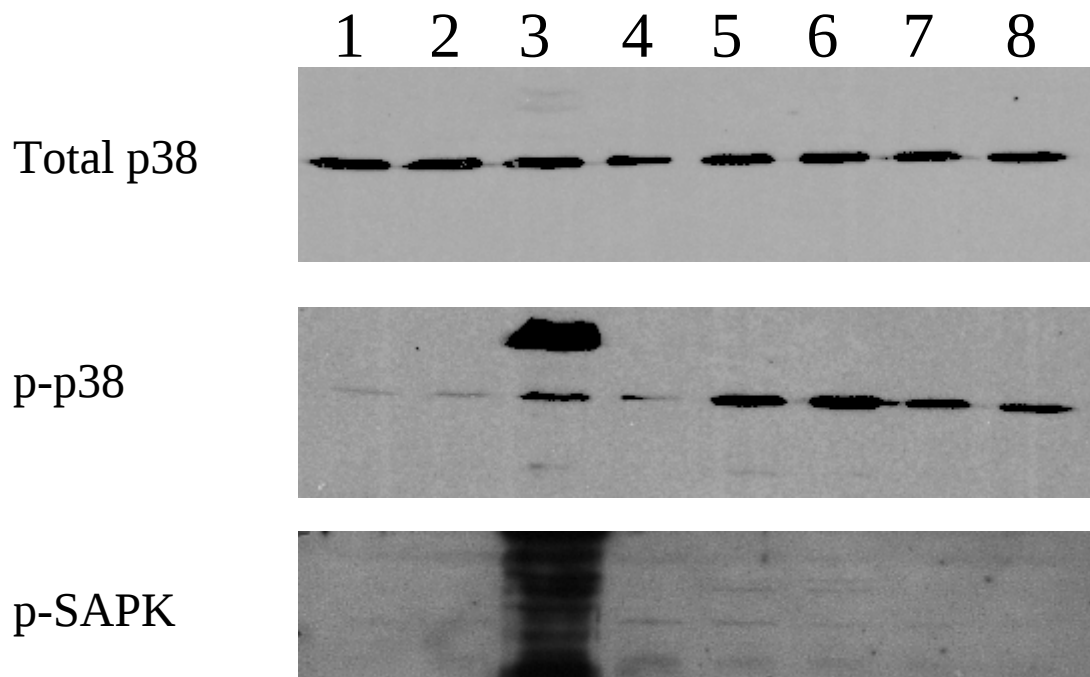


Figure 1: peptide 5-mediated p38 and SAPK activation. Ramos cells were incubated with indicated reagents for 5 minutes for SAPK and 30 minutes for p38. Cellular extracts were prepared and analyzed by immunoblotting using phospho specific antibodies. Lane ;**1**) untreated cells, **2**) naked beads alone , **3**) anti-IgM (30µg/ml) **4**) HB22.7 (60ug/ml) **5**) Bead-bound Peptide **5**) Bead-bound Peptide **44** **7**) Soluble Peptide **5** , **8**) Soluble Peptide **44**. The data is representative of two independent experiments.

In terms of the studies that have been proposed in Aim 3, we wanted to verify binding and physiologic properties of Peptide 5. Since this has recently been done we are now

developing DOTA-conjugated Peptide 5 that will be used in subsequent immuno-PET studies that are described in Aim 3.

Reportable Outcomes

The majority of the data described above is reportable and has recently been published in the International Journal of Peptide Research (appendix 1). The additional data presented above is also reportable but will only be published when verified and additional data has been generated that will facilitate publication.

Conclusion

The studies presented herein demonstrate that a peptide derived from the CDR2 of the anti-CD22 mAb HB22.7 (Peptide 5) binds to CD22 on B lymphocytes, mediates internalization, signal transduction, and killing of lymphoma cells. We also demonstrated that this peptide can be used as a vehicle to deliver pro-apoptotic payload to lymphoma cell cells that enhance the killing potential of the parent mAb and peptide. We believe that these peptides can be developed into exciting new highly effective and less toxic therapeutics for the treatment of lymphoma.

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Authors: David Pearson · Robert T. O'Donnell · Miguel Cerejo · Hayes C. McKnight · Xiaobing Wang · Jan
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Keywords (separated by '-') CD22 - CDR - B-cell - Lymphoma

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CD22-Binding Peptides Derived from Anti-CD22 Ligand Blocking Antibodies Retain the Targeting and Cell Killing Properties of the Parent Antibodies and May Serve as a Drug Delivery Vehicle

David Pearson · Robert T. O'Donnell · Miguel Cerejo · Hayes C. McKnight · Xiaobing Wang · Jan Mařík · Kit Lam · Joseph M. Tuscano

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Abstract CD22 is a B-cell specific membrane glycoprotein that mediates homotypic and heterotypic cell adhesion; it also regulates B-cell receptor (BCR)-mediated signals. Monoclonal antibodies (mAb) directed at the ligand binding domain of CD22 initiate CD22-mediated signal transduction and apoptosis in B-cell lymphomas (NHL). Amino acid analysis of the complimentary determining regions (CDRs) of six different anti-CD22 ligand blocking mAb revealed a high level of sequence conservation. The heavy chain CDRs 1, 2, and 3 are 85, 40, and 38% conserved, respectively; light chain CDRs 1, 2, and 3, are 95, 90 and 90% conserved, respectively. Based on these conserved sequences, five peptides were designed and synthesized. Only the sequence derived from heavy chain CDR2 (Peptide 5) demonstrated significant B-cell binding. Peptide 5 bound to both malignant and primary B-cells with very little T-cell binding. The affinity had a K_m of 5×10^{-6} M. Peptide 5 mediated killing of several NHL cell lines to a degree similar to that of the parent mAb (HB22.7). Peptide 5's loop structure was shown to be crucial for B-cell binding and ligand blocking. Mutational analysis revealed that most Peptide 5 amino acids were critical for B cell binding. Using a CD22 transfected COS cell line, we demonstrated CD22-specific binding and CD22 ligand blocking to a degree similar to HB22.7.

Finally Peptide 5 was used as a vehicle to deliver a pro-apoptotic peptide into NHL cells. Peptide 5 was fused to a BH3 death domain-containing peptide which demonstrated more effective NHL cell killing than the parent peptide.

Keywords CD22 · CDR · B-cell · Lymphoma

Introduction

CD22 (B-lymphocyte cell adhesion molecule, BL-CAM or Siglec-2) is a 140 Kd phosphoglycoprotein on the surface membrane of most B-lymphocytes and B-cell NHL (Law et al. 1994; Dorken et al. 1986). CD22 is a terminal alpha 2, 6 linked lectin member of the immunoglobulin (Ig) superfamily (Engel et al. 1993; Kelm et al. 1994; Stamenkovic et al. 1991). While specific CD22-binding ligands have not been identified, it is known that ligands include sialic acid bearing proteins (Sgroi et al. 1993; Powell et al. 1993; Stamenkovic and Seed 1990; Tedder et al. 1997).

CD22 is intimately involved in the regulation of B-cell function. It has the potential to positively and negatively impact B-cell signaling through its cytoplasmic domain (Sato et al. 1998). Located within the cytoplasmic domains of CD22 are tyrosine based activation motifs (TAMs) and tyrosine based inhibition motifs (TIMs). The TAMs recruit and bind src family tyrosine kinases whereas TIMs contain docking sites for SH2 domains of SHP1 protein tyrosine phosphatase that negatively regulates BCR signaling and activation (Shen et al. 1991; Doody et al. 1995; Matthews et al. 1992; Plutzky et al. 1992; Siminovitch and Neel 1998; Tamir et al. 2000). Studies involving CD22 (–/–) mice support the hypothesis that CD22 has both positive and negative effects on BCR signal transduction (Tedder et al. 1997; Sato et al. 1996).

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The predominant CD22 species expressed on the cell surface consists of seven extracellular Ig-like domains (Stamenkovic and Seed 1990; Torres et al. 1992). Mutation analysis and antibody mapping studies demonstrated that the first and second Ig-like domains serve as the ligand-binding domains of CD22 (Engel et al. 1995; Law et al. 1995). Antibodies that bind to the first two CD22 domains mediate CD22-mediated SAPK and p38 activation, proliferation in primary B-cells, and apoptosis in neoplastic B-cells. HB22-7 is one such ligand blocking anti-CD22 mAb that has demonstrated lymphomacidal activity in human NHL xenograft models (Tuscano et al. 2003). The apoptotic mechanism is mediated by activation of the SAPK pathway after CD22 cross-linking with HB22.7 (Tedder et al. 1997; Tooze et al. 1997; Tuscano et al. 1999; Tuscano et al. 1996). Additionally, CD22 cross-linking leads to phosphorylation of c-jun, which in turn activates AP-1 (Tuscano et al. 1999).

The antigen-binding site of an antibody is primarily formed by six polypeptide loops known as the hypervariable or CDRs. Three of the six loops (L1, L2 and L3) protrude from the variable domain of the light chain (VL) and three (H1, H2 and H3) from the variable domain of the heavy chain (VH) (Al-Lazikani and Lesk 1997). The binding site produced by these loops provides a surface and charge distribution complementary to that of the antigen. Oligopeptides can be designed to mimic the activity of large natural proteins, like antibodies; these peptides have numerous applications for therapeutics and diagnostics.

Previous studies successfully utilized CDRs to identify target-specific peptides (Sharabi et al. 2006). The cDNA and amino acid sequences of the heavy and light chain hypervariable regions were determined for six of the ligand blocking anti-CD22 mAbs. The CDR amino acid sequences within these regions demonstrated a high level of conservation thus providing the rationale for synthesis and characterization of CD22-binding peptides. Presented herein is the initial characterization of these peptides. Peptides were created which retain the targeting and ligand blocking properties of the parent mAb, and have anti-NHL activity. Moreover these peptides were used as vehicles to deliver a pro-apoptotic drug into NHL cells.

Materials and Methods

Peptide Synthesis Chemistry

All chemicals and buffers were either molecular biology, tissue culture grade or higher. Tentagel-S (Rapp Polymere, Tübingen, Germany) was used for the synthesis of bead-bound peptides. Fluorenylmethyloxycarbonyl (Fmoc) amino acids, with standard side chain protecting groups were

obtained from Bachem (Torrance, CA), Advanced ChemTech (Louisville, KY), or Propeptide (Vert-le-Petit, France). Benzotriazol-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate (BOP), diisopropylethylamine (DIEA), diisopropyl carbodiimide (DIC), *N*-hydrobenzotriazole (HOBt), and piperidine were obtained from Advanced ChemTech. Dimethylsulfoxide (DMSO) was purchased from Sigma Chemical Co. (St. Louis, MO). Standard Fmoc chemistry was used in the solid phase peptide synthesis (Stewart and Young 1984; Atherton and Sheppard 1989). Rink resin was used as solid support for the synthesis of soluble peptides. A 3-fold molar excess of each Fmoc amino acid was added to the resin for each coupling reaction. The coupling reaction was initiated with the addition of BOP, DIEA and HOBt. HOBt and DIC were used in some of the syntheses. The columns were tightly capped and mixed by tumbling for 2 h to overnight at room temperature. The ninhydrin test (Kaiser et al. 1969) was used to test for the completion of the coupling reaction. For those coupling reactions determined to be incomplete, fresh BOP, DIEA, and HOBt were added and the reaction was allowed to continue for a few more hours and again tested for completion. Once coupling was complete, the resin was washed with dimethylformamide (DMF). Piperidine (20% in DMF) was then added for deprotection of the *N*-Fmoc group. About 5 min later the piperidine was removed and fresh 20% piperidine was added and incubated for an additional 10 min. The resins were then washed 5 times in DMF and methanol. The resin was then ready for addition of the next amino acid. Once peptide synthesis was completed, the *N*- α -Fmoc group was removed with 20% piperidine, and the side-chain protecting groups were removed with reagent K (trifluoroacetic acid/phenol/water/thiophenol/thanedithol, 82:5:5:5:2.5, v/v/v/w/v; King et al. 1990). Cyclization of the cysteine containing peptides via disulfide bond formation on beads was accomplished by incubating the de-protected peptides with TFA:iodine overnight. The Tentagel beads with covalently linked peptides will be referred to as peptide-beads. Soluble peptides released from rink resin were cyclized using air oxidation by stirring overnight and purified by HPLC.

The Peptide 5 BH3 death domain (peptide 5-DD)-containing peptide was synthesized by Genscript Corp. (Piscataway, NJ), purified and verified via HPLC and mass spectroscopy.

Cell Culture, Primary B-Cell and T-Cell Isolation

Isolation of primary B-cells and T-cells from whole blood was performed by venipuncture into heparinized vacutainers. The blood was diluted 1:1 with sterile PBS, layered over 10 ml of lymphocyte separation media (BioWhittaker, MD); the peripheral blood mononuclear cells (PBMC)

were isolated as previously described (Tuscano et al. 1996). Washed PBMCs were resuspended in RPMI supplemented with 10% FCS and incubated with AET-activated sheep red blood cells (SRBC) for 1 h. B-cells were collected at the interface after centrifugation in lymphocyte separation media. This method consistently produced B-cells that were >90% pure by CD20 FACS analysis. T-cells were isolated by lysing T-cell-bound SRBCs with ACK lysis buffer (BioWhittaker, MD.) for 1 min followed by washing with sterile PBS. This method consistently produced T-cells of >90% purity as assessed by CD3 FACS analysis.

The Ramos, Raji and Jurkat cell lines were obtained from ATCC, and Karpas 422 was obtained from DSMZ (Braunschweig, Germany). All cells and cell lines were maintained in RPMI complete media (Gibco/Invitrogen) supplemented with 10% FCS and 2 mM L-glutamine (Gibco) in the presence of gentamycin, penicillin, and streptomycin. The cell cultures were maintained in a humidified tissue culture incubator 5/95% CO₂/air environment at 37°C. Cultures were split twice weekly to maintain log growth phase.

Peptide Cell Binding Studies

Approximately 50,000 peptide-beads (70 µl of settled beads) were washed with PBS and resuspended in PBS (1 ml) containing 10⁶ cells. Cells were incubated overnight with beads, and shaken gently (100 rpm) at 37°C. The cell-bead mixture was transferred to a 24-well dish and the number of cells bound per bead was determined using an inverted Olympus microscope; at least 25 beads were randomly examined in triplicate.

Peptide-Mediated Cell Killing

Peptide-beads were prepared and incubated with cells (4 × 10⁴ cells/ml) for 4 days. Percent cell killing was quantified by visual examination using trypan blue dye exclusion. Each experiment was done in triplicate and reported as an average of 3 independent experiments. Prism software was used to determine *P*-values. Peptide mediated apoptosis was verified by propidium iodide and FITC-annexin V staining and assessed versus FACS according to the manufacturer's recommendations (Sigma, St. Louis, MO).

Loop Reduction

Peptide-beads containing cyclized peptides were incubated in 50 mM dithiothreitol (DTT) for 15 min at room temperature to reduce the disulfide bond. The beads were then washed 3 times with PBS to remove residual DTT. The beads were resuspended in PBS (50 µl), incubated with the

cells and assessed for binding and cell killing as described above.

Peptide Binding Affinity

Biotinylated and cyclized soluble peptides were incubated with Karpas 422 cells (10⁶/ml) with decreasing concentrations of peptide in PBS/4% FCS on ice for 60 min with equal molar concentration of streptavidin-FITC. Following the incubation, the samples were diluted 10-fold with ice-cold PBS/4% FCS and then fixed with formaldehyde to a final concentration of 1%. The samples were analyzed using a Beckman FacsCaliber Flow Cytometer.

CD22 Ligand Blocking Assay

The CD22 ligand blocking assay was performed as described (Engel et al. 1993). COS cells were transfected by calcium phosphate precipitation with the full-length CD22 cDNA in the CDM8 expression vector. After 48 h the cells were washed twice with ice cold DMEM, pre-treated with CD22 ligand blocking (HB22.7) or non-blocking (HB22.27) mAb or peptides in 1 ml of DMEM for 1 h at 4°C while gently rocking. This was followed by the addition of Jurkat cells (10⁷/ml) for 1 h at 4°C. The non-adherent cells were removed by repeated gentle washes with PBS. The cells were fixed in 3% formaldehyde. The number of adherent Jurkat cells was determined using an inverted phase contrast tissue culture microscope. Each experiment was done in triplicate and the results represent a mean of 2 independent experiments.

Results

Peptide 5 Binds CD22-Positive NHL Cells

CD22-binding peptides were created based on the sequence homology of six independently generated CD22 ligand blocking mAbs. Heavy and light chain variable region sequences of the six blocking mAbs (HB-22.5, 22.7, 22.23, 22.33, 22.13, and HB22.196) were determined (Table 1). The heavy chain CDR 1, 2, and 3 are 85, 40, and 38% conserved, while light chain CDR1, 2, and 3, are 95, 90 and 90% conserved. Initial studies sought to determine if peptides derived from conserved CDR amino acid sequences of CD22 ligand blocking mAbs would bind specifically to B-cells. Five peptides were designed from the CDR sequences with cysteine (C) residues added to N- and C-terminal residues to obtain cyclic constrained structures which are predicted to mimic the CDR loop structure of the parent mAb (Fig. 1). The peptides ranged from 9 to 21 amino acids. Peptides were synthesized in

Table 1

Hybridoma Antibody Variable Heavy Chain Sequence

Hybridoma	CDR1	CDR2	CDR3
HB22.5	SGYSF TIDYTMNW...W I G L L H . P F N G G T S Y N Q K F K G...Y F C A R	GT G R N Y A M D Y W G	
HB22.196	SGYSF I G Y Y M H W...W I G R V N . P N T A . G L T Y N Q R F K D...Y Y C S R	V D Y D D Y G W F F D V W G	
HB22.7	SGFSL S D Y G V N W...W L G I I W . G D G R T D Y N S A L K S...Y Y C A R	A P G N R A M E Y W G	
HB22.33	TGYSI S G Y Y W N W...W M G Y I R . Y D G S N N Y N P S L K N...Y Y C A R	G G I T V A M D Y W G	
HB22.13	SGFTF I D Y Y M N W...W L G F I K N K F N G Y T T E Y N T S V K G...Y Y C A R	G L G R S Y A M D Y W G	
HB22.23	SGFTF S Y Y W M N W...W I A E I R L K S N N Y A T H Y A E S V K G...Y Y C T R	Y D G S S R D Y W G	

HB22 Hybridoma Antibody V Kappa Light Chain Sequence

Hybridoma	CDR1	CDR2	CDR3
HB22.5	DRVTIT CKASQSVT	NDLAW...YYASNRYTGV...FCQQDYSSP	LTFG
HB22.196	ERVTLT CKASENVV	TYMWSW...YGASNRYTGV...CGQGYSPY	YTFG
HB22.7	DRITLT CKASQSVT	NDVAW...YYASNRYTGV...FCQQDYFSP	WTFG
HB22.33	DQASIS CRSQSLSVHSNGN	TYLHW...YKVSNRFSGV...FCQSSTHVP	YTFG
HB22.13	DRVSIT CKASQSVT	NDVTW...YFASNRYTGV...FCQQDYSSP	LTFG
HB22.23	DRVSIT CKASQSVT	NDVTW...YFASNRYTGV...FCQQDYSSP	LTFG

Light Chain HB22-7 Derived Peptide Sequences

Peptide 1 CKASQSVTNDVAC (CDR1)

Peptide 2 CYASNRYTC (CDR2)

Peptide 3 CQQDYRSPLTFC (CDR3)

Heavy Chain HB22-7 Potential Peptide Sequences

Peptide 4 CSDYGVNWVC (CDR1)

Peptide 5 CRSKLASNYDTRGDGW11GLC (CDR2)

Fig. 1 Anti-CD22 CDR amino acid sequences are used to generate cyclized anti-CD22 peptides. Peptide sequence derived from CD22 ligand blocking mAb CDR amino acid sequence conservation. The brackets SS bridges formed through oxidation to cyclize peptides at inserted cysteine amino acids. The CDR from which the peptide was derived is indicated in parentheses

Lineage-Specific Binding

To assess the lymphocyte lineage specificity of Peptide 5 binding, peptide-beads coated with either Peptide 1 or Peptide 5 were incubated for 24 h with Karpas 422, primary B-cells or T-cells with and without pretreatment with the parent HB22.7 mAb. Peptide 5-beads bound more frequently to primary B-cells and Karpas 422 cells compared to Peptide 1 which also preferentially bound primary B-cells, Fig. 3. There was minimal binding of peptide 5-beads to primary T-cells. Consistent with Peptide 5 binding to the CD22 ligand blocking region, pre-incubation with HB22.7 blocked cell binding of Peptide 5 to primary B-cells and Karpas 422 cells, Fig. 3. An isotype matched IgG control antibody had minimal effect on disrupting the binding of B-cells to Peptide 5. Peptide 5 bound primary B-cells with a 5-fold greater frequency than it did to the malignant B-cell line Karpas 422.

Structure and Sequence Requirement for Peptide 5-Mediated B-Cell Binding

To assess whether the loop structure of the CDR-based Peptide 5 influenced B-cell binding, beads containing Peptide 5 was pretreated with DTT to reduce the disulfide bond and disrupt the loop structure. Disruption of the disulfide bond of Peptide 5 with DTT substantially reduced B-cell binding almost to the same degree as did pre-incubation with HB22-7, Fig. 4. This result confirms the requirement for a constrained secondary CDR loop structure and not just the primary amino acid sequence for ligand binding.

We next determined which amino acids were required for B-cell binding by Peptide 5 using an alanine scan technique which exchanged an alanine with each amino

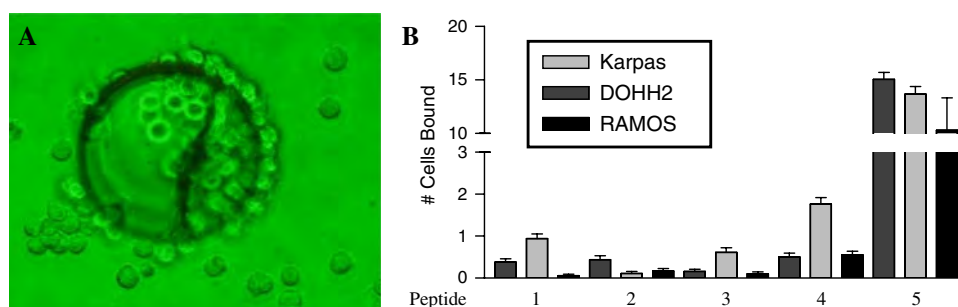


Fig. 2 Anti-CD22 peptides bind several B cell NHL cell lines. (a) Representative binding of Karpas 422 NHL cells to a TentaGel beads bound with Peptide 5. Observed at 10 $\times$ magnification. (b) Screening of the CDR derived peptides on beads for binding of several B-cell

NHL cell lines. The data represents the average of 3 or more independent experiments with at least 25 beads counted per experiment

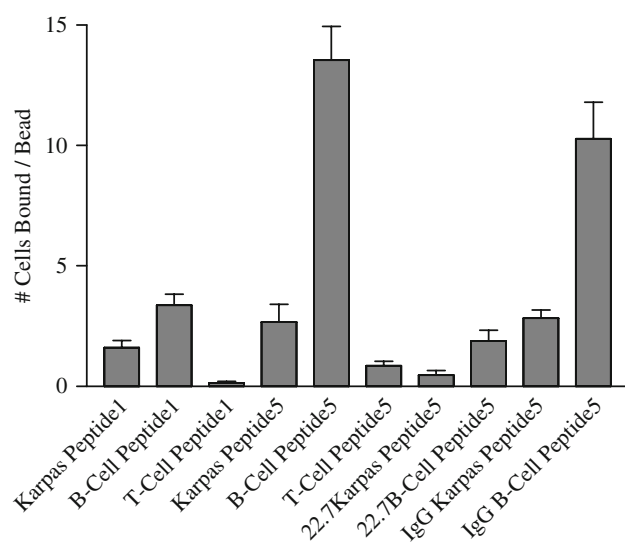


Fig. 3 Cell specific binding by CDR-derived peptides. Primary B- and T- cells along with the B-cell NHL cell line KARPAS 422 were incubated with the indicated peptide-bound beads for 24 h. The average number of cells bound per bead was then determined using an inverted phase microscope. The data represents the average of 3 independent experiments with at least 25 beads counted per experiment

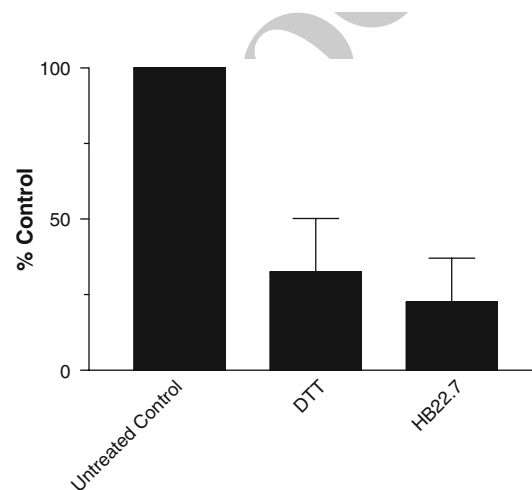


Fig. 4 Cyclization of Peptide 5 is important for cellular binding. Peptide 5-bound beads were treated with DTT to reduce the S-S bonds and linearize the peptide. As a control, KARPAS cells were preincubated with 50 μ g/ml of HB22.7. The number of cells bound per bead was determined as previously described and reported as a percent of control. The data represents the average of 3 independent experiments with at least 25 beads counted per experiment

acid sequentially on Peptide 5. The alanine scan revealed that all but two of the amino acid residues were crucial for B-cell binding. Replacing the tyrosine residue at position 8 or the glycine residue at position 12 with alanine had little effect on cell binding when compared to replacement of other residues, Fig. 5a. The specific role of each required residue in epitope recognition and binding is currently under investigation.

Both N-terminal deletion and C-terminal deletion experiments were performed on Peptide 5 to further delineate important amino acid residues or regions and their role in B-cell binding. Deletion of either the N-terminal or C-terminal amino acid has detrimental effects on Peptide 5 binding, Fig. 5b and c. The terminal deletion analysis is consistent with the alanine scan data in showing

that most amino acids are critical for CD22 binding. Moreover this data is consistent with the observation that the CDR sequences of blocking anti-CD22 mAbs are highly conserved and thus critical for CD22 binding.

Peptide 5 Blocks CD22-CD22 Ligand Binding

The CDR sequences were derived from mAbs that specifically block CD22 ligand binding. Therefore, the capacity of Peptide 5 to block CD22-CD22 ligand binding was assessed next using a cell-binding and ligand blocking assay. A previously developed assay used CD22-transfected COS cells and CD22 ligand-bearing Jurkat cells to monitor CD22 ligand binding and ligand blocking. In this study, CD22-transfected COS cells were incubated with Jurkat cells with or without soluble Peptide 5, or Peptide 1, the CD22 ligand blocking mAb HB22.7 or non-blocking mAb HB22.27. Consistent with previous reports (Engel

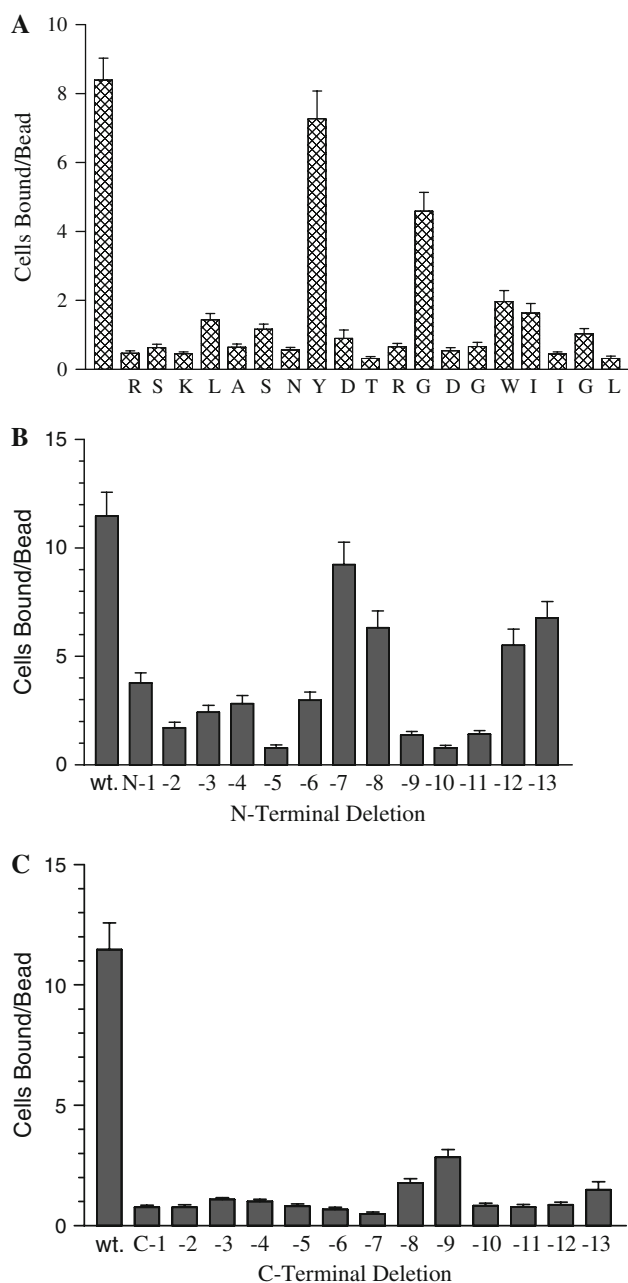


Fig. 5 Structural requirements that mediate the binding of Peptide 5 to B cells. **(a)** Alanine mutational walk of Peptide 5. Peptides derived from Peptide 5 were synthesized sequentially substituting alanine at individual amino acid positions. The binding of KARPAS 422 cells to the peptide-bound beads was determined. **(b)** N- and C-terminal. **(c)** deletion analysis of Peptide 5. Peptides derived from Peptide 5 were synthesized sequentially deleting at the N- and C-terminal amino acid positions. The binding of KARPAS cells to the peptide-bound beads was determined. The data are the average of at least 3 independent experiments

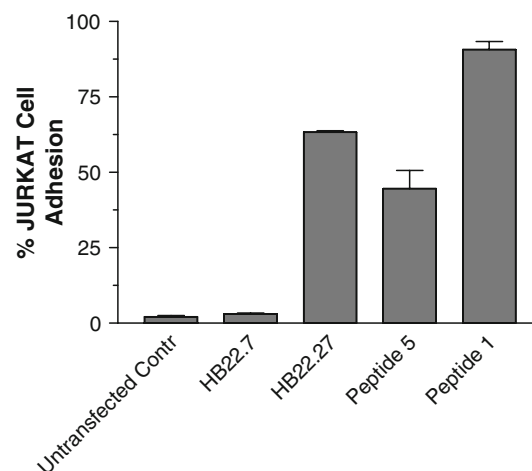


Fig. 6 CD22 ligand blocking assay. COS cells were transiently transfected with a CD22 cDNA and incubated with CD22-ligand bearing Jurkat cells, washed, fixed and adherent cells counted with and without the presence of indicated reagents. The number of bound Jurkat cells per transfected cell was determined microscopically. The data are the average of at least two independent experiments done in duplicate

Peptide 1 blocked only 35 and 10%, respectively, of CD22-mediated binding, Fig. 6. Reduction of the loop structure by pre-incubation of Peptide 5 with DTT reduced its blocking ability to 10%, confirming that the loop structure is required for epitope binding and ligand blocking (data not shown).

Peptide Binding Constants

The affinity of Peptide 5 and 1 was determined by flow cytometry-based Scatchard analysis (Gordon 1995), Fig. 7. To assess the potential to utilize Peptide 5 in flow-based assays soluble Peptide 5 was biotinylated and compared with HB22.7 by FACS analysis of binding to Karpas 422 cells, Fig. 7a. When compared to the streptavidin-FITC control and HB22.7-FITC, Peptide 5 had intermediate binding. In the Scatchard analysis Peptide 5 displayed classical sigmoidal binding to NHL cells with saturation occurring at a peptide concentration of approximately 0.1 mM. Peptide 5 had a K_d of 5×10^{-6} M; Peptide 1 had a very low binding affinity consistent with the previous analysis and thus the K_d was not determined. Peptide 5 has approximately 100–1000 times less affinity than the parent antibody HB22.7 (Tuscano et al. 2003).

Peptide 5-Mediated Cytotoxicity

Since Peptide 5 epitope binding and ligand blocking properties are similar to the parent mAbs, we examined Peptide 5-mediated killing of NHL cells. Peptide

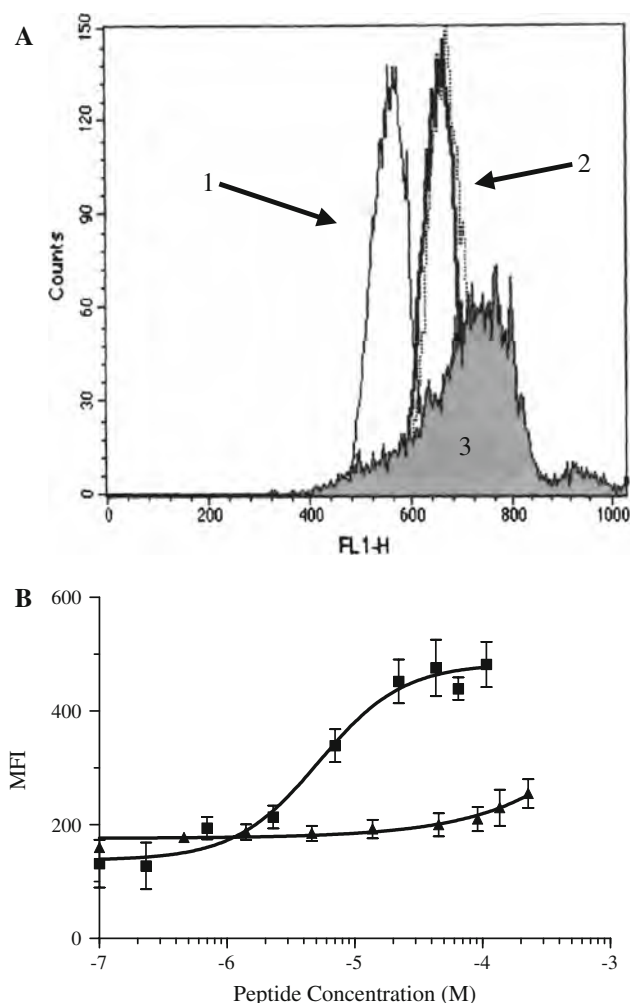


Fig. 7 Soluble Peptide 5 binding can be detected by FACS and used to assess binding affinity. (a) Biotinylated Peptide 5 binds Karpas 422 detected by streptavidin-FITC (Dorken et al. 1986) and has intermediate binding when compared to streptavidin-FITC alone (Law et al. 1994) or HB22.7-FITC (Engel et al. 1993). (b) FACS-based Scatchard analysis was used to determine the binding affinity (K_d) of Peptide 5 (■) or Peptide 1 (▲). Increasing concentrations of the peptides were incubated with the primary B-cells and detection was via streptavidin-FITC

5-mediated NHL cell killing was assessed using the Burkitt's NHL cell line, Ramos. Ramos cells were incubated with 50 $\mu\text{g}/\text{ml}$ of HB22.7 or an equimolar amount of soluble Peptide 5 or 1 for 3 days. The number of viable cells was determined by trypan blue exclusion, Fig. 8. HB22.7 and Peptide 5 killed approximately 30 and 28% of Ramos cells, respectively. In contrast, Peptide 1 had little effect on Ramos cell viability. As expected, CD22 negative primary T-cells are unaffected by HB22.7 or Peptide 5 (data not shown). Propidium iodide and annexin-mediated apoptosis detection assays demonstrated that approximately one third (or 10%) of Peptide 5-mediated killing could be attributed to apoptosis (data not shown).

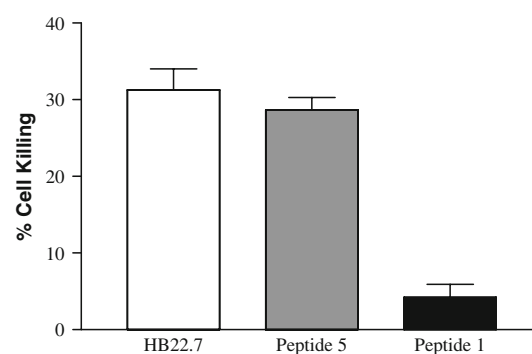


Fig. 8 Peptide 5 has lymphomacidal properties. The Ramos B cells were incubated with soluble Peptide 5 (1 $\mu\text{g}/\text{cc}$), HB22.7 (60 $\mu\text{g}/\text{cc}$), or anti-IgM (30 $\mu\text{g}/\text{cc}$). Cell viability was determined using trypan blue exclusion. The data are the average of at least three independent experiments

Next Peptide 5 was used as a vehicle to mediate targeting and entry of NHL cytotoxics by fusing Peptide 5 with a 21 amino acid peptide that contains the pro-apoptotic BH3 death domain sequence found in the pro-apoptotic protein BAD (Peptide 5-BAD) (Moreau et al. 2003), Fig. 9a. The ability of the fusion peptide to mediate targeted NHL cell killing was assessed by trypan blue exclusion. The killing potential was assessed by incubating Peptide 5-BAD with B-cell NHL lines (Ramos, Raji, and DOHH2) and a T-cell line (Jurkat) and comparing this with equimolar concentrations of HB22.7 and anti-IgM, Fig. 9b. This analysis demonstrated targeted B-cell NHL killing and a dose responsive effect in Ramos and DOHH2 cells. Next a more complete examination of the dose response effect of Peptide 5-BAD was examined by titrating the concentration of Peptide 5-BAD from 0.02 up to 22 μM and assessing for cytotoxic effects with Ramos B cells, Fig. 9c. This demonstrated a consistent dose responsive effect, and more effective killing when compared to an equimolar concentration of the parent mAb, HB22.7.

Discussion

Several anti-CD22 mAb including HB22.7, HB22.23, and HB22.33, effectively block the interaction of CD22 with its ligand (Engel et al. 1993). In vitro studies demonstrated that cross-linking of CD22 with blocking mAbs results in a 3 to 5-fold increase in SAPK activity with subsequent induction of apoptosis (Tuscano et al. 1999). In pre-clinical NHL models this has translated into effective lymphomacidal therapy (Tuscano et al. 2003) and is the basis for a new humanized antibody that will soon be evaluated in human patients with NHL. The CDR regions of all the blocking mAbs were sequenced and aligned. Several of the CDR sequences from independently generated hybridomas



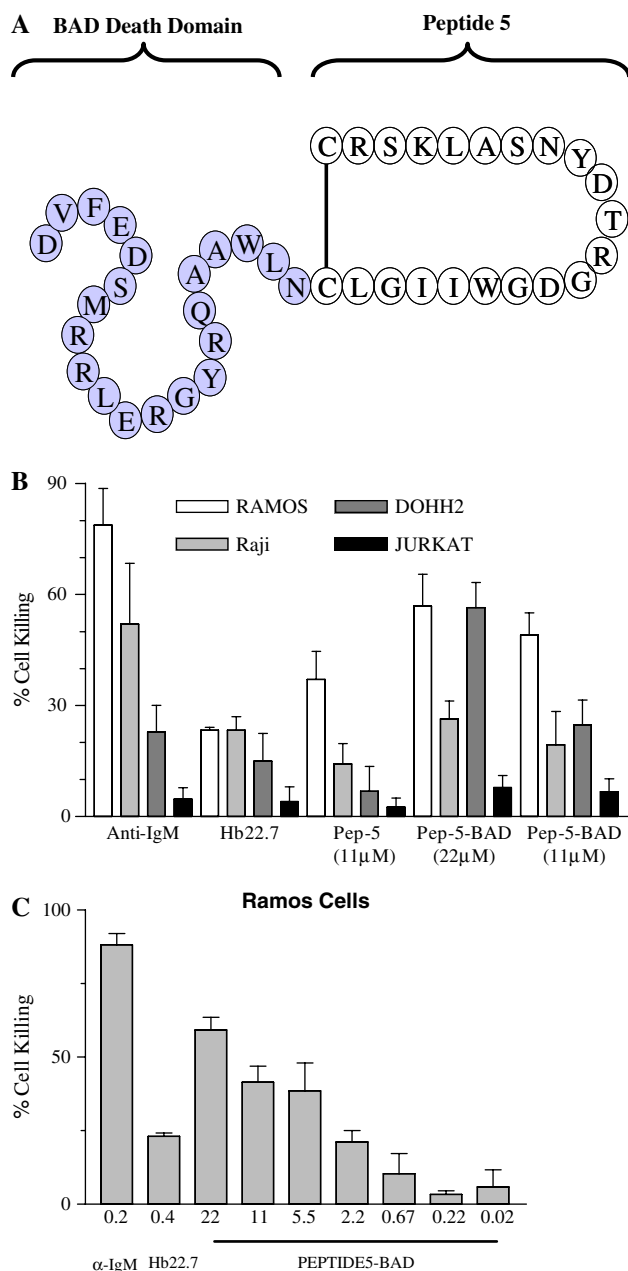


Fig. 9 The fusion peptide, Peptide 5-BAD has lymphomacidal activity. (a) The fusion of the BH3-containing death domain of BAD with the amino acid sequence of Peptide 5. (b) Equimolar amounts of Peptide 5, Peptide 5-BAD, HB22.7, or anti-IgM were incubated with three B, and one T cell NHL cell lines. Cell viability was determined using trypan blue exclusion. The data are the average of at least three independent experiments. (c) The killing effects of Peptide 5 were dose responsive. Increasing concentrations of Peptide 5-BAD were incubated with the Ramos B cell line and compared to HB22.7 and anti-IgM. Cell viability was determined using trypan blue exclusion. The data are the average of at least three independent experiments

CD22-mediated signal transduction, mediate B-cell entry, and thus could be developed as a vehicle for NHL-targeted therapeutics.

This peptide approach has been used previously to produce a virus-neutralizing micro-antibody (Heap et al. 2005). Another CDR-mimetic peptide has been developed to target and effectively neutralize TNF- α and its apoptotic effect in L929 cells (Qin et al. 2005). CDR-mimetic peptides have several advantages over mAb including relatively low cost, lack of antigenicity, stability, good tissue permeability (Florence et al. 2003), and the potential to be easily manipulated. Peptides can have similar binding activities of the intact mAb from which they were derived (Takasaki et al. 1997).

In this report, we demonstrate that CDR-based peptides derived from the anti-CD22 ligand blocking mAb are capable of binding CD22 with resultant lymphomacidal activity. Previously described combinatorial chemistry techniques were used to effectively present and screen CDR based peptides in primary B and T-cells, and B-cell NHL cell lines. Peptide 5 was extensively studied due to its superior binding to Karpas 422 cells (B-cell NHL), and normal primary B-cells when compared to the four other synthesized CDR-based peptides, Fig. 2. Binding studies revealed Peptide 5 to be relatively B-cell specific with only minimal T-cell binding (Fig. 3). Pre-incubation of B cells with HB22.7 abrogated Peptide 5-mediated binding which is consistent with the hypothesis that Peptide 5 binds to the same CD22 epitope as one of the parent mAbs, HB22.7. Structural examination revealed that the Peptide 5 loop structure and that all 21 amino acids of Peptide 5 appears to be required to achieve cellular specificity and binding to CD22. Cysteine residues were added at both ends of the peptide for cyclization to mimic the CDR structure. Loop reduction with DTT disrupts the disulfide bonds necessary for binding to CD22, Fig. 4. Consequently, secondary structure of Peptide 5 appears crucial for B-cell binding. Next the alanine scan mutational analysis and the N- and C-terminal deletion analysis demonstrated that all but two amino acids were critical for CD22 binding (Fig. 5). The non-blocking CD22 mAb (HB22.27) and blocking CD22 mAb (HB22.7) differ dramatically in the percent inhibition of ligand binding; they have been previously shown to bind different regions of CD22. Next a formal analysis of CD22 ligand blocking was done to verify that Peptide 5 binds to domains 1 and 2 of CD22 and blocks CD22 ligand binding. When compared to HB22.7 and HB22.27, Peptide 5 has intermediate blocking activity, whereas Peptide 1 demonstrated very little CD22 ligand blocking activity (Fig. 6). This supports the hypothesis that Peptide 5 binds CD22 domains 1 and 2 and at least partially blocks CD22 ligand binding. The small size of Peptide 5 and the fact that HB22.7 contains 12 CD22-binding CDRs may account for the inferior blocking capability of Peptide 5.

The CD22-binding affinity of Peptide 5 was assessed using a flow-based Scatchard analysis which demonstrated a K_d of 5×10^{-6} M (Fig. 7). While this is considerably lower than what has been measured for HB22.7 (10^{-9} M), it is consistent with the affinity of other CDR-mimetic peptides. The difference can be, in part accounted for by the increased number of CDRs within the parent blocking mAbs. Studies utilizing peptidomimetic libraries are currently being used to improve the affinity of Peptide 5.

Based on previous data with HB22.7, we hypothesized that CD22 ligand blocking is required for CD22-mediated lymphomacidal activity. Our studies reveal that Peptide 5 has similar lymphomacidal effects when compared to HB22.7 despite some difference in its ability to block CD22 ligand binding, Fig. 8. One of the advantages of peptide-based therapeutics is that they are easily manipulated to modify affinity and specificity. In addition, they can be used as vehicles to carry cytotoxic payload. CD22 is a unique therapeutic target as it is B-cell specific, found on the majority of B-cell NHL, and is internalized once bound (Tedder et al. 1997).

We harnessed the death-promoting alpha helical properties of the BH3 domain of BAD by fusing it to Peptide 5 which will promote B cell internalization. Previous studies have used this approach by fusing the BH3 domain to the internalizing antennapedia (ANT) domain (Li et al. 2007). This study demonstrated Bcl-2 independent pro-apoptotic effects; however the ANT domain is not tissue specific. Treatment of Ramos NHL cells with Peptide 5-BAD resulted in dose responsive lymphomacidal activity that was more effective than the parent mAb, HB22.7, Fig. 9. Studies that specifically examine the mechanism by which Peptide 5-BAD mediates lymphomacidal activity are ongoing.

MAB-based therapeutics employ a cell surface targeting strategy which has been met with much success as evidenced by the FDA approval of Rituxan (anti-CD20), Herceptin (anti-Her2 Neu), Mylotarg (anti-CD33), Campath (anti-CD52), Erbitux (anti-EGFR) amongst others. There are, however, limitations to mAb-based therapeutics due to their large size which may limit tumor penetration. Furthermore, nuclear medicine imaging of the distribution of indium-111 labeled mAb demonstrates that they are frequently taken up by reticuloendothelial organs such as the liver, spleen, and bone marrow. Peptides offer the advantage of greater tissue penetration due to their low molecular weight and potentially greater access to the target cell interior (Privé and Melnick 2006). Their small size also allows for efficient modification and isolation. Peptides elicit less of an immune response in vivo than do mAbs (Hernandez et al. 2004). In addition, previous studies demonstrated that CD22-mAb binding mediates rapid internalization (Haas et al. 2006). Peptide 5 shares the

same binding and physiological properties of the parent mAbs which makes it an excellent candidate for a future anti-CD22-based therapeutic. Exemplified by Peptide 5-BAD, these peptides and their optimized derivatives may be easily manipulated and serve as a vehicle that will specifically deliver cytotoxics to the malignant or autoimmune B-cell interior.

In conclusion, we created peptides that mimic the CDR binding domains of CD22 ligand blocking mAbs. Peptide 5 targets B-cell NHL, blocks CD22 ligand binding, and mediates lymphomacidal activity which is enhanced when fused to a death-promoting peptide. In fact, we demonstrated that by fusing the death promoting peptide (BH3) to Peptide 5 we can enhance its lymphomacidal properties beyond that of the parent mAb. This approach utilizes a mechanism that circumvents the apoptotic inhibitory properties of Bcl-2 over-expression which is often found in B-cell NHL and may form the basis for a new and exciting drug for treatment of NHL.

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