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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 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. Within year 2 we have identified several other peptides that are more effective than peptide 5 and we have begun to characterize their signaling, cytotoxic and apoptotic potential. In addition we have demonstrated that phosphatase inhibition augments the effectiveness of these peptides. Studies that will evaluate the effectiveness of these peptides in vivo are ongoing.					
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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 NH₂-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) 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 within the first year of the granting period included identification and initial characterization of several CD22-binding peptides which has been summarized in the year 1 progress report and recent publication (7).

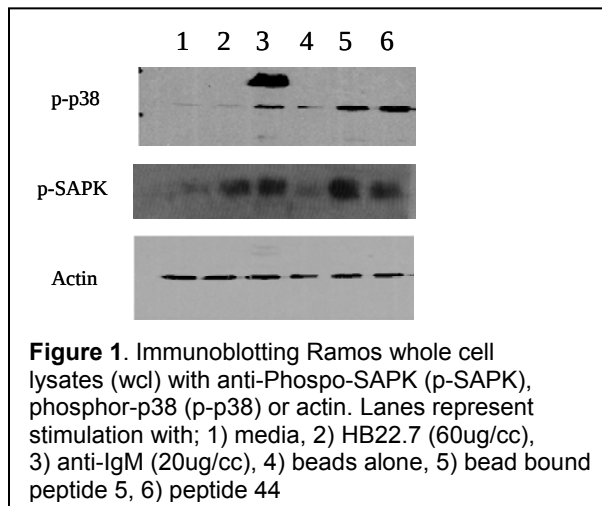
Below the research accomplishments for year two will be summarized and organized based on the proposed aims and goals as outlined in the Statement of Work (SOW).

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 tonsillar 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.

With regard to goal 2, significant progress has been made in this area. We examined the signaling pathways shown to be involved in CD22-mediated signaling including the stress activated kinase (SAPK) and p38 (3). Several immunoblotting (IB) experiments were done that demonstrated that both peptide 5 (previously 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.



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**

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

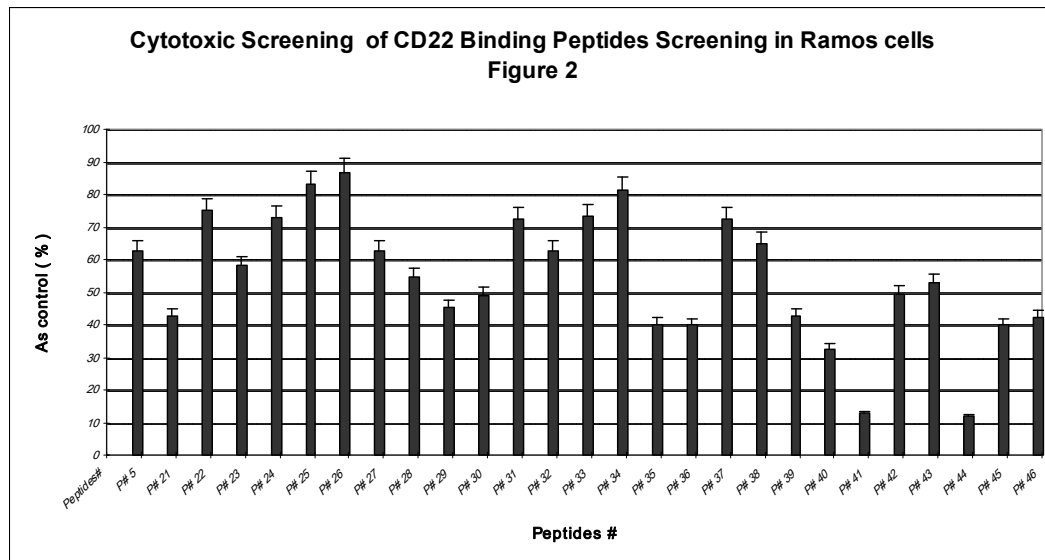


Figure 2. CD22-binding peptides identified during the mutational analysis of peptide 5. Cell killing was assessed via trypan blue exclusion and reported as a percent of untreated control. Assays were done in triplicate with error bars representing standard deviation.

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

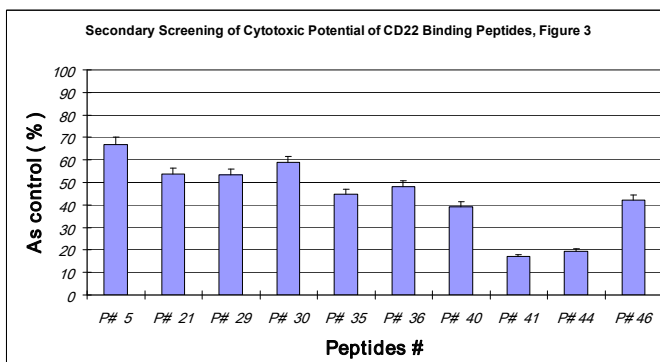


Figure 3. Selected CD22-binding peptides were again screened for their potential to kill Ramos B cells. Killing was assessed as described in figure 2

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 cytotoxicity 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.

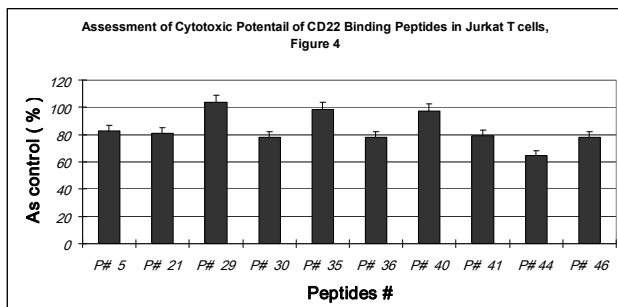


Figure 4. Selected CD22-binding peptides were screened for cytotoxic potential in Jurkat T cells. Killing was assessed as described in figure 2.

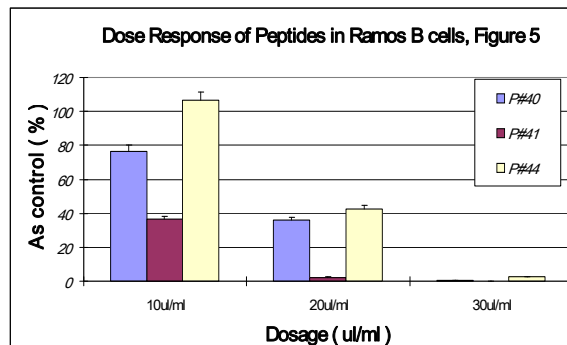
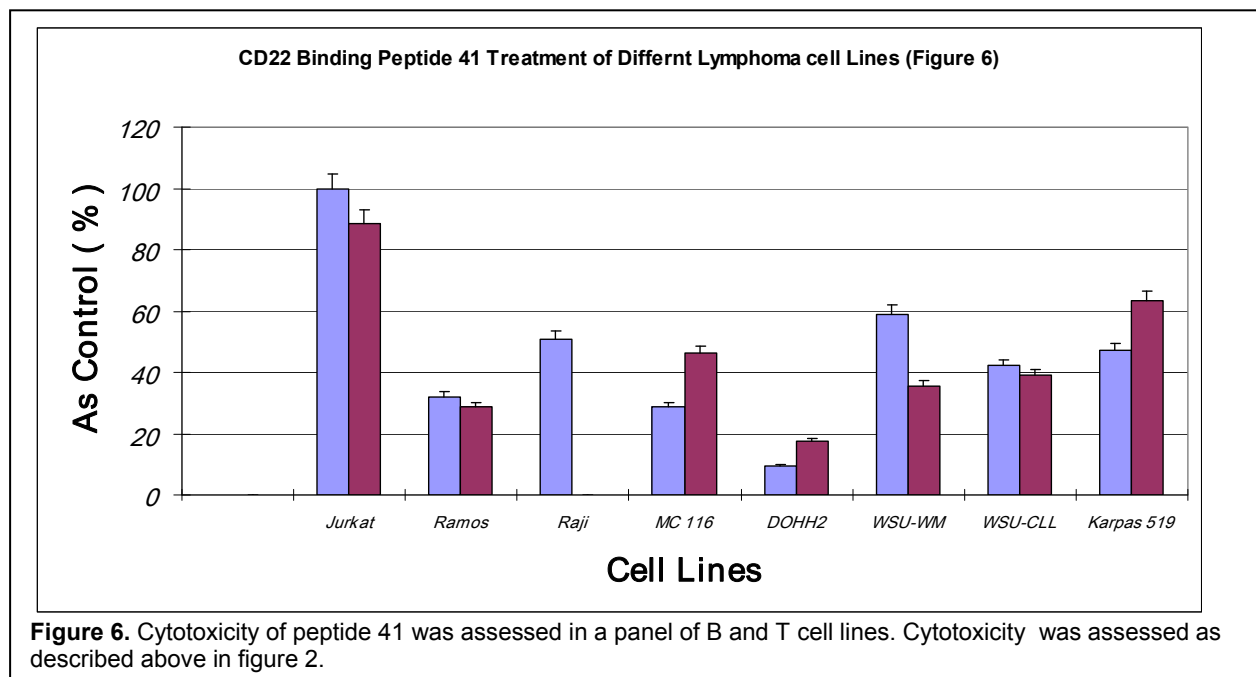
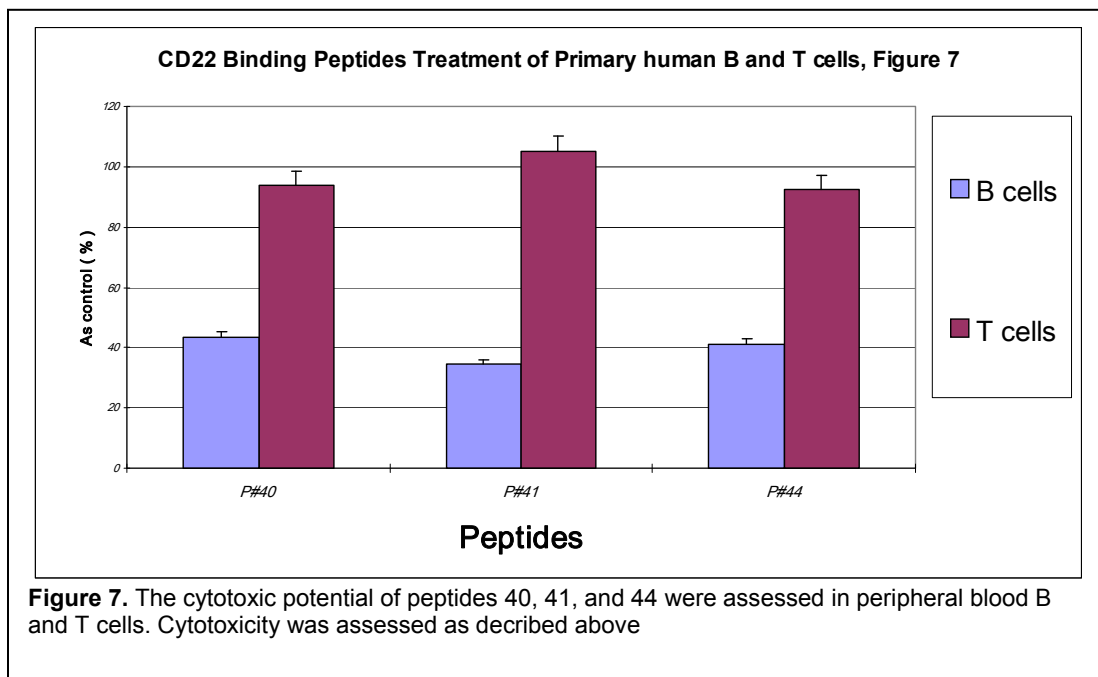


Figure 5. Dose responsive effect of peptides 40, 41, and 44 in Ramos B cells. Cytotoxicity was assessed as described in figure 2

The breadth of cytotoxicity 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.



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.

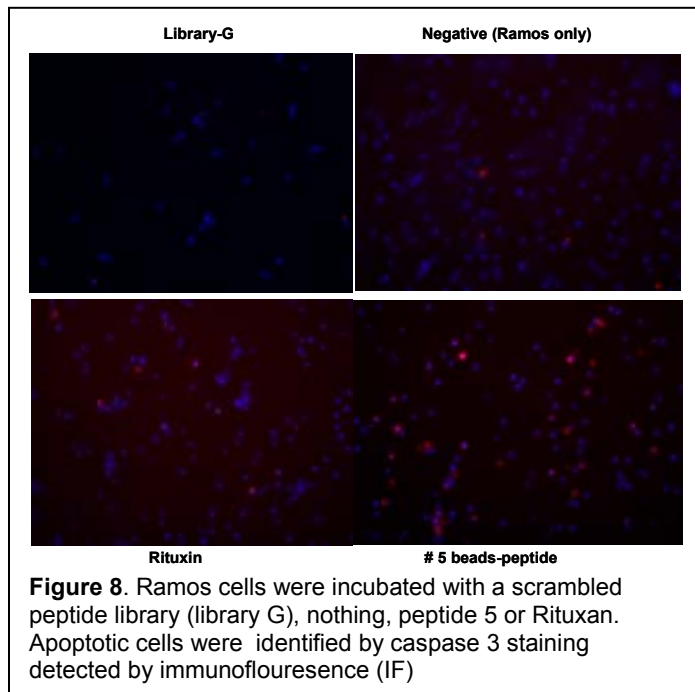


These peptides were also effective at killing primary B cells and B cell specificity was confirmed.

Goal:

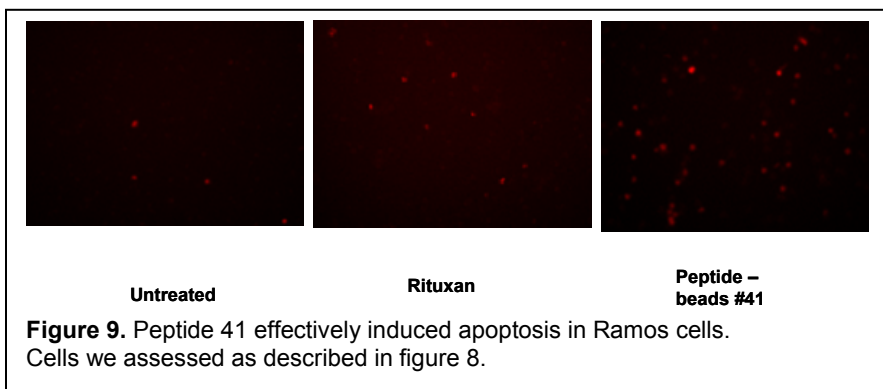
4. Promising peptides that initiate signal transduction and mediate apoptosis will be further assessed in vivo for their lymphomacidal properties using a nude mouse xenograft model.

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



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

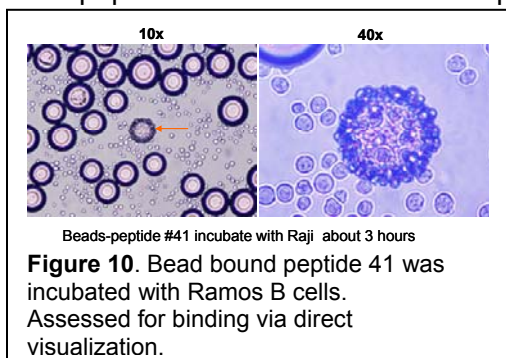
Next we assessed the apoptotic potential of peptide 41, figure 9.



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 have 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.

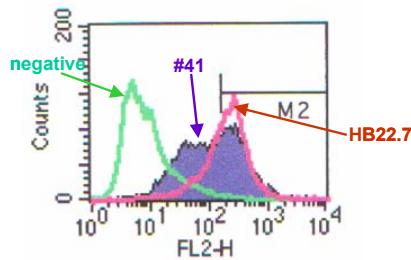


Figure 11. Ramos B cells were either incubated with anti-mouse FITC (negative), HB22.7 + anti-mouse FITC (HB22.7) or pre-incubated with peptide 41, washed and then incubated with HB22.7 + anti-mouse FITC (#41)

This demonstrated that peptide 41 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.

plate A (24 well) Ramos cells 6×10^4 /ml/well, incubated with immobilized peptide for 24 hours, then transferred medium to plate B with fresh Ramos cells and incubate the plate for other 2 days at 37°C

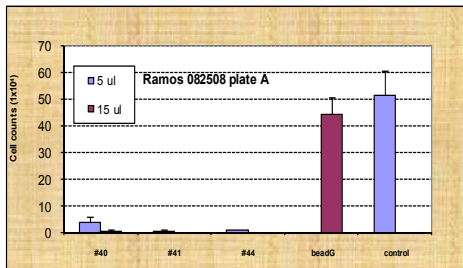


Plate B (24 well) Ramos cells 1×10^5 /ml/well, remove medium, transfer medium from the plate A and incubate the plate for 2 days at 37°C

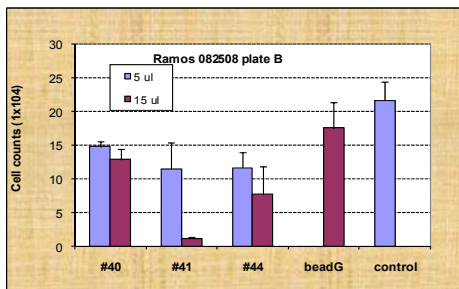


Figure 12. Supernatant from Ramos cells that had been incubated with immobilized peptide 41 was incubated with fresh Ramos cells and assessed for cytotoxicity as described above

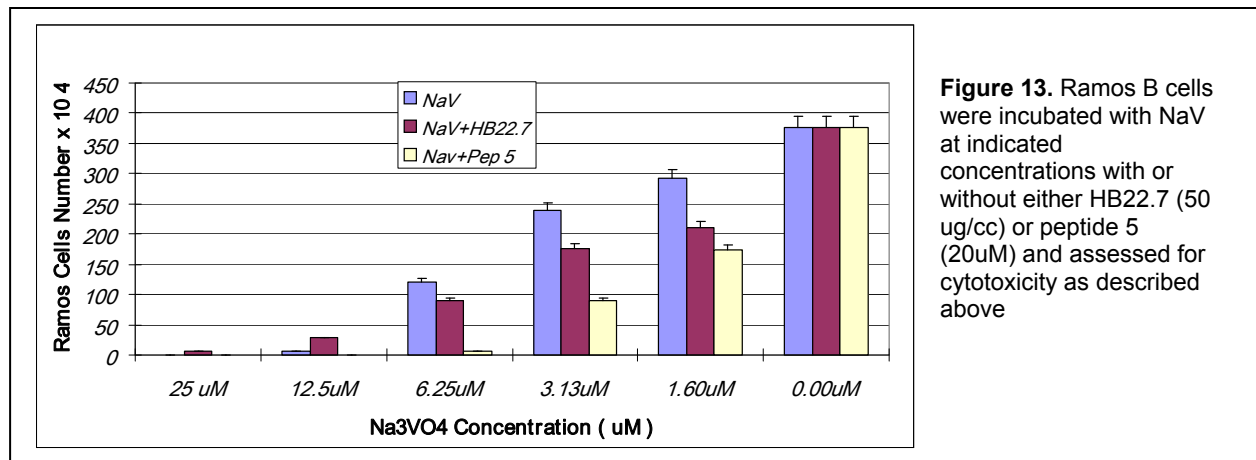
This demonstrated that peptide 41 mediated the secretion of a soluble factor that had cytotoxic potential in fresh Ramos cells.

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.

Previous studies in our lab demonstrated that phosphatase inhibition with sodium orthovanadate (NaV) could augment CD22-mediated 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



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.

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).

We have begun to develop the DOTA conjugated peptides that will facilitate the initial PET scan studies

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).

III. Annual Report Summary/Key Research Accomplishments

- 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.
- We have begun to develop DOTA-conjugated peptide 5 and 41 in anticipation of immuno-PET studies.

IV. Reportable Outcomes

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 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. Currently underway, or being planned are studies looking at the effectiveness of these peptides in a nude mouse model of human lymphoma, *in vivo* targeting using immuno-PET scanning, and the development of additional modifications of the currently described peptides using combinatorial peptide libraries. 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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