AD_____

Award Number: W81XWH-07-1-0471

TITLE: TARGETED LYMPHOMA CELL DEATH BY NOVEL SIGNAL TRANSDUCTION MODIFICATIONS

PRINCIPAL INVESTIGATOR: JOSEPH TUSCANO, M.D.

CONTRACTING ORGANIZATION: University of California, Davis, Davis, CA 95616

REPORT DATE: July 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2011	Final	15 JUN 2007 - 14 JUN 2011

4. TITLE AND SUBTITLE
TARGETED LYMPHOMA CELL DEATH BY NOVEL SIGNAL TRANSDUCTION MODIFICATIONS

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0471

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
JOSEPH TUSCANO, M.D.

E-Mail: joseph.tuscano@ucdmc.ucdavis.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, Davis
Davis, CA 95616

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The proposed research set to; 1) create and characterize CD22-binding peptides that initiate signal transduction and apoptosis in non-Hodgkin's lymphoma (NHL), 2) optimize CD22-mediated signal transduction and lymphomacidal properties of ligand blocking anti-CD22 monoclonal antibodies (mAbs) and peptides with CD22-specific phosphatase inhibition and 3) correlate mAb-mediated and anti-CD22 peptide-mediated in vivo physiologic changes, efficacy, and tumor targeting using advanced immuno-positron emission topography (i-PET) and FDG-PET imaging technology. Since funding we have identified five peptides that are based on CDR's of anti-CD22 mAbs. Peptide 5 has been characterized and described in the annual report for year 1 and 2. Because peptide 5 was not target specific it could not be developed further. We then created reagents in year 3 to optimize targeting (CD22 + epithelial cell line and a new combinatorial peptide library). Within year 4 we identified addition peptides that one of which was B cell and CD22 specific (peptide #8). This peptide was found to induce effective cell killing in the lymphoma cell line, Ramos. We have now begun to characterize the specificity, signaling, cytotoxic and apoptotic potential.

15. SUBJECT TERMS
CD22, lymphoma, peptides

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	33	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

I. Introduction

CD22 is a B-lymphocyte-specific glycoprotein that can function as an adhesion molecule capable of binding multiple hematopoietic cell types; it can also transduce 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 (mAb) that bind the two NH_2-terminal immunoglobulin domains of CD22; these mAb specifically block the interaction of CD22 with its ligand. CD22-blocking mAb are highly effective at inducing proliferation of primary B-cells but the CD22 blocking mAb produce apoptotic responses in neoplastic B-cells (1-3). Our lab and others have demonstrated that ligand blocking mAbs have *distinct* functional properties. We identified anti-CD22 mAbs that are unique and functionally distinguishable from other anti-B-cell, and even other anti-CD22 mAb (4-6). In fact, the NCI has approved and funded the humanization of the anti-CD22 blocking mAb, HB22.7 through the Rapid Access Intervention Drug (RAID) Program. Humanized HB22.7 could become an exciting new therapy for patients with CD22-positive non-Hodgkin's lymphoma (NHL), much as rituximab (Rituxan) is an option to patients with CD20-positive NHL.

By sequencing the heavy and light chain variable regions of five anti-CD22 blocking mAbs, we identified highly conserved complementary determining regions (CDRs) amino acid sequences that bind CD22, and initiate CD22-mediated signal transduction. Anti-CD22 peptides were created based on the CDRs. We hypothesize that these unique peptides derived from the anti-CD22 mAb CDRs can be effective therapy against NHL and autoimmune disease. Furthermore, we hypothesize that the peptides that initiate signaling and enter B-cell NHL will be the cornerstone for development of a CD22-based drug delivery system. These novel, new anti-CD22 peptides may be even more effective than their parent mAbs, and the "next step" toward a new generation of effective anti-NHL drugs.

In addition, our understanding of CD22-mediated signal transduction allows us to demonstrate that phosphatase inhibition can lead to enhanced CD22-mediated signals, apoptosis, and lymphomacidal effects in human NHL xenografts.

We also have the capacity to use small animal immuno-positron emission tomography (iPET). IPET is a new, sophisticated imaging system that can facilitate our understanding of the NHL-targeting of these new drugs, and to rapidly enhance new drug development. Therefore, our Specific Aims are to:

II. Body

Progress will be organized based on the year completed and the goals outlined with the statement of work.

Year 1 (July 2007-July 2008)

Since initiation of funding in 2007 we 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 and is represented in the published manuscript (appendix 1), and summarized below.

We demonstrated 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).

Year 2 (July 2008-July 2009)

Below the research accomplishments for year two will be summarized and organized based on the proposed aims and goals as outlined in the Statement of Work (SOW).

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.*

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 tonsilar B-cells. –* ***Completed year 1***

2. *The physiologic effects of high affinity peptides: initiation of signal transduction, and effects on cell growth and apoptosis, will be studied-****Partially completed year1 and 2***

With regard to goal 2, significant progress has been made in this area. We examined the signaling pathways shown to be involved in CD22-mediatede signaling including the stress activated kinase (SAPK) and p38 (3). Multiple immunoblotting (IB) experiments were done that demonstrated that both peptide 5 (previous characterized) and peptide 44 both activate the SAPK and p38 pathways, figure 1. The IB was repeated three times with figure 1 being representative of all three experiments.

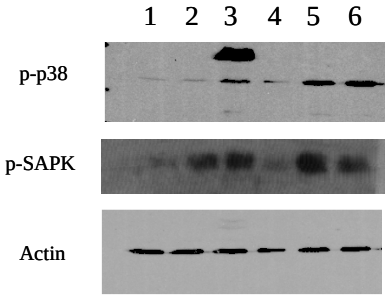


Figure 1. Immunoblotting Ramos whole cell lysates (wcl) with anti-Phospo-SAPK (p-SAPK), phosphor-p38 (p-p38) or actin. Lanes represent stimulation with; 1) media, 2) HB22.7 (60ug/cc), 3) anti-IgM (20ug/cc), 4) beads alone, 5) bead bound peptide 5, 6) peptide 44

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. -**Completed year 1 and year 2***

As proposed from the mutational analysis (7) we screened peptides for their ability to kill lymphoma cells, figure 2.

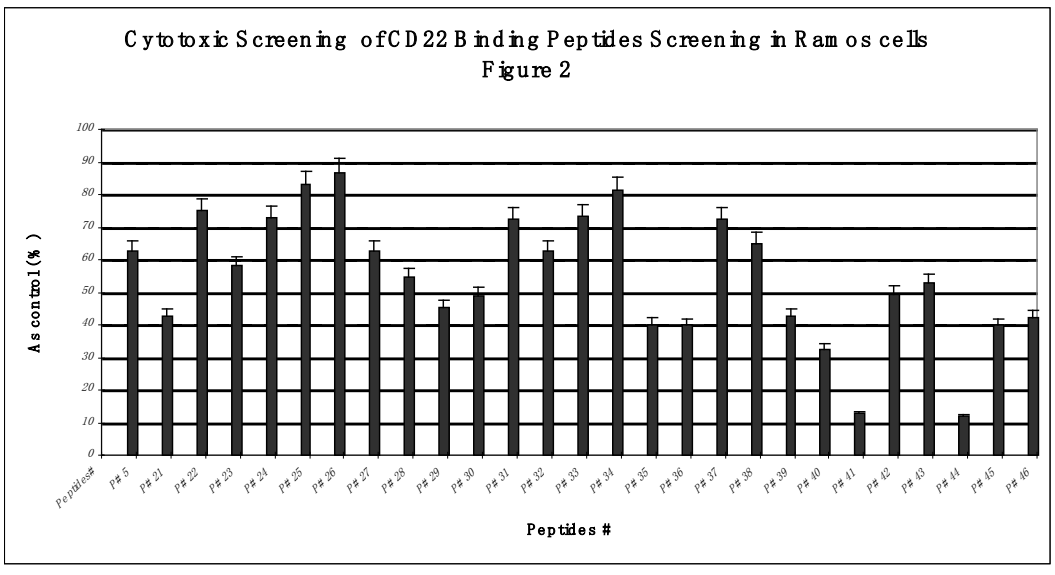


Figure 2. CD22-binding peptides identified during the mutational analysis of peptide 5. Cell killing was assessed via trypan blue exclusion and reported as a percent of untreated control. Assays were done in triplicate with error bars representing standard deviation.

Promising peptides we selected based on their killing potential and a confirmatory cytotoxicity assay was done, figure 3.

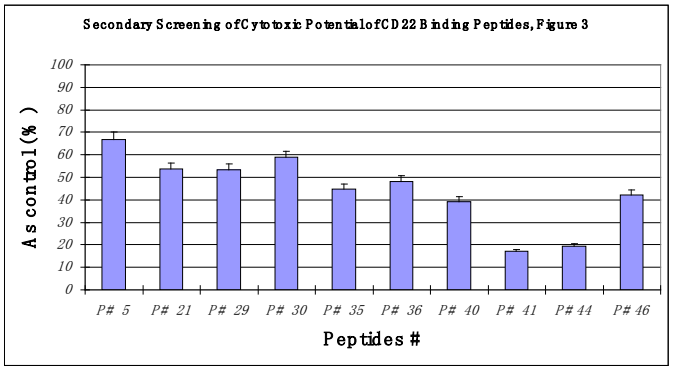


Figure 3. Selected CD22-binding peptides were again screened for their potential to kill Ramos B cells. Killing was assessed as described in figure 2

Several promising peptides were identified that had greater killing potential than the parent, peptide 5. We next assessed if the killing was B cell specific, by assessing the killing potential in a malignant T cell line, Jurkat, figure 4. While the peptides did demonstrate some cytotoxicty in T cells it was not of the same magnitude as that observed in B cells.

We next examined escalating doses of the three most promising peptides (40, 41, 44) in the Ramos B cell line, Figure 5. This demonstrated that all three peptides demonstrated a dose responsive effect.

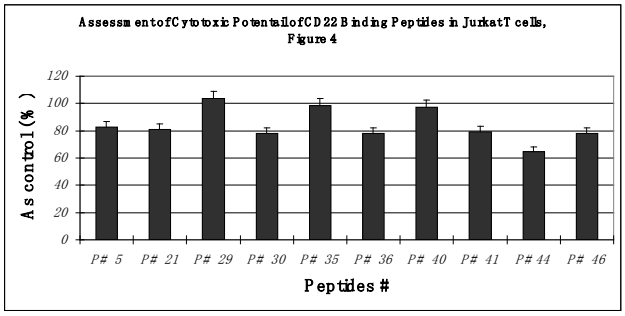


Figure 4. Selected CD22-binding peptides were screened for cytotoxic potential in Jukat T cells. Killing was assessed as described in figure 2.

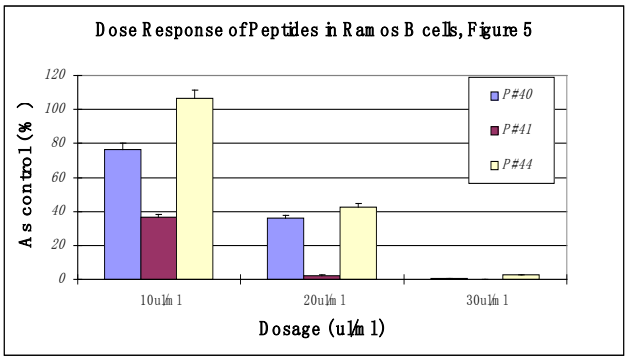


Figure 5. Dose responsive effect of peptides 40, 41, and 44 in Ramos B cells. Cytotoxicity was assessed as described in figure 2

The breadth of cytotoxicty of the most promising peptide (#41) was then assessed in cell lines that representing the major subtypes of lymphoma (Burkitts:Raji/Ramos, Follicular:MC116/Dohh2, Lymphoplasmacytic:WSU-WM, Chronic lymphocytic leukemia (CLL):WSU-CLL, mantle cell:Karpas 519, figure 6.

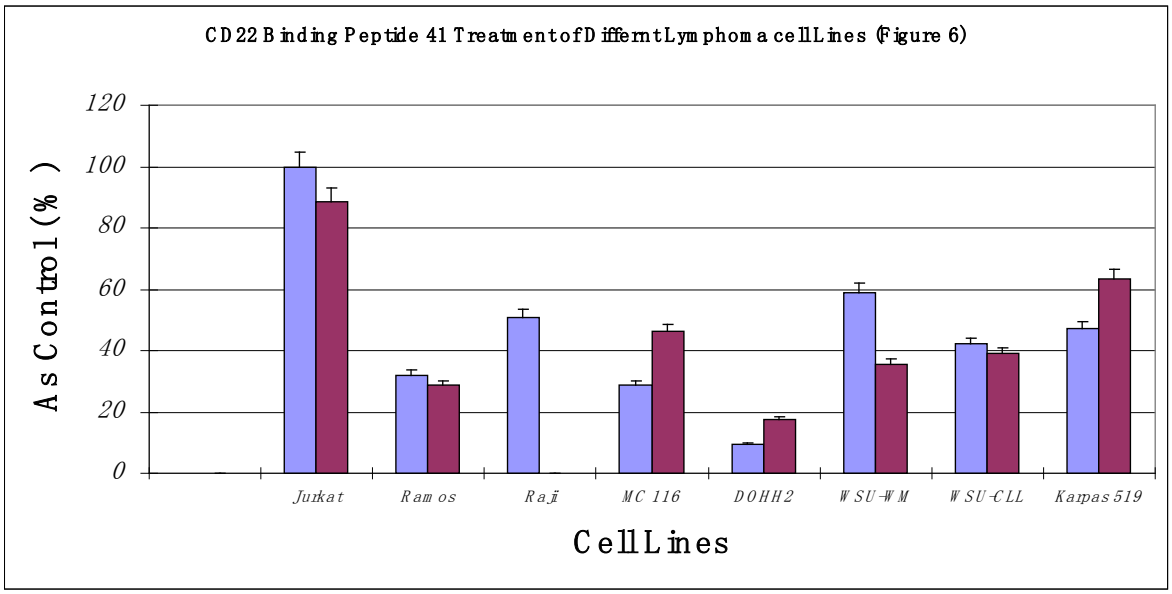


Figure 6. Cytotoxicity of peptide 41 was assessed in a panel of B and T cell lines. Cytotoxicity was assessed as described above in figure 2.

Peptide #41 was effective at killing a number of different B cell lines and confirmed enhanced killing in B versus T cells. The cytotoxic potential of peptides 40, 41, and 44 in primary B and T cells was then assessed, figure 7.

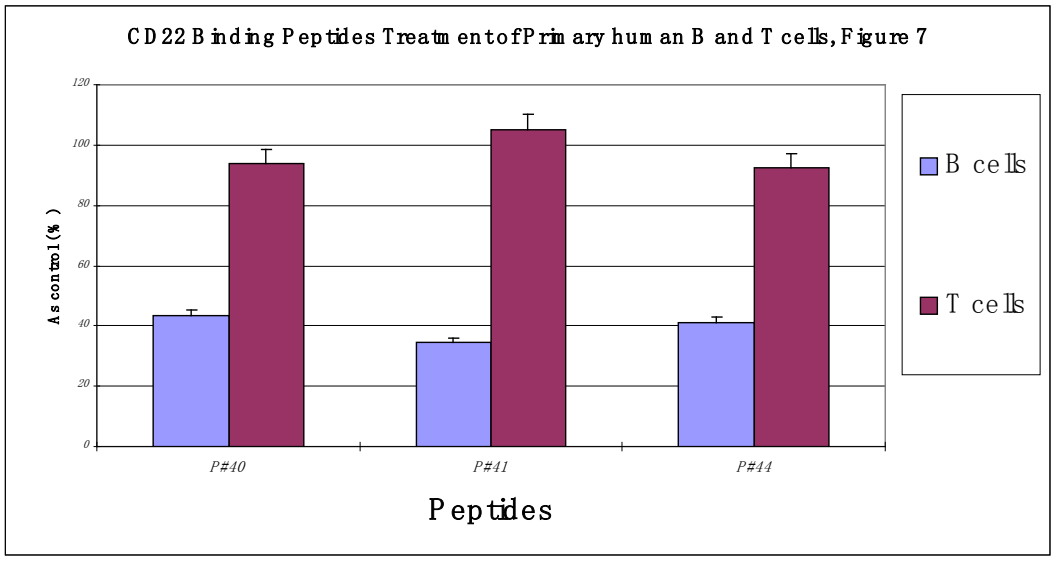


Figure 7. The cytotoxic potential of peptides 40, 41, and 44 were assessed in peripheral blood B and T cells. Cytotoxicity was assessed as decribed above

These peptides were also effective at killing primary B cells and B cell specificity was confirmed.

Goal: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-****Partially completed year 2***

We next assessed the ability of several of the peptides to mediate apoptosis in the Ramos B cell line, figure 8

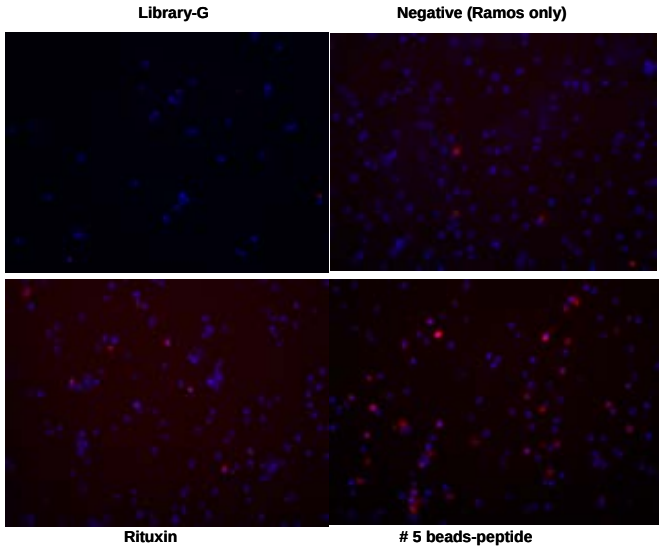


Figure 8. Ramos cells were incubated with a scrambled peptide library (library G), nothing, peptide 5 or Rituxan. Apoptotic cells were identified by caspase 3 staining detected by immunoflouresence (IF)

This demonstrated effective induction of apoptosis with peptide 5 which compared favorably to the targeted NHL therapeutic, Rituxan**.**

Next we assessed the apoptotic potential of peptide 41, figure 9.

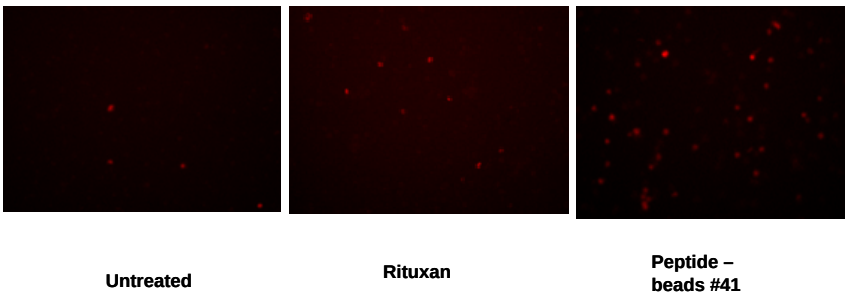
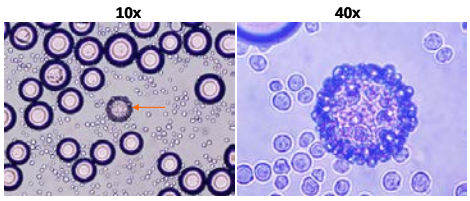


Figure 9. Peptide 41 effectively induced apoptosis in Ramos cells. Cells we assessed as described in figure 8.

This demonstrated that both peptide 5 and peptide 41 effectively induced apoptosis to a greater extent than Rituxan.

While the binding potential of peptide 5 has previously been assessed, the binding potential of peptide 41 has not. Thus bead-bound peptide 41 was assessed for its potential to bind Ramos B cells, figure 10.

This result was surprising as the majority of Ramos cells did not bind to the peptide 41 coated beads, but repeated studies has confirmed that it had B cell-specific cytotoxic effects and mediated apoptosis. Previous studies have demonstrated that peptide 5 blocked the binding of the anti-CD22 mAb HB22.7 to B cells. Thus we used a flow cytometry-based assay to examine the effects of peptide 41 on HB22.7 binding to B cells, figure 11.



Beads-peptide #41 incubate with Raji about 3 hours

Figure 10. Bead bound peptide 41 was incubated with Ramos B cells. Assessed for binding via direct visualization.

This demonstrated that peptide 41 still partially blocked the binding of HB22.7 to Ramos B cells suggesting that it may be transiently binding to the CD22 ligand binding domain. We then hypothesized that peptide 41 may be mediating apoptosis by transiently binding to B cells (? CD22), and mediating the secretion of a pro-apoptotic soluble factor. To test this hypothesis we used immobilized peptide 41 to mediate apoptosis in Ramos cells and then recovered the supernatant and subsequently incubated it with fresh Ramos B cells, figure 12.

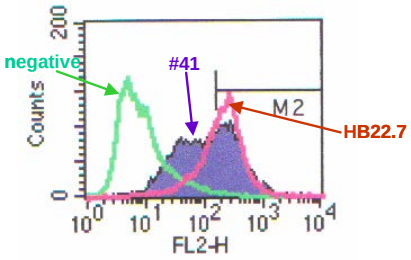


Figure 11. Ramos B cells were either incubated with anti-mouse FITC (negative), HB22.7 + anti-mouse FITC (HB22.7) or pre-incubated with peptide 41, washed and then incubated with HB22.7 + anti-mouse FITC (#41)

This demonstrated that peptide 41 mediated the secretion of a soluble factor that had cytotoxic potential in fresh Ramos cells.

plate A (24 well) Ramos cells $6x10^4$/ml/well, incubated with immobilized peptide for 24 hours, then transfered medium to plate B with fresh Ramos cellsand incubate the plate for other 2 days at 37^0C

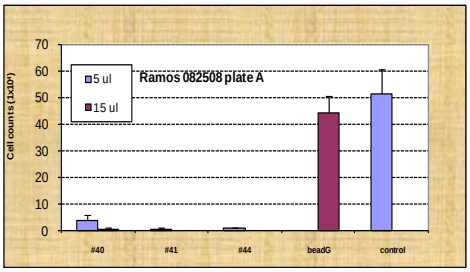


Plate B (24 well) Ramos cells $1x10^5$/ml/well, remove medium, transfer medium from the plate A and incubate the plate for 2 days at 37^0C

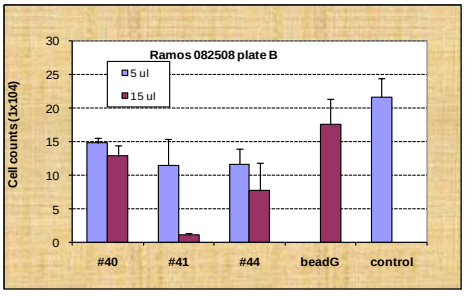


Figure 12. Supernatant from Ramos cells that had been incubated with immobilized peptide 41 was incubated with fresh Ramos cells and assessed for cytotoxicity as described above

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.*

Goals for Aim II are*:*

1. To analyze CD22-mediated signal transduction and apoptosis manipulated by tyrosine phosphatase inhibition in vitro. ***Completed-year 2***

Previous studies in our lab demonstrated that phosphatase inhibition with sodium orthovanidate (NaV) could augment CD22-medited signal transduction, cytotoxicity and in vivo efficacy of the anti-CD22 mAb HB22.7 (8, 9). As proposed we examined the effects of phosphatase inhibition on the cytotoxic potential of peptide 5, figure 13

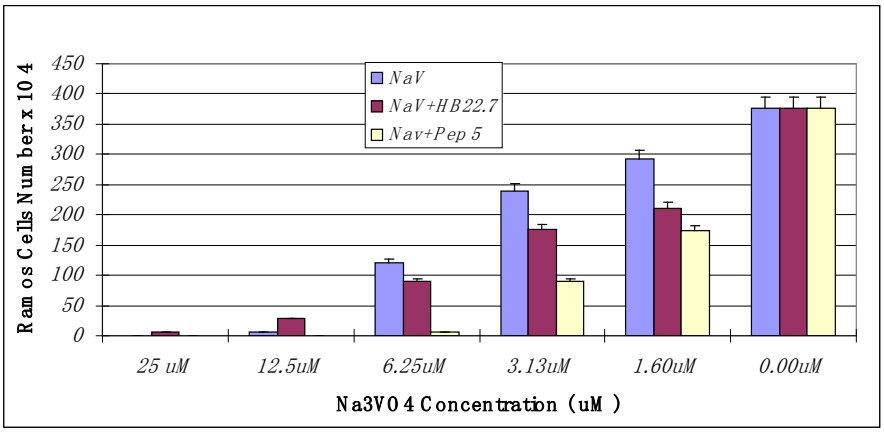


Figure 13. Ramos B cells were incubated with NaV at indicated concentrations with or without either HB22.7 (50 ug/cc) or peptide 5 (20uM) and assessed for cytotoxicity as described above

2. To assess the efficacy of combining phosphatase inhibitor(s) with the anti-CD22 ligand blocking mAb and peptides in human NHL xenograft models.-**Completed Year 1 and 2.**

Year 3 (July 2009-July 2010)

Below the research accomplishments for year three will be summarized and organized based on the proposed aims and goals as outlined in the Statement of Work (SOW).

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.*

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 tonsilar B-cells. –* ***Completed year 1***

2. *The physiologic effects of high affinity peptides: initiation of signal transduction, and effects on cell growth and apoptosis, will be studied.-**Completed year 2***

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. -**Completed year 1***

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. **–Partially completed years 1-3***

As proposed and in an attempt to identify peptides with higher affinity and better apoptotic and lymphomacidal activity we used the one bead, one peptide combinatorial library developed by Dr. Kit Lam. Previous studies with these CD22-binding peptides demonstrated that amino acid number 9 and 21 could be changed to alanine without consequence to CD22 binding. Thus these were strategic sites for modification by the peptide library to potentially improve affinity and killing. A specific library was created for each specific aminio acid site (Aa9 and Aa21) as previously described and proposed.
The library screening involved incubation of the peptide-coated beads with the CD22-positive cell line Ramos
(figure 14).

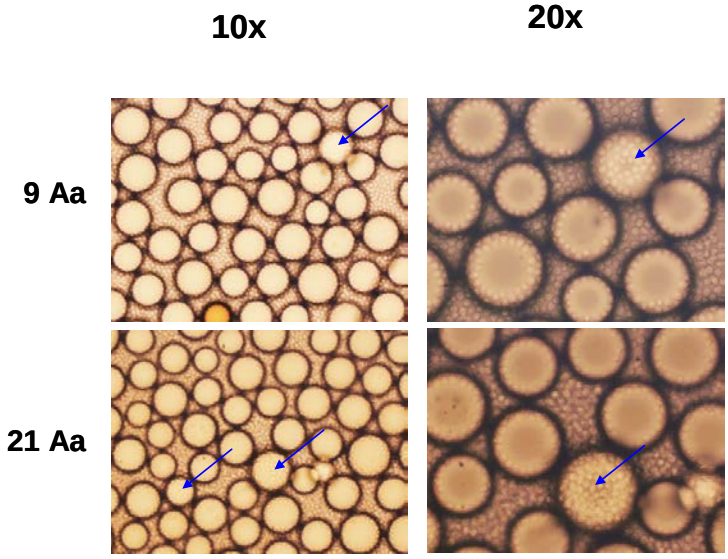


Figure 14. Ramos B cells were incubated with the site-specific peptide library and beads that bound to Ramos cells are indicated (blue arrows) and were manually selected for further analysis.

These peptides were identified and produced in larger quantities to examine their cytotoxic potential. This was done by incubating with Ramos cells for 3 days and assessing cell viability (figure 15).

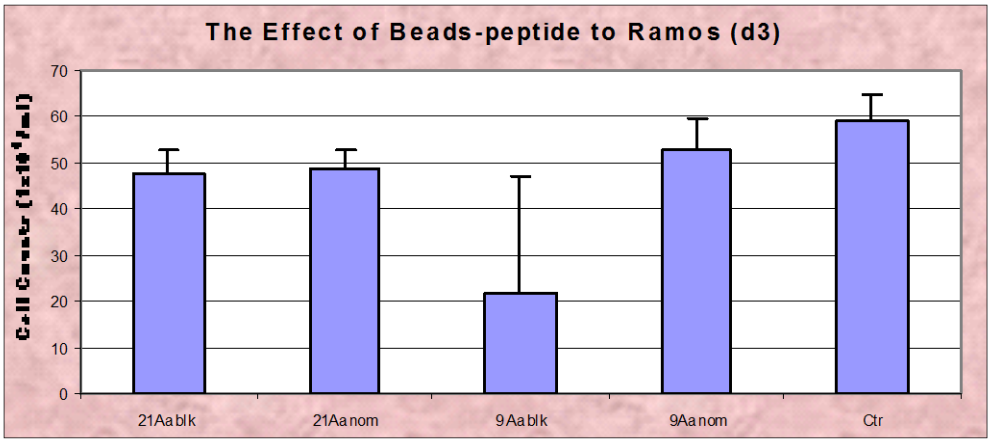


Figure 15. Aa 9 and Aa21 peptide cell killing assay. 20ug/cc of each peptide was incubated with Ramos cells for 3 days and assessed for viability using trypan blue exclusion. The results represent the average of 3 replicate experiments, with the error bars representing the standard deviation (SD).

These results suggest that peptide Aa9 has significant killing potential although the SD was relatively high. These studies are currently being repeated with higher peptide doses and several CD22 positive NHL cell lines.

We next wanted to assess the potential of peptide 9Aa to kill a number different CD22 positive cell lines, (figure 16).

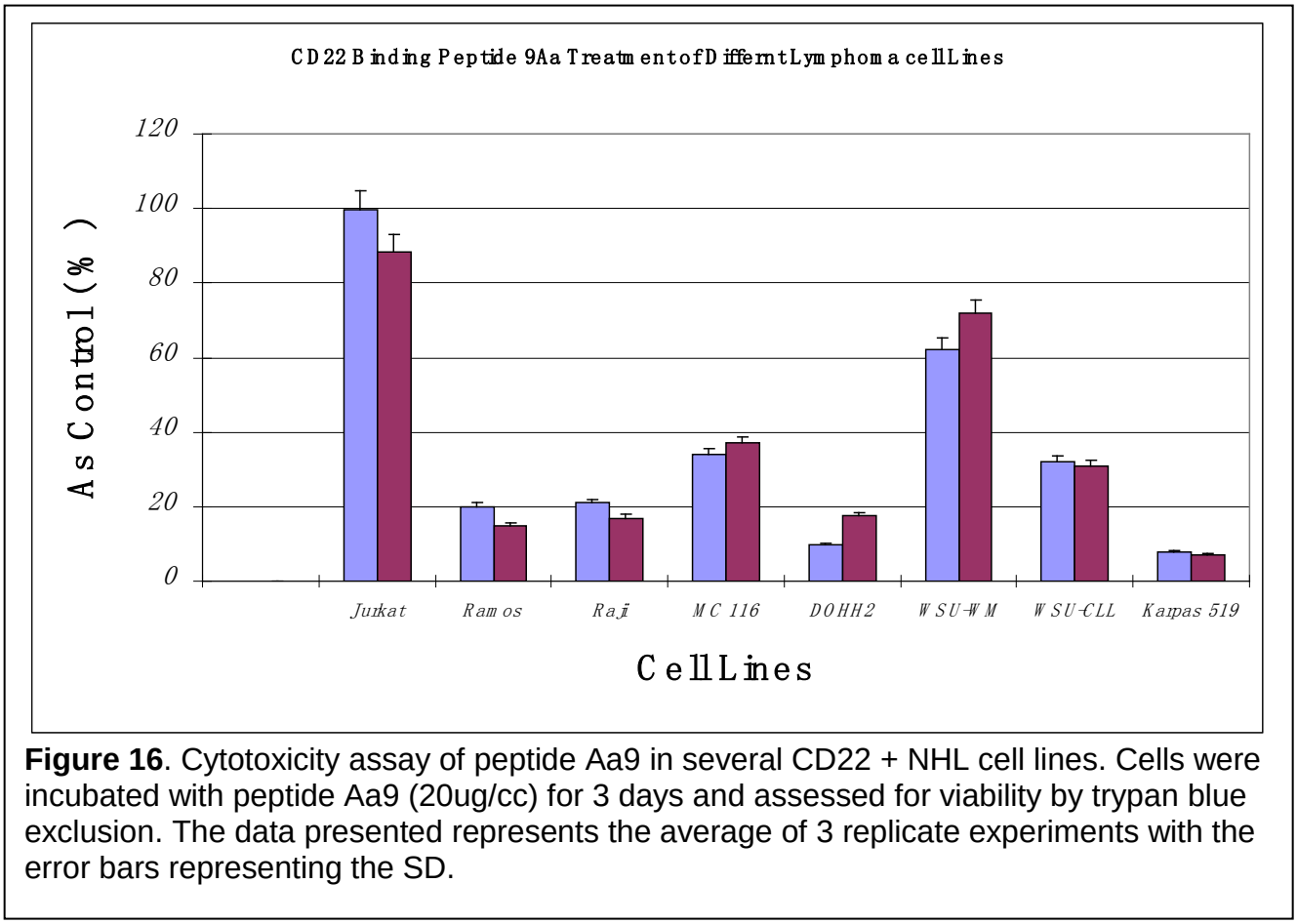


Figure 16. Cytotoxicity assay of peptide Aa9 in several CD22 + NHL cell lines. Cells were incubated with peptide Aa9 (20ug/cc) for 3 days and assessed for viability by trypan blue exclusion. The data presented represents the average of 3 replicate experiments with the error bars representing the SD.

This data verified the cytotoxic activity of 9Aa modification of peptide 5. This lack of significant killing of the CD22 negative cell line, Jurkat, suggested tissue (B cell) specificity. However we next looked at the tissue specificity of the Aa9 peptide in several CD22 negative cell lines. We found that when we examined binding to a number of solid tumor cell lines (including breast, lung and prostate) peptide Aa9 also bound to these cell lines, suggesting that is was less specific. We then compared the binding of peptide Aa9 to peptide 5 and found that while it may have killed NHL cell lines more effectively it was less NHL-specific. We then consulted with our collaborator Dr. Kit Lam and we agreed that more NHL-, and CD22-specific peptides were needed ***before the project could move forward***. To achieve this goal a considerable amount of time and effort wsa dedicated to the development CD22 specific cell line to better select CD22-binding peptides. This would be done by stably tranfecting CD22 into a cell line that did not normally express CD22 allowing us to have a CD22 postive and negative cell lines that would allow for more efficient selection of CD22-specific peptides. We did this first by subcloning CD22-encoding cDNA (obtained from Dr. John Kehrl NIAID, NIH) into the pCDNA eukaryotic expression vector. After sequence verification this vector was transfected into the CD22 negative cell line 293T cells. The transfected cells were then selected by G418 resistance. Subcloned cell lines with high levels of CD22 surface expression were selected by CD22-directed FACS-based cell sorting. This process was completed by the end of year 3 and peptide 5 library modified at Aa 9 and Aa21 will be

used to select peptides that are CD22-specific and with greater affinity. Having CD22 positive and negative cell lines will allow for identification of peptides within the library that will specifically bind to CD22. Nonspecific binding peptides will be eliminated but removing those that bind to the parent untransfected (CD22 negative) 293 cells. Again this will potentially allow for selection modifications to peptide 5 that specifically bind CD22 with greater affinity and more specificity.

Year 4 (July 2010-July 2011)

Below the research accomplishments for year four will be summarized and organized based on the proposed aims and goals as outlined in the Statement of Work (SOW).

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.*

The goals of Aim I are:

5. *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 tonsilar B-cells. –* ***Completed year 1***

6. *The physiologic effects of high affinity peptides: initiation of signal transduction, and effects on cell growth and apoptosis, will be studied.-****Completed year 2***

7. *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. -****Completed year 1***

8. *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.*

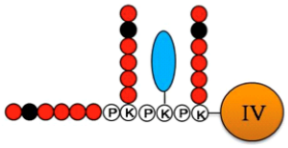
As described in the summary of year 3 in an attempt to identify peptides with higher affinity and better apoptotic and lymphomacidal activity we used the one bead, one peptide combinatorial library developed by Dr. Kit Lam as well as the development of a CD22 specific cell line.

Within year 4 there were several technical issues that hampered progress. Several of the libraries that were initially used were found to be degraded. This was noted by finding unusual binding patterns and subsequently sequencing the corresponding peptide. The known peptide/cholesterol backbone was not present and this suggested degradation. The library had to be resynthesized which took nearly 10 weeks. After the library was resynthesized it was screened against the CD22+ cell lines Ramos. 37

positive peptide beads were identified. Those were subsequently screened with the CD22 negative cell line 293. Those that bound were eliminated (7 peptide beads), leaving 30 candidates. The 30 candidate peptide beads were then screened with the CD22 + 293 cells and 10 beads bound and were selected for sequencing. The sequence of 6 peptides could be accurately determined (figure 17).

Cholesterol-Peptide Hybrid Library Screening Results for Cell Adhesion Against CD22+ 293 (also no binding to CD22- cells)

No.	Library	Cell Line	X_6	Φ_5	X_4	X_3	X_2	X_1
1	LOR 4	293	Phe(4me)	?	Phe(3Cl)	D	Aic	Thi
2	LOR 4	293	Phe(4me)	?	D	?	?	No signal
3	LOR 4	293	A	W	D	W	Thi	f
4	LOR 4	293	I	I	D	I	e	A
5	LOR 4	293	Y	Phe(3Cl)	D	I	N	W
6	LOR 4	293	Dpr	L	D	Y	q	f
7	LOR 4	293	D	No signal	HoPhe	V	No signal	D
8	LOR 4	293	P	Phe(3Cl)	q	A	Aic	D
9	LOR 4	293	R/P	?	No signal	No signal	No signal	No signal
10	LOR 4	293	I/W	?	t	A	HoPhe	Thi



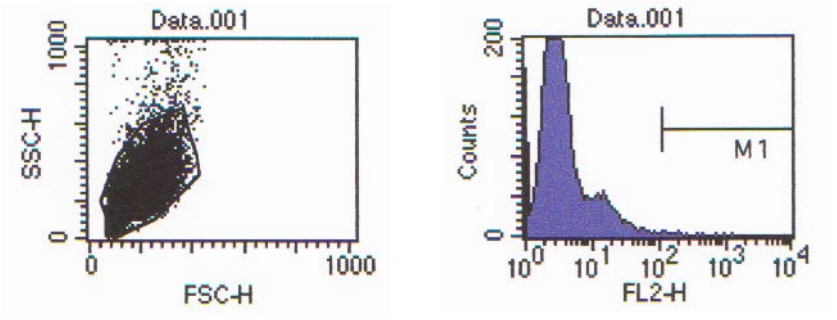
Blue oval: Cholesterol
Φ_5: Fixed for hydrophobicity
?: unsure
Boxed: consensus

Figure 17. LOR 4 peptide bead library screen sequence analysis. Positive selection from Ramos cells, negative selection from 293 cells, then positive selection from CD22+ 293 cells.

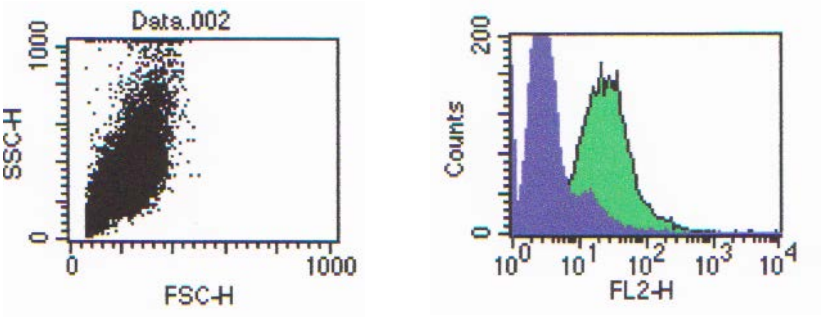
The sequence from peptides 3-6, 8 and 10 could be accurately determined and were considered viable candidates. These peptides were then synthesized, biotinylated and assessed for binding to the CD22 + cell line Ramos. Only peptide #8 bound to Ramos cells (figure 18).

Flow Cytometric analysis of Peptide 8 Binding to the Lymphoma Cell Line, Ramos

Ramos
+PE staining



Ramos
+HB22.7
+PE staining



Ramos
Peptide 8-biotin
+PE staining

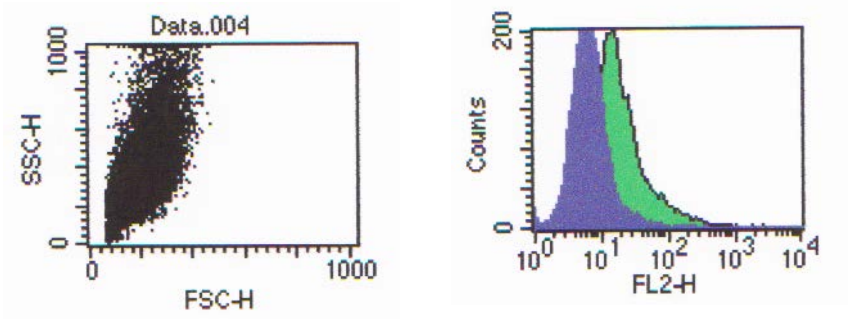


Figure 18. Peptide 8 was biotinlyated and assessed for binding by flow cytometry using streptavidin-PE. Biotinylated HB22.7 was used as a positive control.

Peptide #8 was then assessed for in vitro cytotoxicity in the cell line Ramos, (figure 19).

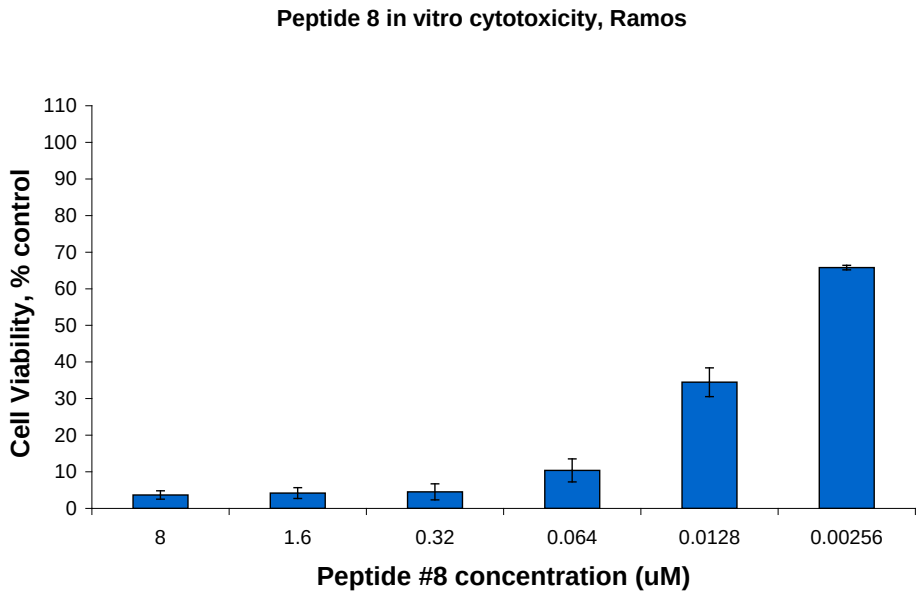


Figure 19. Cytotoxicity assay of peptide #8 in CD22 + NHL cell line ramos. Cells were incubated with peptide #8 for 3 days and assessed for viability by trypan blue exclusion. The data presented represents the average of 3 replicate experiments with the error bars representing the SD

We next assessed the binding of peptide #8 in multiple tissue types and found that peptide #8 specifically bound to B cells and B cell NHL cell lines and did not bind to other tissues types (including lung, colon, breast cancer and T cell lymphoma cell lines) assessed by flow cytometry (data not shown).

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.*

Goals for Aim II are*:*

*1. To analyze CD22-mediated signal transduction and apoptosis manipulated by tyrosine phosphatase inhibition in vitro.-**Completed for peptide 5, and partially completed for higher affinity CD22-binding peptides under development (peptide #8).***

2. To assess the efficacy of combining phosphatase inhibitor(s) with the anti-CD22 ligand blocking mAb and peptides in human NHL xenograft models.-**Completed for peptide 5, pending for peptide 8**

III. Final Report Summary/Key Research Accomplishments

Years 1 and 2

- Peptides 5 and 44 were found to activate the SAPK and p38 signal transduction pathways.
- Based on the mutational analysis of CD22 binding peptide 5 several additional peptides were identified that effectively kill lymphoma cells.
- These peptides (#40, 41, and 44) were shown to preferentially kill B cells, and their cytotoxic effects were dose responsive.
- The cytotoxic effects of peptide 41 was active in several NHL cell lines that represent diverse NHL subtypes.
- Peptides 40, 41, and 44 killed normal as well as malignant B cells.
- Peptide 41 induced apoptosis in malignant B cells approximately to same degree as peptide 5 and considerably better than Rituxan.
- We found that peptide 41 partially blocked binding of the anti-CD22 mAb HB22.7 and thus likely binds to the same CD22 epitope and only binds transiently.
- We demonstrated that peptide 41 mediated the production of pro-apoptotic soluble factors.
- We demonstrated that phosphatase inhibition augmented the cytotoxic potential of peptide 5.

Year 3

- Used the combinatorial peptide library to identify modified peptide 5 that bound to multiple B cell NHL cell lines.
- Identified peptide 5 derivative Aa9 that had significant apoptotic potential in a number of NHL cell lines
- Found that modified peptide 5, Aa9 had less tissue specificity which necessitated the development of more specific peptides which required development of CD22 positive epithelial cell lines as well as new peptide libraries.
- Developed a CD22 transfected cell line that has a high level of CD22 surface expression that was used to screen the combinatorial library for more specific CD22-binding peptides

Year 4

- Re-synthesized a cholesterol-based combinatorial peptide library (LOR4)
- Screened LOR4 peptide library using CD22+ Ramos cells, CD22 negative and CD22 transfected/positive 293 cells
- Identified 10 candidate peptide beads. Of these 6 could be sequenced and re-synthesized.
- One of the candidate peptides (#8) specifically bound to B cell NHL and did not bind to other cell types
- Peptide 8 was effective at killing the CD22+ B cell line Ramos.

IV. Reportable Outcomes

Pearson D., O'Donnell RO, Cerejo M, McKnight HC, Wang X, Lam KS, and Joseph M. Tuscano. 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. *Int J Pept Res Ther* (2008) 14:237–246

Currently there are no additional publications. The data presented above is reportable but will only be published when verified and additional data has been generated that will facilitate publication. All subsequent publications will acknowledge the DOD Investigator-Initiated Research Award Number (W81XWH-07-1-0471).

V. Conclusion

The studies presented herein demonstrate that a peptide derived from 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 (work completed in year 1). Studies completed in year 2 identified additional peptides (#40, 41, and 44) that were developed from the mutational analysis of peptide 5 that have been found to be even more effective at killing lymphoma cells and inducing apoptosis. Interestingly these new peptides appear to mediate their cytotoxic effects by inducing malignant B cells to produce pro-apoptotic soluble factors.

A considerable amount of time was spent using the focused combinatorial peptide library system to develop modifications of peptide 5 that had higher affinity and greater apoptotic potential. While this resulted in identification of peptides (Aa9) that had significant apoptotic potential in multiple NHL cell lines, they had less tissue specificity making them unsuitable for further development. Subsequent studies focused on the development of CD22 specific cell lines that would allow for better selection of CD22-binding peptides that are more specific. The development of these cell lines was completed and a new combinatorial library developed (Year 3). In year 4 technical issues required re-synthesis of the peptide library, but once this was completed, extensive screening identified 10 candidate CD22 binding peptides. Of these we could obtain complete sequence on 6. Of the 6 one (peptide 8) bound and effectively killed Ramos cells. Because the peptides that were initially developed (#5 and Aa9) were not suitable for further development as well as other technical difficulties described above, we were unable to complete Aim II, goal 2, and Aim III. Despite the completion funding studies are ongoing in our lab to complete all the objectives proposed in the grant, including examination of cytotoxicity in other NHL cell lines, *in vivo* efficacy analysis in NHL xenograft models and immune-PET studies.

VI. Literature Citations

1. Sato, S., Tuscano, J. M., Inaoki, M., and Tedder, T. F. (1998) *Semin Immuno* **10**(4)**,** 287-97.
2. Tuscano, J., Engel, P., Tedder, T. F., and Kehrl, J. H. (1996) *Blood* **87**(11)**,** 4723-30.
3. Tuscano, J. M., Riva, A., Toscano, S. N., Tedder, T. F., and Kehrl, J. H. (1999) *Blood* **94**(4)**,** 1382-92.
4. Tuscano, TM, O'Donnell RT, Miers LA, Kroger LA, Kukis DL, Lamborn KR, Tedder TF and DeNardo DL. *Blood* 101(9), 3641-3647, 2003.
5. Law, C. L., Aruffo, A., Chandran, K. A., Doty, R. T., and Clark, E. A. (1995) Ig domains 1 and 2 of murine CD22 constitute the ligand-binding domain and bind multiple sialylated ligands expressed on B and T cells. *J Immunol* 155(7), 3368-76
6. O'Donnell RT, Pearson D, McKnight HC, Ma YP, Tuscano JM. Treatment of non-Hodgkin's lymphoma xenografts with the HB22.7 anti-CD22 monoclonal antibody and phosphatase inhibitors improves efficacy. *Cancer Immunol Immunother.* 2009 Mar 18
7. Pearson D., O'Donnell RO, Cerejo M, McKnight HC, Wang X, Lam KS, and Joseph M. Tuscano. 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. Int J Pept Res Ther (2008) 14:237–246
8. O'Donnell RT, Pearson D, McKnight HC, Ma YP, Tuscano JM. Treatment of non-Hodgkin's lymphoma xenografts with the HB22.7 anti-CD22 monoclonal antibody and phosphatase inhibitors improves efficacy. *Cancer Immunol Immunother.* 2009 Mar 18.
9. O'Donnell RT, Pearson D, McKnight HC, Ma YP, Tuscano JM. Phosphatase inhibition augments anti-CD22-mediated signaling and cytotoxicity in non-hodgkin's lymphoma cells. *Leuk Res.* 2009 Feb 21.

VII. Personnel receiving pay from the research effort

1. Hayes McKingh-technician (year 1)
2. David Pearson-senior technician (year 1)
3. Yunpen Ma-technician (years 2 and 3)
4. Cheng Xiong-technician (years 3 and 4)
5. Robert O'Donnel-co-PI (years 1-4)
6. Joseph Tuscano-PI (years 1-4)

Appendix I

Int J Pept Res Ther (2008) 14:237–246
DOI 10.1007/s10989-008-9138-z

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 Mařik · Kit S. Lam · Joseph M. Tuscano

Accepted: 10 July 2008 / Published online: 1 August 2008

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 Km 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

D. Pearson · R. T. O'Donnell · M. Cerejo · H. C. McKnight · X. Wang · J. Mařik · K. S. Lam · J. M. Tuscano (✉)
Division of Hematology and Oncology, Department of Internal Medicine, University of California Davis Cancer Center, 4501 X Street, Suite 3016, Sacramento, CA 95630, USA
e-mail: joseph.tuscano@ucdmc.ucdavis.edu

R. T. O'Donnell · J. M. Tuscano
Northern California Veterans Administration Healthcare System, Sacramento, CA, USA

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).

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. TentalGel-S (Rapp Polymere, Tubingen, 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. Dimethlylsulfoxide (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. 1970) 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/w/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% CO_2/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 10^6 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 (4×10^4 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 (10^6/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 (10^7/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 CDR sequence alignment from hybridomas that produce CD22 ligand blocking antibodies

Hybridoma Antibody Variable Heavy Chain Sequence

Hybridoma	CDR1	CDR2	CDR3
HB22.5	SGYSF	TDYTMNW… W I GLLH. PFNGLG TS YNQKFKG….	YFCAR GTGRN YAMDY WG
HB22.196	SGYSF	I GYYMHW… W I GRVN.PNTA. G LT YNQRFKD ….	YYCSR VDYDDYG WFFDVWG
HB22.7	SGFSL	SDYGVNW… WLG I IW..GD G R TD YNSALKS….	YYCAR APGNR AMEY WG
HB22.33	TGYSI	SGYYWNW…WMGY IR..YD GLS NN YNPSLKN….	YYCAR GGITV AMDY WG
HB22.13	SGFTF	I DYYMNW… WLGF IKNKFNGYTTE YNTSVKG….	YYCAR GLGRS YAMDY WG
HB22.23	SGFTF	SYYWMNW… W I AE IRLKSNNYATH YAESVKG….	YYCTR YDGSSR DY WG

HB22 Hybridoma Antibody V Kappa Light Chain Sequence

Hybridoma	CDR1	CDR2	CDR3
HB22.5	DRVTIT CKASQTVT	NDLAW…..YY ASNRYT GV….FC	QQDYSSP LTFG
HB22.196	ERVTLT CKAS ENVV	TYVSW….YG ASNRYT GV….CG	QQGYSYP Y TFG
HB22.7	DRITLT CKASQSVT	NDVAW…..YY ASNRYT GV….FC	QQDYRSP WTFG
HB22.33	DQASIS CRSSQSLVHSNGNTYLHW….YK	VSNRFS GV…FC	SQSTHVP Y TFG
HB22.13	DRVSIT CKASQSVT	NDVTW…..YF ASNRYT GV…..FC	QQDYSSP LTFG
HB22.23	DRVSIT CKASQSVT	NDVTW…..YF ASNRYT GV…..FC	QQDYSSP 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 CLGIIWGDGRTDYNSALKSRC (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 in indicated in parentheses

solid phase on TentaGel resin, cyclized and screened for cell binding while they remained covalently linked to the beads. This highly reproducible method has been used successfully to screen peptide libraries for cell binding by microscopy, Fig. 2a. Karpas 422, Ramos, and DOHH2 NHL cells were incubated with peptide-coated beads representing the various CDR sequences, Fig. 2b. Peptide 5 had greater binding frequency than did Peptides 1–4. Peptide 5 had a 5-fold greater number of bound cells than did Peptides1–3; Peptide 4 demonstrated an intermediate level of binding. Furthermore, Peptide 5 had the greatest binding frequency to the Karpas 422 cell line which is consistent with relative increased CD22 expression level in this cell line (data not shown).

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

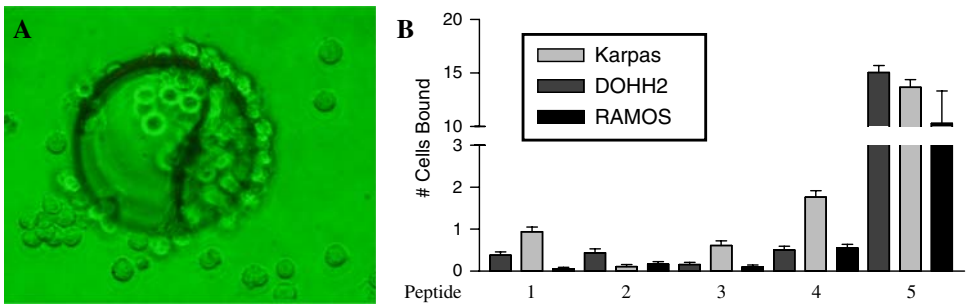


Fig. 2 Anti-CD22 peptides bind several B cell NHL cell lines. (**a**) Representative binding of Karpas 422 NHL cells to a TentaGel beads bound with Peptide 5. Observed at 10× magnification. (**b**) Screening of the CDR derived peptides on beads for binding of several B-cell NHL cell lines. The data represents the average of 3 or more independent experiments with at least 25 beads counted per experiment

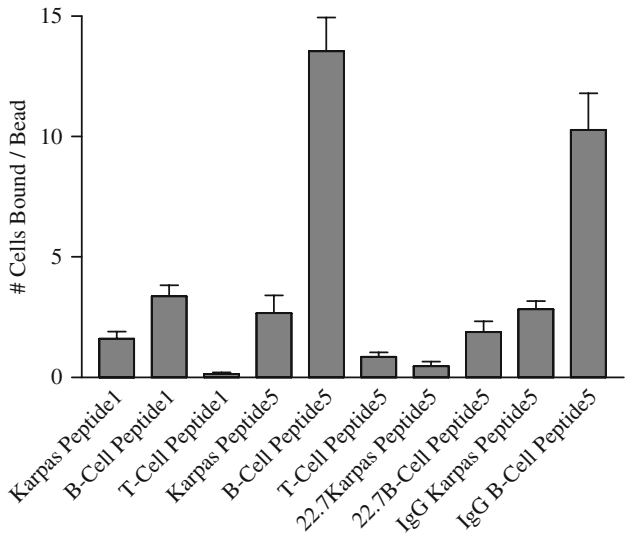


Fig. 3 Cell specific binding by CDR-derived peptides. Primary B- and T- cells along with the B-cell NHL cell line KARPAS 422 were incubated with the indicated peptide-bound beads for 24 h. The average number of cells bound per bead was then determined using an inverted phase microscope. The data represents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

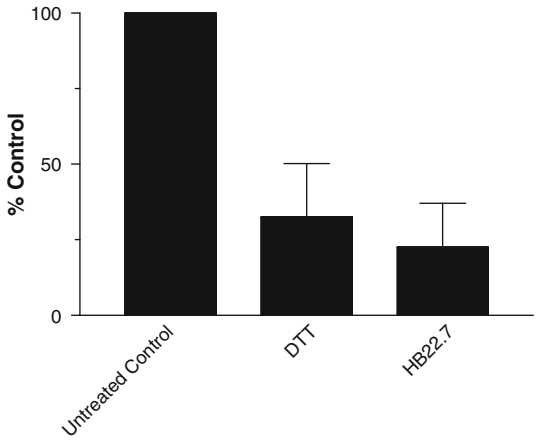


Fig. 4 Cyclization of Peptide 5 is important for cellular binding. Peptide 5-bound beads were treated with DTT to reduce the S–S bonds and linearize the peptide. As a control, KARPAS cells were preincubated with 50 μg/ml of HB22.7. The number of cells bound per bead was determined as previously described 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

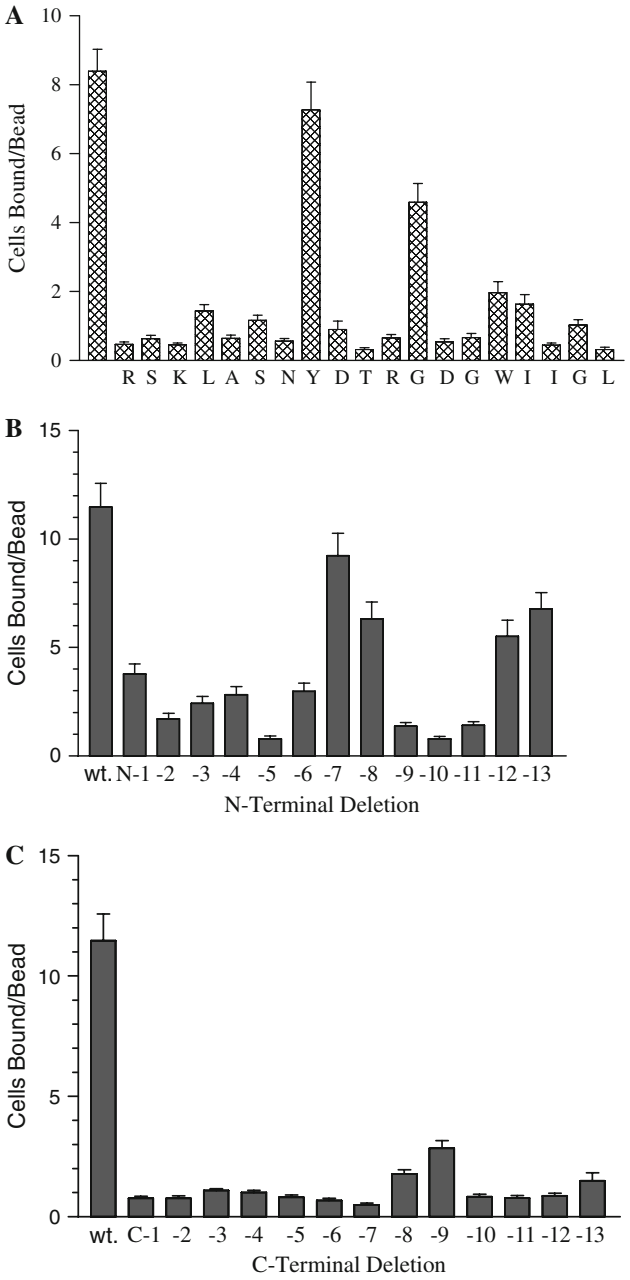


Fig. 5 Structural requirements that mediate the binding of Peptide 5 to B cells. (**a**) Alanine mutational walk of Peptide 5. Peptides derived from Peptide 5 were synthesized sequentially substituting alanine at individual amino acid positions. The binding of KARPAS 422 cells to the peptide-bound beads 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

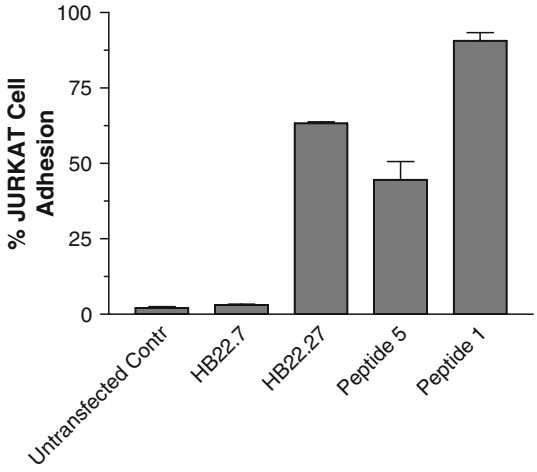


Fig. 6 CD22 ligand blocking assay. COS cells were transiently transfected with a CD22 cDNA and incubated with CD22-ligand bearing Jurkat cells, washed, fixed and adherent cells counted with and without the presence of indicated reagents. The number of bound Jurkat cells per transfected cell was determined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

et al. 1993), HB22.7 blocked up to 95% of CD22 mediated binding to its ligand, Fig. 6. An equimolar concentration of Peptide 5 blocks approximately 50% of CD22 mediated cell attachment. The non-blocking HB22.27 mAb and 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 Cytotoxocity

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

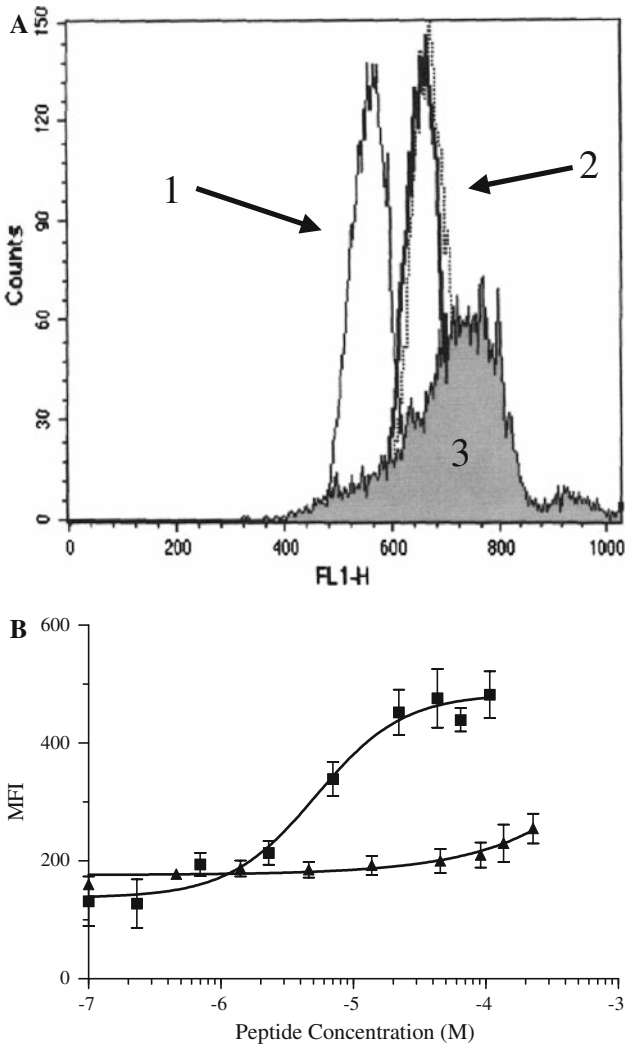


Fig. 7 Soluble Peptide 5 binding can be detected by FACS and used to assess binding affinity. (**a**) Biotinylated Peptide 5 binds Karpas 422 detected by streptavidin-FITC (Dorken et al. 1986) and has intermediate binding when compared to streptavidin-FITC alone (Law et al. 1994) or HB22.7-FITC (Engel 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 strepavidin-FITC

5-mediated NHL cell killing was assessed using the Burkitt's NHL cell line, Ramos. Ramos cells were incubated with 50 μg/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).

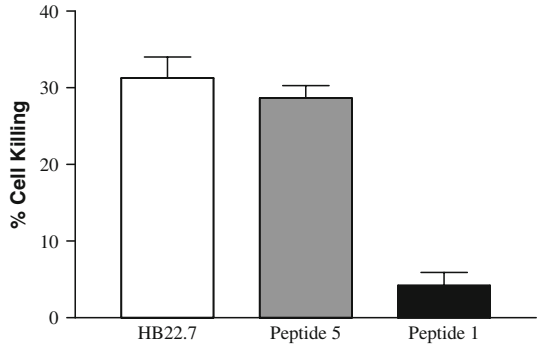


Fig. 8 Peptide 5 has lymphomacidal properties. The Ramos B cells were incubated with soluble Peptide 5 (1 μg/cc), HB22.7 (60 μg/cc), or anti-IgM (30 μg/cc). Cell viability was determined using trypan blue exclusion. The data are the average of at least three independent experiments

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

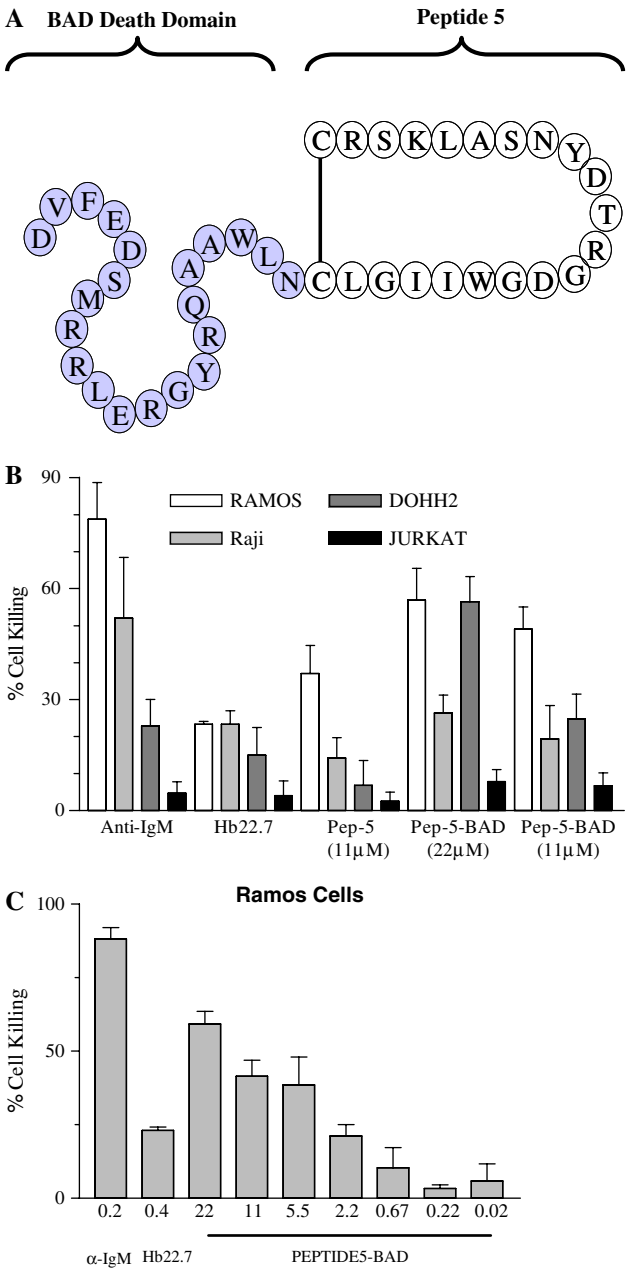


Fig. 9 The fusion peptide, Peptide 5-BAD has lymphomacidal activity. (**a**) The fusion of the BH3-containing death domain of BAD with the amino acid sequence of Peptide 5. (**b**) Equimolar amounts of Peptide 5, Peptide 5-BAD, HB22.7, or anti-IgM were incubated with three B, and one T cell NHL cell 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

had a remarkable degree of sequence homology. On this basis, we developed peptides based on this sequence homology that would specifically target CD22, initiate 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. 2006). 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.

Acknowledgements This work was supported by the Leukemia and Lymphoma Society Translational Research Award, the Schwedler Foundation and DOD grant # 21262678.

References

Al-Lazikani B, Lesk AM (1997) Chothia C: standard conformations for the canonical structures of immunoglobulins. J Mol Biol 273(4):927–948

Atherton E, Sheppard RC (1989) Solid phase peptide synthesis. IRL Press, Oxford

Doody GM, Justement LB, Delibrias CC, Matthews RJ, Lin J, Thomas ML, Fearon DT (1995) A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. Science 269(5221):242–244

Dorken B, Moldenhauer G, Pezzutto A, Schwartz R, Feller A, Kiesel S, Nadler LM (1986) HD39 (B3), a B lineage-restricted antigen whose cell surface expression is limited to resting and activated human B lymphocytes. J Immunol 136(12):4470–4479

Engel P, Nojima Y, Rothstein D, Zhou LJ, Wilson GL, Kehrl JH, Tedder TF (1993) The same epitope on CD22 of B lymphocytes mediates the adhesion of erythrocytes, T and B lymphocytes, neutrophils and monocytes. J Immunol 150:4719–4732

Engel P, Wagner N, Miller AS, Tedder TF (1995) Identification of the ligand-binding domains of CD22, a member of the immunoglobulin superfamily that uniquely binds a sialic acid-dependent ligand. J Exp Med 181(4):1581–1586

Florence C, Florence R, Patrick M, Cedric B, Thierry C, Claude G, Jean-Claude M, Martinie P, Daniel P, Bernard P, Michael K, Roger L, Anthony R (2003) Biochemical. Biophys Res Commun 307:198–205

Gordon IL (1995) Scatchard analysis of fluorescent concanavalin. A binding to lymphocytes. Cytometry 20(3):238–244

Haas KM, Sen S, Sanford IG, Miller AS, Poe JC, Tedder TF (2006) CD22 ligand binding regulates normal and malignant B lymphocyte survival in vivo. J Immunol 177(5):3063–3073

Heap CJ, Wang Y, Pinheiro TJT, Reading SA, Jennings KR, Dimmock NJ (2005) Analysis of a17-amino acid residue, virus neutralizing microantibody. J Gen Virol 86:1791–1800

Hernandez J, Schoeder K, Blondelle SE, Pons FG, Lone YC, Simora A, Langlade-Demoyen P, Wilson DB, Zanetti M (2004) Antigenicity and immunogenicity of peptide analogues of a low affinity peptide of the human telomerase reverse transcriptase tumor antigen. Eur J Immunol 34(8):2331–2341

Kaiser E, Colescott RL, Bossinger CD, Cook PI (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal Biochem 34(2):595–598

Kelm S, Pelz A, Schauer R, Filbin MT, Tang S, de Bellard ME, Schnaar RL, Mahoney JA, Hartnell A, Bradfield P et al (1994) Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. Curr Biol 4(11):965–972

King DS, Fields CG, Fields GB (1990) A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. Int J Pept Protein Res 36(3):255–266

Law CL, Craxton A, Otipoby KL, Sidorenko SP, Klaus SJ, Clark EC (1994) Regulation of signalling through B-lymphocyte antigen receptors by cell–cell interaction molecules. Immunol Today 15:442

Law CL, Aruffo A, Chandran KA, Doty RT, Clark EA (1995) Ig domains 1 and 2 of murine CD22 constitute the ligand-binding domain and bind multiple sialylated ligands expressed on B and T cells. J Immunol 155(7):3368–3376

Li R, Boehm AL, Miranda MB, Shangary S, Grandis JR, Johnson DE (2007) Targeting antiapoptotic Bcl-2 family members with cell-permeable BH3 peptides induces apoptosis signaling and death in head and neck squamous cell carcinoma cells. Neoplasia 9(10):801–811

Matthews RJ, Bowne DB, Flores E, Thomas ML (1992) Characterization of hematopoietic intracellular protein tyrosine phosphatases: description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences. Mol Cell Biol 12(5):2396–2405

Moreau C, Cartron PF, Hunt A, Meflah K, Green DR, Evan G, Vallette FM, Juin P (2003) Minimal BH3 peptides promote cell death by antagonizing anti-apoptotic proteins. J Biol Chem 278(21):19426–19435 Epub 2003 Mar

Plutzky J, Neel BG, Rosenberg RD, Eddy RL, Byers MG, Jani-Sait S, Shows TB (1992) Chromosomal localization of an SH2-containing tyrosine phosphatase (PTPN6). Genomics 13(3):869–872

Powell LD, Sgroi D, Sjoberg ER, Stamenkovic I, Varki A (1993) Natural ligands of the B cell adhesion molecule CD22 beta carry N-linked oligosaccharides with alpha-2, 6-linked sialic acids that are required for recognition. J Biol Chem 268(10):7019–7027

Privé GG, Melnick A (2006) Specific peptides for the therapeutic targeting of oncogenes. Curr Opin Genet Dev 16(1):71–77 Epub 2005

Qin W, Feng J, Zhou Lin Y-L, Shen B (2006) Fusion protein of CDR mimetic peptide with Fc inhibit TNF-alpha induced cytotoxicity. Mol Immunol 43(6):660–666

Sato S, Miller AS, Inaoki M, Bock CB, Jansen PJ, Tang ML, Tedder TF (1996) CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. Immunity 5(6):551–562

Sato S, Tuscano JM, Inaoki M, Tedder TF (1998) CD22 negatively and positively regulates signal transduction through the B lymphocyte antigen receptor. Semin Immunol 10(4):287–297

Sgroi D, Varki A, Braesch-Andersen S, Stamenkovic I (1993) CD22, a B cell-specific immunoglobulin superfamily member, is a sialic acid-binding lectin. J Biol Chem 268(10):7011–7018

Sharabi A, Zinger H, Zborowsky M, Sthoeger ZM, Mozes E (2006) A peptide based on the complementarity-determining region 1 of an autoantibody ameliorates lupus by up-regulating CD4 + CD25 + cells and TGF-beta. Proc Natl Acad Sci USA 103(23):8810–8815. Epub 2006 May

Shen SH, Bastien L, Posner BI, Chretien P (1991) A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases. Nature 352(6337):736–739

Siminovitch KA, Neel BG (1998) Regulation of B cell signal transduction by SH2-containing protein-tyrosine phosphatases. Semin Immunol 10(4):329–347

Stamenkovic I, Seed B (1990) The B-cell antigen CD22 mediates monocyte and erythrocyte adhesion. Nature 345(6270):74–77

Stamenkovic I, Sgroi D, Aruffo A, Sy MS, Anderson T (1991) The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and alpha 2-6 sialyltransferase, CD75, on B cells. Cell 66(6):1133–1144

Stewart JM, Young JD (1984) Solid phase peptide synthesis. Pierce Chemical CO., Rockford

Takasaki W, Kajino Y, Kajino K, Murali R, Greene MI (1997) Structure-based design and characterization of exocyclic peptidomimetics that inhibit TNF alpha binding to its receptor. Nat Biotechnol 15(12):1266–1270

Tamir I, Dal Porto JM, Cambier JC (2000) Cytoplasmic protein tyrosine phosphatases SHP-1 and SHP-2: regulators of B cell signal transduction. Curr Opin Immunol 12(3):307–315

Tedder TF, Tuscano J, Sato S, Kehrl JH (1997) CD22, a B lymphocyte-specific adhesion molecule that regulates antigen receptor signaling. Annu Rev Immunol 15:481–504

Tooze RM, Doody GM, Fearon DT (1997) Counterregulation by the coreceptors CD19 and CD22 of MAP kinase activation by membrane immunoglobulin. Immunity 7(1):59–67

Torres RM, Law CL, Santos-Argumedo L, Kirkham PA, Grabstein K, Parkhouse RM, Clark EA (1992) Identification and characterization of the murine homologue of CD22, a B lymphocyte-restricted adhesion molecule. J Immunol 149(8):2641–2649

Tuscano J, Engel P, Tedder TF, Kehrl JH (1996) Engagement of the adhesion receptor CD22 triggers a potent stimulatory signal for B cells and blocking CD22/CD22L interactions impairs T-cell proliferation. Blood 87(11):4723–4730

Tuscano JM, Riva A, Toscano SN, Tedder TF, Kehrl JH (1999) CD22 cross-linking generates B-cell antigen receptor-independent signals that activate the JNK/SAPK signaling cascade. Blood 94(4):1382–1392

Tuscano JM, O'Donnell RT, Miers LA, Kroger LA, Kukis DL, Lamborn KR, Tedder TF, DeNardo GL (2003) Anti-CD22 ligand-blocking antibody HB22.7 has independent lymphomacidal properties and augments the efficacy of 90Y-DOTA-peptide-Lym-1 in lymphoma xenografts. Blood 101(9):3641–3647