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Active Conformation of Laminin Peptide 11

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13. ABSTRACT (Maximum 200 Words) We made significant advances in elucidating the structure of the peptide 11 binding domain of LBP. Phage display studies indicated that three sequence domains in the mid and C-terminal domains are involved. Homology modeling the LBP using the crystal structure of bacterial S2 allowed us to obtain an idea of the overall fold of LBP. Structural studies of LBP require that it is expressed in a recombinant system. We have accomplished this for the full length protein and several fragments. Unfortunately, fragments including the C-terminal domain were not folded well, and the full length rLBP was not stable for sufficient time in solution for NMR structure determination. Therefore, we have started to work on structure determination by X-ray crystallography. Synthesis of the candidate 16 YIGSR mimetic was accomplished but was so inefficient that we have switched to an alternative mimetic structure. LBP was shown to have sulphydryl oxidase activity, leading us to compare the bioactivity of peptide 11 monomer and dimer. The most active species was found to be the dimer. Several lines of evidence indicate that there is an induced conformational change upon YIGSR binding to LBP. Therefore the bound conformation of the peptide is required for mimetic design.				
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FOREWORD

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Introduction

Many clinical studies on breast cancer and other solid tumors show strong positive correlations of high expression of the 67 kDa laminin binding protein (LBP) with poor prognosis^{1,2,3,4,5,6,7,8,9,10,11}, and recently, SAGE studies have confirmed this correlation for colon carcinoma. The 67 kDa LBP has been shown to be a legitimate target for cancer therapy by the demonstration that reduction of tumor cell expression of the 67 kDa LBP, brought about by anti-sense or antibody approaches does inhibit tumor metastasis in mice^{12,13,14}. However, current limitations with gene and antibody therapies restrict the use of these approaches for long term clinical applications. Our approach is to use the active conformation of the matrix ligand for the 67 kDa LBP as a template for the design of orally compatible anti-metastatic drugs. This ligand has been identified as a nine amino acid sequence from laminin-1, CDPGYIGSR, and is known as peptide 11^{15,16}. The actual mimetics are designed to represent only the YIGSR region of bound peptide 11, because YIGSR is known to be the minimal active sequence¹⁶ and synthesis of organic mimetics for longer sequences than this is daunting. The ability of synthetic peptide 11 or YIGSR to block tumor cell invasion and metastasis depends on its ability to interfere with the interactions of the 67 kDa LBP with basement membrane laminin-1^{15,16}. The 67 kDa LBP (a dimer from the 37 kDa LBP gene product) evolved from the S2 ribosomal class of proteins¹⁷. A recent study of its evolutionary genomics revealed that a unique evolution of the protein occurred in the C-terminal domain in parallel with the appearance in multicellular organisms of laminin and laminin-like molecules¹⁷. This C-terminal domain has been indicated by us and others as the matrix ligand binding domain^{18,19,20,21}. The half-life of peptide 11 (and YIGSR) in the bloodstream is in the order of minutes, consistent with rapid proteolysis²². Therefore, in order to develop useful therapeutics, either the biological half-life of peptide 11 must be very significantly extended, or else it is essential to mimic the properties of peptide 11 using non-peptide compounds. The overall goal of this research project is to design accurate mimetics using template-based approaches, and to evaluate their anti-invasive and anti-metastatic activity. If we are successful in synthesizing mimetics with good anti-metastatic activity, this will provide a "proof of concept" for our structure-based design approach, and the best mimetics should be effective lead compounds suitable for going into combinatorial chemistry programs to provide the most effective derivatives.

There are three specific aims, which should allow us to accomplish the goal of this research project:

Aim 1 In collaboration with Drs. V. Copié and E. A. Dratz, of the Chemistry and Biochemistry Department at MSU¹, to determine the relevant structural characteristics of the ligand binding domain of the LBP.

Aim 2 In collaboration with Dr. W. Todd Wipke, Professor of Chemistry and Biochemistry, UCSC², to undertake structure-based design of non-peptide mimetics for the active conformation of peptide 11 using INVENTON, an artificially intelligent computer program for the design of structural mimetic compounds.

Aim 3 In collaboration with Dr. J. Konopelski, UCSC, to synthesize the most promising structures derived by the INVENTON program, and to evaluate the activities of the new compounds in inhibiting tumor cell invasion *in vitro*, and metastasis in experimental animals.

¹ MSU = Montana State University

² UCSC = University of California, Santa Cruz

Body of Report

This is a highly collaborative project with contributions from five different laboratories and two different institutions. It follows that the "statement of work" is complex, and for convenience of the reader, we re-iterate it below.

STATEMENT OF WORK:

Technical Objective (aim) 1

- Task 1: Months 1-15: Determine the LBP residues, which interact with peptide 11.
- Task 2: Months 1-18: Express the ligand binding domain of the LBP in *E. coli*, conduct multidimensional NMR experiments to determine structural information relevant to drug design.
- Task 3: Months 1-15: Provide all relevant information from Tasks 1-2 of this proposal, along with the fully refined bound peptide 11 conformation (**derived from work carried out exclusively on our NIH award**), to Dr. Wipke to improve the peptide 11 template used by INVENTON for the design of peptide mimics.

Technical Objective (aim) 2

- Task 4: Months 6-18: Using the artificially intelligent program, INVENTON, design mimics of the LBP-bound conformation of peptide 11 (actually the YIGSR domain from this structure).
- Task 5: Months 12-18: Integrate new information coming from tasks 1-3 into the drug design template used by INVENTON.
- Task 6: Months 6-18: Evaluate the output structures from INVENTON for potential drug lead compounds. Synthesize the most approachable of these.
- Task 7: Months 6-18: Work with Dr. Wipe's group in providing heuristic rules for determining the relative ease of synthesis for output structures from INVENTON.
- Task 8: Months 12-22: Provide Dr. Starkey's group with mimetic compounds for limited preclinical tests.

Technical Objective (aim) 3

- Task 9: Months 1-12: Test informative analogs of peptide 11 for anti-invasive and anti-metastatic activity.
- Task 10: Months 12-24: Test mimetic compounds for 1) tissue culture toxicity, 2) anti-invasive activity and 3) anti-metastatic activity.

Progress on Technical Objective 1, task 1.

Experimental Methods and Procedures

The methodology originally outlined in the grant application was restricted to mapping the contact residues in the LBP by using our photo-crosslinking biotinylated analog of peptide 11²³. The derivatized LBP and its cleavage fragments would be isolated using monovalent avidin matrices, and final analysis would be carried out using mass spectrometry sequencing techniques. The proposed methodology for isolating derivatized LBP and cleavage products relied on the use of commercially available monovalent avidin matrices. Unfortunately, these gave very poor yields and commercial anti-biotin antibodies were found to exhibit very low affinities. We, therefore, made our own rabbit anti-biotin antibodies for this work. Appropriate antibody specificity was sought using immunizing antigens consisting of biotin and biotinylated peptide 11 crosslinked to an irrelevant protein (KLH - keyhole limpet hemocyanin). After removal of KLH specific responses, we tested the antibodies by Western blot analysis. Good titers were found to biotinylated proteins and derivatized LBP. The antibody still exhibited unwanted cross reactions and was further purified over a peptide 11 column to provide a highly specific reagent capable of isolating derivatized LBP from detergent extracts of tumor cell membranes. Yields of derivatized LBP from whole cell membranes were still relatively low, and we decided to utilize rLBP for these experiments.

Limited proteolysis experiments were carried out on isolated recombinant LBP using trypsin, V8 and elastase. Trypsin and V8 proteases both yielded useful sized fragments, which were purified by HPLC. The masses were determined by mass spectrometry and N-terminal sequenced. Overall, few cleavage sites were found in the N-terminal third of native rLBP monomer suggesting that the core of the protein includes much of this sequence. The highly conserved "EASY" sequence is cleaved at residue 136, there is a cleavage site at residue 220 and several in the terminal sequence containing the DWS repeats. Three informative domains were cloned and expressed. Binding to laminin-1 was demonstrated by ELISA for the full length construct, fragment 1-220, and fragment 137-230. Only limited binding of fragment 200-295 to laminin-1 could be demonstrated, and preliminary NMR experiments suggest that this isolated domain may not be well folded/stable in the monomer state. A manuscript describing these studies is in preparation.

Because of the initial difficulties with isolating sufficient derivatized LBP from tumor cell membranes, we carried out a series of experiments using phage display mapping to identify sequences within the LBP, which interact with peptide 11. This alternative experimental approach cannot provide the detail to identify the actual contact residues, but, nonetheless, provided very useful preliminary data identifying the interacting sequences. The results of this work were published in the Journal of Molecular Biology. Reprints of this manuscript have already been forwarded with a previous report, and the reader is referred to them for detailed methods, results and discussion.

Assumptions

We anticipated that results from the phage display experiments would identify sequences within the LBP, which contain the contact residues for peptide 11.

Results

Specifically eluted phage populations exhibited three classes of mimitopes for different regions in the cDNA derived amino acid sequence of the 67 kDa laminin binding protein (LBP). These regions were 1)

a palindromic sequence known as peptide G, 2) a predicted helical domain corresponding to LBP residues 205-229, and 3) TEDWS -containing C-terminal repeats. All elution conditions also yielded phage with putative heparin binding sequences. We modeled the LBP²⁰⁵⁻²²⁹ domain, which we demonstrated by circular dichroism (CD) to have a helical secondary structure, and determined that this region likely possesses heparin binding characteristics located to one side of the helix, while the opposite side may contain a hydrophobic patch where peptide 11 could bind. Using Elisa plate assays, we demonstrated that peptide 11 and heparan sulfate both individually bound to synthetic LBP²⁰⁵⁻²²⁹ peptide. We also demonstrated that synthetic PATEDWSA peptide could inhibit tumor cell adhesion to laminin-1. These data support the proposal that the 67 kDa LBP can bind the β -1 laminin chain at the peptide 11 region, and suggest that heparan sulfate is a likely alternate ligand for the binding interactions. Our results also confirm previous data¹⁹ suggesting that the most C-terminal region of the LBP, which contains the TEDWS repeats, is involved in cell adhesion to laminin-1, and we specifically indicated the repeat sequence in that activity.

Discussion and Recommendations

These experiments indicate that peptide 11 interacts with three different sequences domains in the 67 kDa LBP. The data also suggests that heparin/heparan sulfate is an alternate ligand for the 67 kDa LBP. As expected, the ligand binding domain of the LBP appears to be quite complex. Any structures developed for the active conformation of peptide 11, and for the ligand binding domain of the LBP, need to be compatible with these data on peptide 11 interacting sequences.

Progress on Technical Objective 1, task 2.

Experimental Methods and Procedures

Expression of the ligand binding domain of the LBP in *E. coli*:

This work is being carried out in collaboration with Dr. Valérie Copié at Montana State University. The first step in conducting the NMR structural studies of the ligand binding domain of the LBP was to express this domain in bacteria. This allows for heavy isotope labeling of the domain at reasonable cost. The coding region for the full length expression product was obtained from our mammalian vector and initially cloned into the pTrcHis B prokaryotic expression vector (Invitrogen). Top 10 *E. coli* cells (Invitrogen) were successfully transformed, and they produced a protein of the correct molecular weight which stained positively with our anti-LBP antibody in a Western blot. However, the Trp promoter in this vector is not particularly

efficient, and only modest yields of the LBP were obtained.

A second vector was then tried. This is the pET-30 vector from Novagen which utilizes the more efficient T7 promoter system. This time we used CD41 *E. coli* cells which have been successfully used in our hands to produce isotopically labeled peptides. On induction with IPTG (Isopropyl- β -D-thiogalactopyranoside), large quantities of the expressed LBP were obtained (Figure 1), and the same was the case for the C135 LBP ligand binding domain. We utilized the N-terminal poly-His

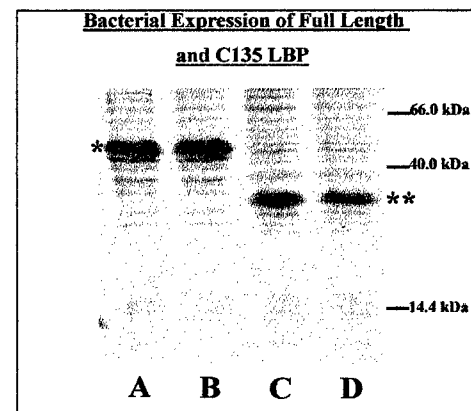


Figure 1. Whole bacterial cell lysates resolved on Coomassie Blue stained SDS-PAGE gels. Asterisks indicate the positions of the recombinant LBP products.

tag for isolation of the expressed recombinant protein by Ni affinity (Figure 2). The pET-30 vector contains two N-terminal protease cleavage sites for removing the poly-His tag. Closest to the expressed

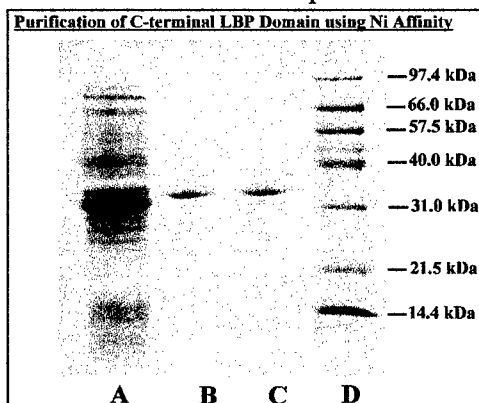


Figure 2. Purification of C135 LBP expression product using Ni affinity. SDS-PAGE gel stained with Coomassie Blue. Lane A = whole cell lysate, lanes B and C = imidazole eluate from the Ni column, lane D = molecular weight markers.

protein sequence is an enterokinase site. We abandoned this site when we could not achieve cleavage efficiencies greater than 50%. The more distant thrombin cleavage site worked very well with close to 100% efficiency (Figure 3). Biotinylated thrombin is used for the cleavage, and it is easily removed from the expressed protein preparation over a streptavidin column. Free poly-His sequence is removed by a second pass over the Ni column. While, the pET-30 vector system worked well for our protein, the expression product contained too many extra residues. Therefore we eventually utilized a modification of this vector, pET-15b which does not contain an enterokinase cleavage site, and which has the thrombin cleavage site immediately N-terminal to the expressed protein sequence. The molecular weight of the isolated expressed C135 ligand binding domain was checked by time of flight MALDI (matrix assisted laser desorption) mass spectrometry, and the success of refolding the domain by circular dichroism (CD) spectroscopy.

The recombinant C135 domain labelled well with ^{15}N , a prerequisite for NMR studies. However, while this domain remained well folded at low and medium concentrations, at the high concentrations needed for NMR structure determination, partial aggregation was noted. Comparisons between native and reduced gels indicated that aggregation was largely driven by disulfide bond formation occurring between LBP molecules. We mutated the two Cys residues in the LBP to Ala. The Cys minus expression product did not aggregate as badly as the wild type protein, however, aggregation still made NMR work problematical. Two other approaches were used to improve the solubility status of the LBP products. The limited proteolysis experiments were used to identify more cohesive domains for expression, and LBP domains have been expressed as mosaics with the streptococcal protein G GB1 domain to induce solubility in the experimental protein sequence²⁴. Well resolved spectra have been obtained from some (but not all) of the domains identified by limited proteolysis.

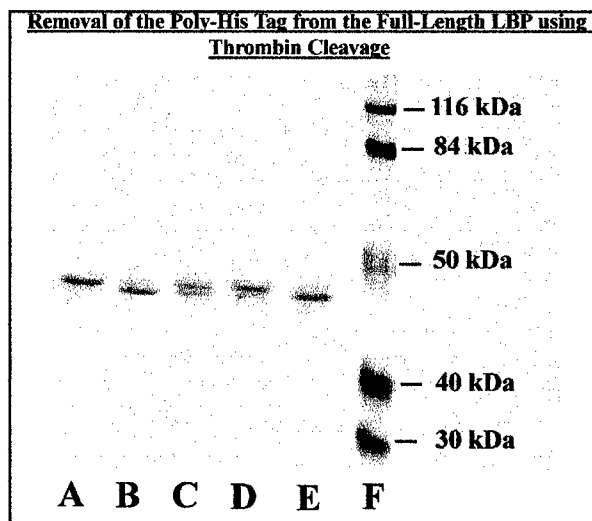


Figure 3. Full length LBP bacterial product resolved on a Coomassie Blue stained SDS-PAGE gel. Lane A = uncut product, lanes B-D = product partially cleaved by thrombin, lane E = fully cleaved product, lane F = molecular weight standards.

Assumptions

The only assumption being made for this task is that the bacterial monomeric protein will function like the high molecular weight form of the protein. The only published data indicate that, for binding to laminin-1, this is true²⁸. Unsupported statements (no data given) in the literature question this finding (46). Therefore, we conducted a series of control binding experiments with the monomer. These studies indicated that the recombinant monomer bound to laminin-1 in a qualitatively similar way to the shed 67 kDa LBP. However, the avidity of binding was greater for the 67 kDa LBP.

Results

As discussed in the "Methods" section, we have succeeded in generating efficient bacterial expression systems for full length LBP, the C135 ligand binding domain (Figure 1), and several of the fragments identified via limited proteolysis. The expressed proteins are readily purified over Ni columns using imidazole elution (Figure 2), and the poly-His sequence is efficiently removed using thrombin (Figure 3). After refolding the initially denatured bacterial expression product, most of the CD spectra (Figure 4) showed no evidence of random coil structure indicating adequate refolding. The LBP CD spectrum is dominated by an alpha helical profile. Since the majority of the predicted alpha helix is in the C-terminal domain of the protein, we expect a largely alpha helical profile for the C135 LBP domain also. Figure 5 shows the mass spectra obtained for the isolated C135 LBP domain. While full length rLBP and cloned fragments 137-230 and 1-220 were clearly shown to bind to laminin-1 in ELISA assays, the most C-terminal fragment, 200-295 appeared to be unstructured as an isolated fragment. This does not bode well for obtaining the structure of this important region by NMR techniques.

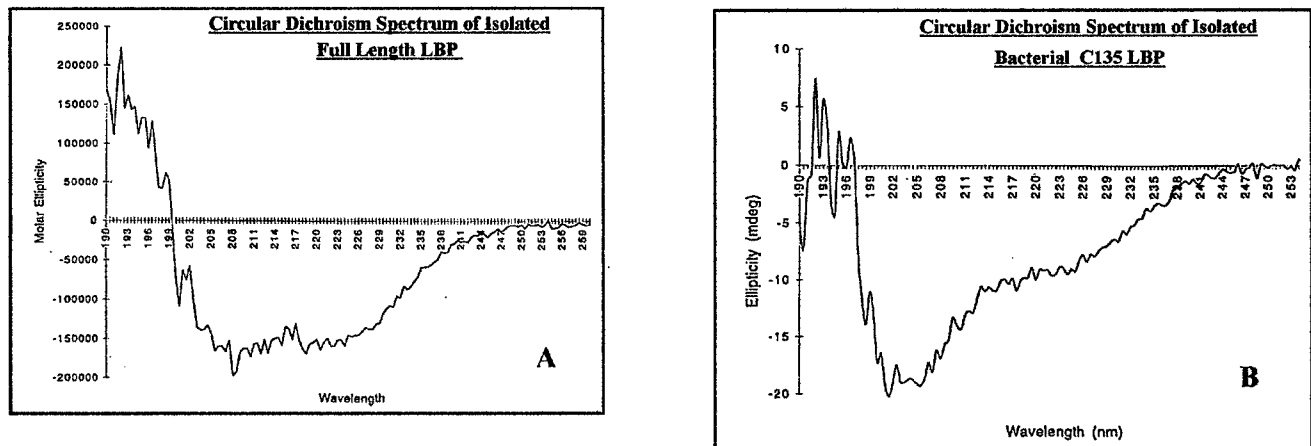


Figure 4. Circular Dichroism Spectra for (A) isolated mammalian 67 kDa LBP, and (B) recombinant C135 LBP matrix ligand binding domain.

As mentioned earlier in the introduction, evolutionary studies indicate that the LBP evolved from the early S2 ribosomal protein such that the sequence characteristics of the highly conserved modern protein emerged and stabilized at the time when tissues and laminin-like extracellular matrix molecules first appeared. During the last year of this award, the crystal structure of *Thermus thermophilus* S2 ribosomal protein was solved as part of the work on the structure of the whole bacterial ribosome. Extensive Clustal alignment studies suggested that the LBP could be aligned well with the S2 proteins provided that two

long highly charged helical domains in the s2 proteins were assumed to have been deleted in the LBP proteins (Figure 7). This would make sense as these helices associate with ribosomal RNA, and this activity is likely lost in the LBP proteins. Also, of course, the S2 proteins lack the most terminal domain of the LBP proteins. Using several different programs, we produced homology models of the N-terminal, and central domains of the LBP model. The models are consistent with the data derived from our limited proteolysis studies. Also the peptide G domain is surface exposed as would be predicted by its extracellular binding activity. A manuscript describing the homology modeling work is in preparation at this time.

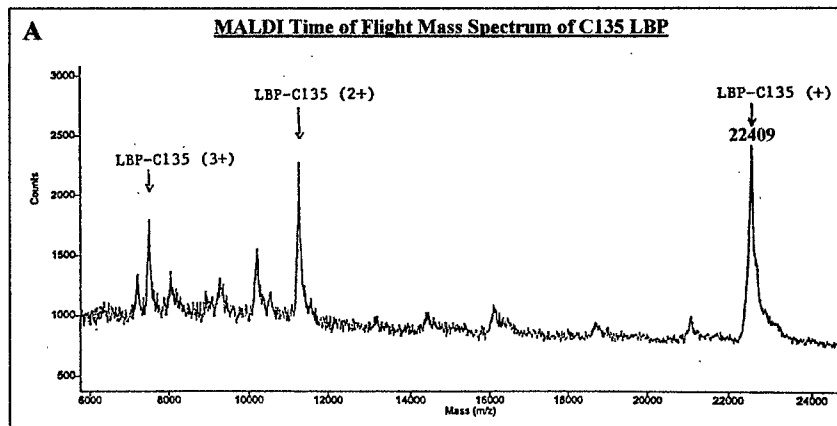


Figure 5. Mass Spectrometry analysis of the C135 LBP domain.

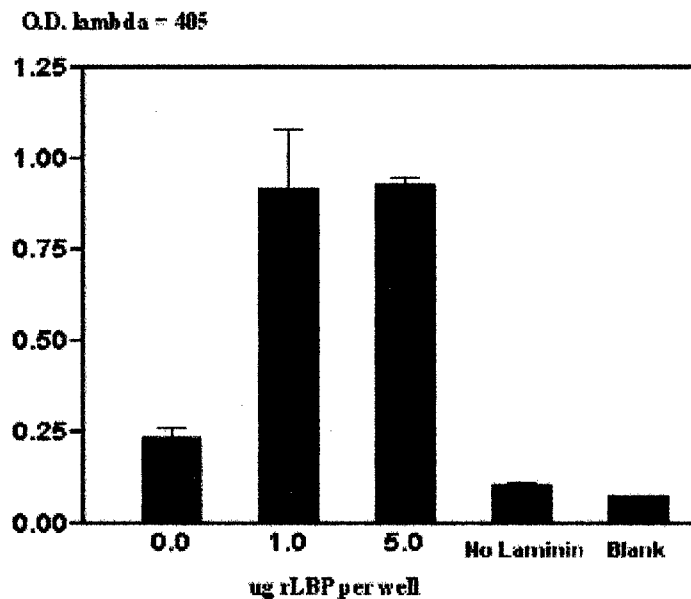


Figure 6. ELISA assay showing binding of laminin-1 to rLBP.

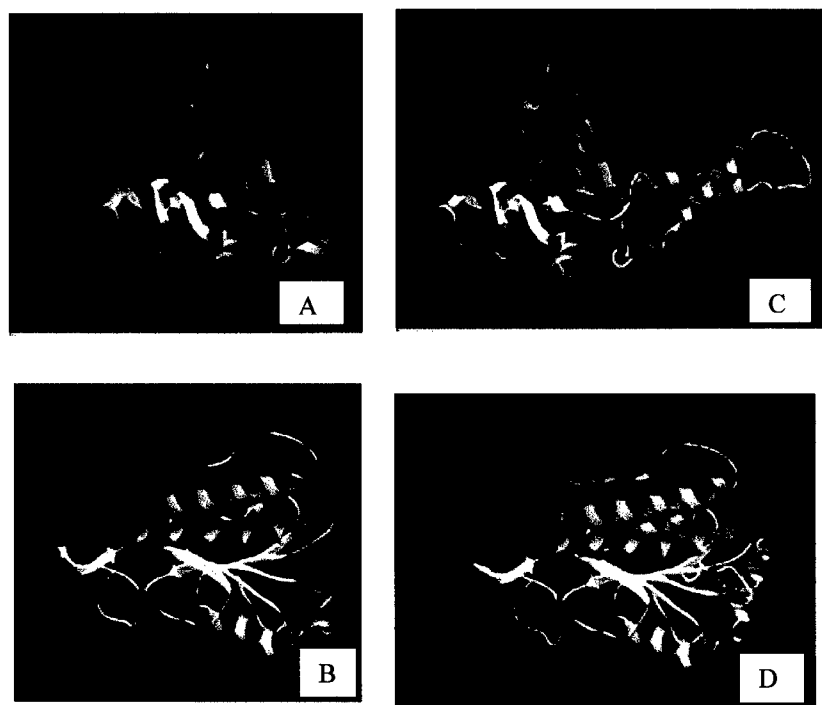


Figure 7. Homology modeling of the N-terminal domains of rLBP from the bacterial S2 template.

Panels A,B are LBP and panels C and D are the crystal structure of bacterial ribosomal protein S2. Horizontal panels are matched for orientation.

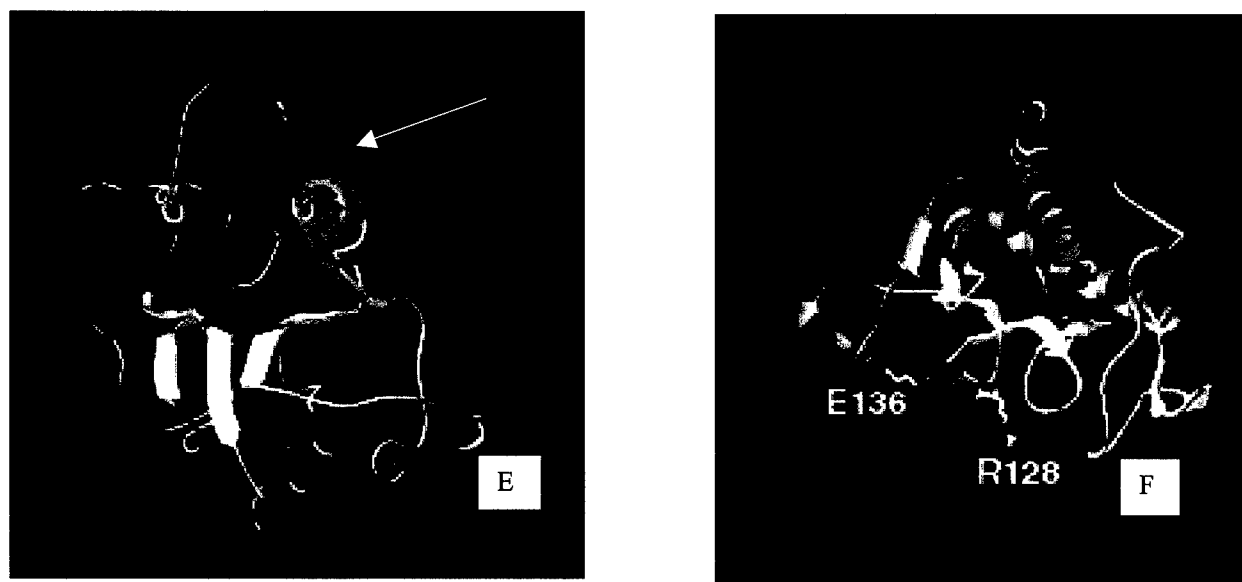


Figure 8. Homology modeling of the N-terminal domains of rLBP from the bacterial S2 template.

Panel E shows to putative peptide G domain (arrow) on the model of rLBP, and panel F shows the position of important cleavage sites.

We now believe that the behavior of the rLBP, and critical expressed fragments of this protein, will preclude obtaining sufficient structural information for elucidating the structure of its laminin binding domain using only NMR approaches. Therefore, we initiated another collaboration with an X-ray crystallographer, Dr. Martin Lawrence at MSU, to evaluate crystallography as a possible approach.

For this work, we cloned the full-length gene encoding hamster LBP into the pQE31 (Qiagen) expression vector as a fusion with an N-terminus 6xHis tag to facilitate Ni- or Co-affinity purification. The pQE31 vector is known to produce large quantities of recombinant proteins. Including the six His residues, the expressed protein contains 16 additional amino acids at the N-terminus but these extra residues are not anticipated to affect the crystallization and may actually improve the solubility of the protein. Initial attempts to obtain a sufficient quantity of "well-behaved," soluble, recombinant LBP in order to pursue a crystal structure were unsuccessful. The majority of the problems encountered have now been solved, and it is feasible for us to obtain quantities of protein suitable for screening crystal conditions. Previously, only small quantities of soluble protein could be purified from the Top10 F' and JM109 F' *E. coli* strains under native conditions even though the protein was highly over-expressed at nearly 80 mg protein/L culture. In an attempt to recover usable protein from inclusion bodies, refolding studies were undertaken following purification under denaturing conditions. All refolding attempts were unsuccessful as precipitation occurred, and the large amount of soluble protein that was recovered resolved at a molecular weight of greater than 600-kDa in size exclusion chromatography. This strongly suggested that nonspecific aggregation was occurring. Attempts to utilize protein in inclusion bodies were abandoned, and ways to maximize the yield of soluble protein purified under native conditions were sought.

We decided to supplement the purification buffers with detergents because LBP is membrane-associated on cells, its primary sequence is fairly hydrophobic, and it resolves at its expected molecular weight in SDS-PAGE despite being highly aggregated in solution following refolding attempts. After experimenting with a number of detergents, it was determined that addition of CHAPS produced the greatest yield of native, soluble protein. Furthermore, CHAPS has the advantage over many detergents in that it is easily exchangeable. Following expression at 37°C and a standard His-tagged protein purification protocol under native conditions (Qiagen), with 10 mM CHAPS supplementing the purification buffers, it is possible to obtain approximately 2 mg soluble protein/L of culture. This yield has since increased to approximately 8 mg protein/L culture by decreasing the expression temperature to 30°C.

A second purification step is required to remove the aggregated, yet soluble LBP material, and some other minor contaminants that remain following the metal affinity purification. Size exclusion chromatography with a Superdex75 column works well for this step because the size of LBP is within the separation range, and because it allows for simultaneous buffer exchange into a minimal buffer suitable for initiating hanging drop crystallization experiments under a wide variety of conditions. Not only does performing the initial affinity purification with CHAPS greatly increase the yield of native, soluble protein, it also allows for recovery of protein of the expected molecular weight following the second purification step. A sizable amount of aggregated protein still elutes in the void volume, but by performing this purification step immediately after metal affinity purification, the majority of the protein is of the expected monomer size. As expected, the apparent molecular weight of the monomer-sized

protein decreases as it is exchanged into buffer with decreasing CHAPS concentration in the Superdex75 column. This indicates that fewer detergent molecules interact with the protein as the CHAPS concentration goes down. Most important is the fact that the monomer-sized protein separated from the aggregated material on this column has a half-life that is greatly extended. This protein is still all monomer after 24 hours, even in CHAPS-free buffer containing only 5 mM tris or tris-maleate with 50 mM sodium chloride at pH 7.5. This demonstrates that there is sufficient time to concentrate the protein and get it into hanging drop crystallization experiments under a wide variety of buffer conditions.

Another problem we faced with obtaining LBP suitable for crystallization was concentrating to high enough concentrations without losing most of the protein to precipitation or binding to the molecular separation membrane. Whatever method is used, it is difficult to concentrate LBP much higher than 6 to 8 mg/ml without inducing precipitation. However, many proteins have been crystallized near this concentration range, so it is not anticipated that this will hamper crystallization efforts. We have identified two methods that minimize the loss of protein, and these will be used to concentrate LBP to around 6 mg/ml, as determined by Bradford assay with BSA standards. The first method that works is hydro-extraction through a dialysis membrane with PEG. Though this method is very gentle and very effective, it can not be used immediately before entering into hanging drop experiments because it is possible that some small molecular weight PEG molecules (the nominal molecular weight of PEG is an average) may pass through the dialysis tubing. This is undesirable because crystallization parameters need to be tightly controlled so that they are highly reproducible and the amount of PEG, a common precipitant, needs to be known. However, it is possible to use this method of concentration prior to the second round of purification to reduce the number of runs that must be made on the Superdex75 column, since buffer exchange also is occurring at this time. Following this purification/buffer exchange step, peak fractions containing monomer-sized protein are pooled and must be concentrated by an alternate method for subsequent crystallization experiments. After experimenting with a number of different spin concentrators, we have found that the Millipore spin columns with Biomax membranes with MWCO of 10-kDa or 30-kDa allow for fairly rapid concentration with minimal protein loss.

In summary, we can now obtain well-behaved, monomer-sized LBP in quantities sufficient for initiating a large-scale search for crystallization conditions.

We have submitted a research grant proposal to the State of Montana Competitive Research Grant Program to complete the crystal trials. If successful in generating quality crystals, we will submit an application to the NIH to solve the LBP structure by X-ray crystallography.

Discussion and Recommendations

Given the difficulties encountered with obtaining full length or C-terminal rLBP sufficiently stable and concentrated for NMR structure determination, and given the relative success in producing material suitable for X-ray crystal trials, we now feel that X-ray crystallography is likely to be the best way to solve the structure of this difficult protein. The fact that we can't keep isolated recombinant monomer unaggregated in solution for much beyond 24 hours is a serious problem for biological NMR studies. It is only a minor annoyance for crystal trials, however.

Progress on Technical Objective 1, task 3.

Experimental Methods and Procedures

Although structural work on peptide 11 is exclusively supported by our NIH award, the data it provides is used in this Army project. Therefore, relevant findings are provided here.

Information about the orientation of required amino acid sidechains in the peptide 11 template is critical to the design of active peptido-mimetics. Unfortunately, there are very few NMR crosspeaks to the required Tyr, Ile or Arg sidechains in aqueous solution. We took advantage of the fact that the relatively viscous DMSO solvent would slow down the motion of the peptide and allow for better definition of individual structure conformations in NMR experiments.

In this solvent we found a sufficient number of long range cross-peaks to allow for structure determination of the shorter YIGSR peptide. The two-dimensional NMR spectra run in DMSO provided a greatly increased number of crosspeaks, with several additional $i,i+2$ backbone NOEs over those seen in the aqueous spectra. $i,i+2$ backbone NOE crosspeaks were found for C-terminal amide to serine, arginine to glycine and serine to isoleucine. The NMR spectra suggested that a water molecule stabilizes the YIGSR peptide by interacting with the tyrosine and the serine hydroxyl residues. Considering all our data, particularly the earlier structure: function studies with numerous peptide 11 analogs³⁰, we concluded that the DMSO structure for the YIGSR peptide was likely different from the receptor bound conformation in water. This could either result from "induced fit" on binding, or from DMSO structures being inherently different from the aqueous ones.

Assumptions

We assumed that the structure of the peptides in DMSO would be similar enough to the aqueous structures to provide useful information. The validity of this assumption varies with different peptide sequences, but we suspect that the assumption may not hold for situation for peptide 11 or YIGSR. However, if the assumption is good, then there must be induced fit of the docking YIGSR sidechains upon receptor binding. Knowing that such a possibility exists may greatly facilitate optimization of YIGSR mimetics in the future.

Results

It appears that our structure: function studies have provided the most useful information to date for mimetic design. The DMSO structures for YIGSR fail to present the required sidechains in the manner expected from the earlier structure: function work. Our most important findings are that both YIGSR and its inactive analog, YIGSK, have similar conformational preferences in water, DMSO and aqueous TFE. Thus, it is almost certain that there is an induced fit of the peptide upon binding to the LBP protein.

Discussion and Recommendations

Our additional work on this objective has served to highlight the importance of the earlier structure: function studies on various peptide 11 analogs. It also highlights the importance of determining the structure of the ligand binding domain of the LBP protein. We are pursuing the NMR studies to do this on the ACS award, and we have added an X-ray crystallographic collaboration.

Progress on Technical Objective 2, tasks 4 and 5.

Experimental Methods and Procedures

Structural information from the full peptide 11 sequence is utilized because the minimal active domain, YIGSR, is too small to provide sufficient NMR crosspeaks. However, only the structure coordinates etc. for the YIGSR region are used in the drug design process. This provides a reasonably sized template for the design of non-peptide mimetics. Design of potential mimetics is carried out using the artificially intelligent program, INVENTON. The pharmacophore hypothesis provides the computer program with information as to what aspects of the peptide were likely important in recognition and binding with the LBP. The receptor (LBP) sees the shape of the peptide, and interacts with its electron density and dipolar nature. For template-based design, INVENTON uses the field of the template. Clearly, candidate structures must have chemically stable functionality. They must also survive the human digestive system to allow for oral therapy.

Now that we are working to obtain an NMR and/or X-ray structure defining the ligand binding domain of the LBP, we will also be able to utilize characteristics of the LBP binding pocket in mimetic design. This was the original way INVENTON was used. The computer algorithms design specific mimics for the detailed bound structure completely automatically using FASM, fragment assembly for construction. After all structures are ranked, individual candidates are examined and molecular dynamics simulations are performed to evaluate flexibility and the ability of the molecule to retain the desired conformation. Dr. Konopelski was responsible for evaluating the probable ease of synthesis of mimetics, and for choosing the actual synthetic approaches.

Assumptions

The main assumption in this part of the work is that the program INVENTON will design mimics which are at least as good as those designed by a human chemist. Our experience with the program working on other projects is that this is the case. Furthermore, the program is far more innovative, producing structures pharmaceutical chemists would not because of the biases in their training and professional experience.

Results

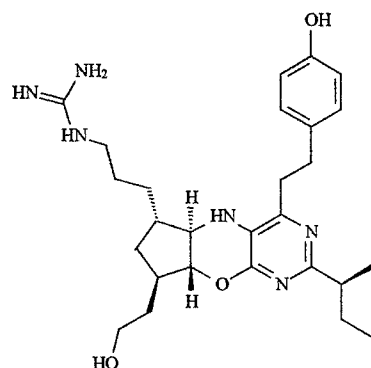
Dr. Wipke's group has been working on refining the operation of the INVENTON program, in particular attempting to automate aspects of ranking the prolific output of mimetic designs for ease of synthesis. We have experienced the problems of designing synthetic pathways for INVENTON's innovative designs. Indeed, this is the reason why this project could not be completed on time. Candidate 16 looked quite approachable and turned out to be very challenging.

Discussion and Recommendations

We have a successful path to the synthesis of the first mimetic designed by INVENTON (candidate 16). Unfortunately, the major yields in this synthesis were of the incorrect chiralities, so amounts of candidate 16 useful for preclinical testing could not be produced. Also, Dr. Konopelski has withdrawn from this project because of work pressures with his new appointment as department head. Nevertheless, we do anticipate completing the synthesis of a somewhat simpler compound where the chirality can be forced. Upon obtaining appropriate funding, this work will be completed at MSU in collaboration with members of the Chemistry Department. Once sufficient compound is available, for the preclinical work

to be completed in the Starkey lab, we will be able to ascertain us whether we are close to our goal of providing a sufficiently active lead compound, or whether major modifications will be needed.

Progress on Technical Objective 2, tasks 6, 7 and 8.



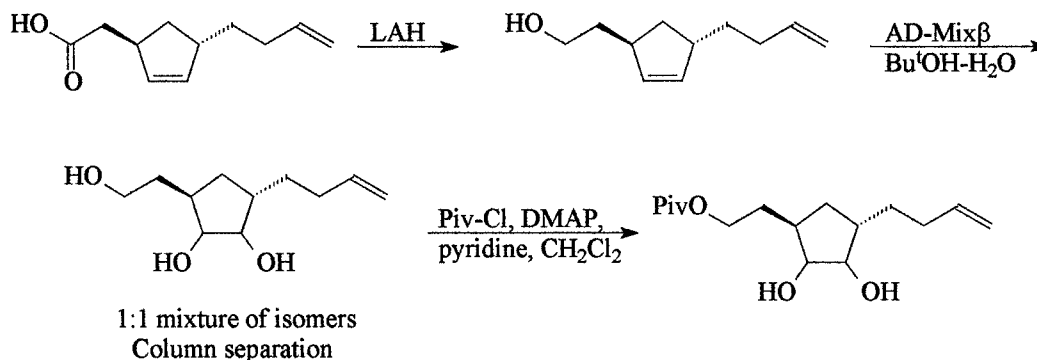
Candidate 16

The Konopelski team at UCSC continues toward the production of sufficient **candidate 16** YIGSR mimetic for preclinical testing in the Starkey lab. This compound was initially designed by the Wipke team using the INVENTON suite of programs. The synthetic work was approached from a drug discovery point of view, with the emphasis on the production of a series of structurally related compounds that can afford excellent structure/activity data after testing. The challenge of forming the tricyclic core structure has finally been met, and the last few steps to producing testable quantities of the desired

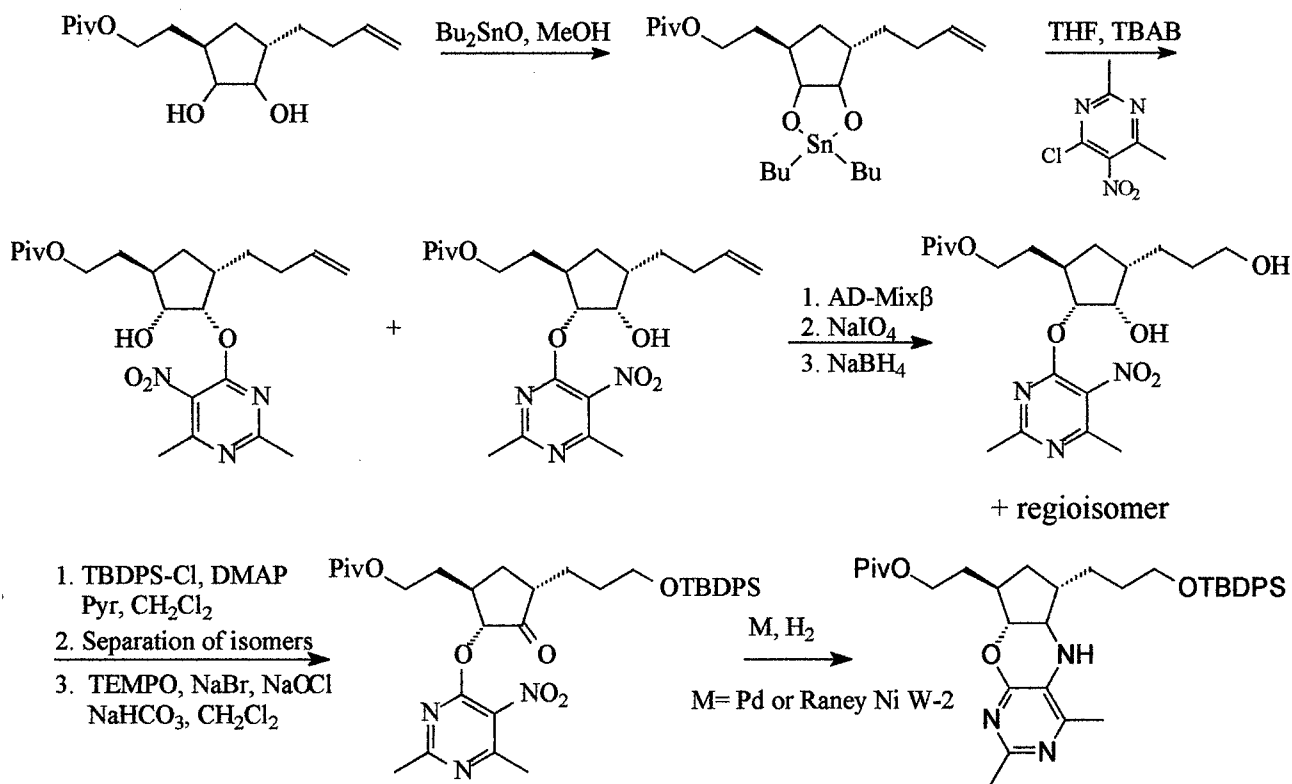
material are currently underway. INVENTON designs mimetics *de novo* without the bias resulting from the experience of a pharmaceutical chemist. Judging the ease of synthesis of such compounds is problematical even for a very experienced person. Candidate 16 proved to be surprisingly challenging, and, as indicated in the earlier reports, the first approaches did not work. Below is an outline of the successful synthetic design.

Synthesis of the Carbocyclic Unit

The cyclopentane ring of the desired compound derives from a known compound. Dihydroxylation affords a 1:1 mixture of isomers, which can be separated by column chromatography.

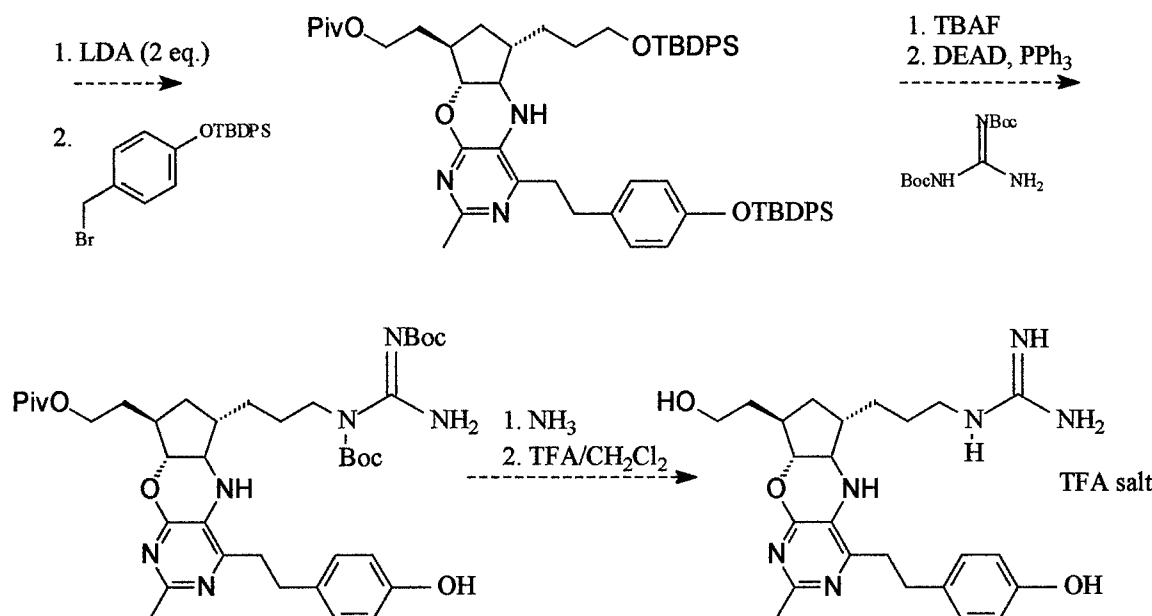


Coupling to the Pyrimidine Unit and Formation of the Tricyclic Core



Independently, the isomers are reacted with a pyrimidine unit through the formation of a tin ketal, which renders a bound oxygen nucleophilic. The reaction affords a mixture of regioisomers which cannot be separated without further equilibration. After much experimentation, a suitable procedure for elaboration of the alkene side chain to the alcohol of desired length and formation of the cyclopentanone group was discovered. Further experimentation was required to obtain the desired selectivity in reduction of the nitro group of the pyrimidine ring to the corresponding amine. This reaction is accompanied by condensation of the amine with the ketone to afford the imine, which is reduced to the desired morpholine ring.

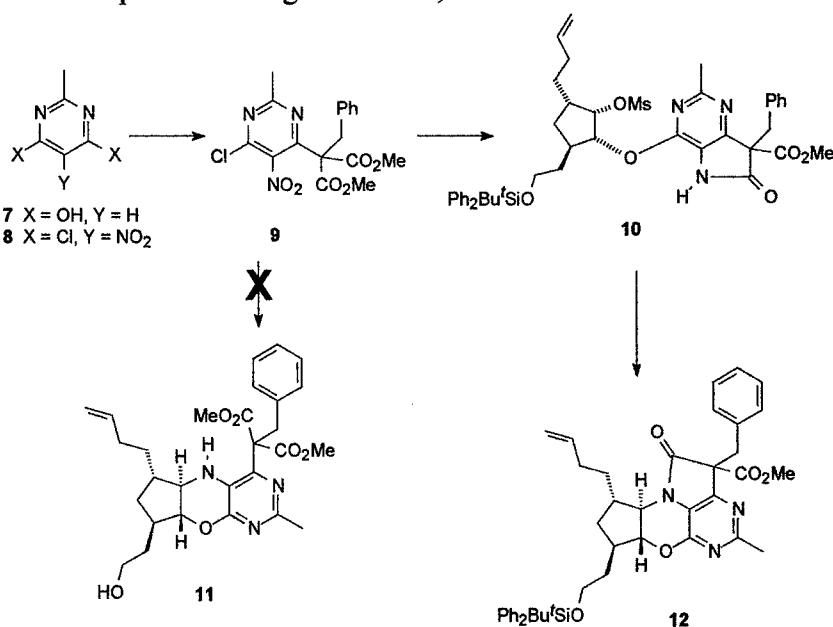
Final Elaboration to the Desired Product



Synthesis of Candidate 16 is completed by introduction of the tyrosine side chain and elaboration of the arginine side chain.

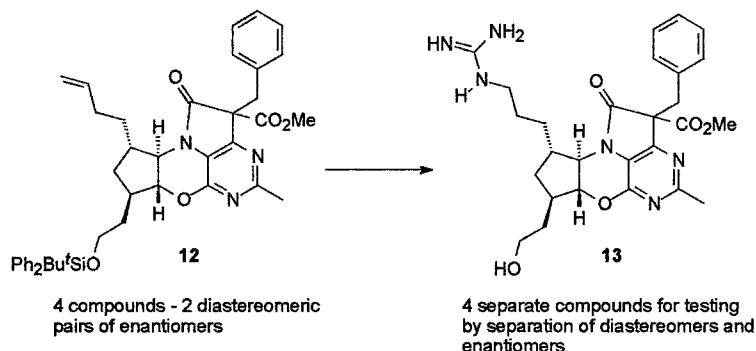
Synthesis of a Series of Structurally Related Compounds to Refine the Optimal Geometry for Anti-invasive Activity

Results from the Starkey lab suggested that the isoleucine side chain may act to reduce the conformational flexibility of the tyrosine aromatic ring, thereby positioning the phenol functionality in the proper space for binding. Given that **candidate 16** provides a rigid scaffold, the isoleucine side chain is not strictly required. Therefore, we prepared our first compound for testing from commercially available **7** as indicated in the diagram to the left. Mesylate formation and reduction of the nitro group affords lactam **10** as a separable mixture of diastereomers rather than tricycle **11**. Treatment of **10** with base affords tetracyclic **12**, which has the desired scaffold in place *and holds the tyrosine side chain more rigidly than does candidate 16*. Removal of the protective groups and elaboration of the guanidine functionality will afford one of



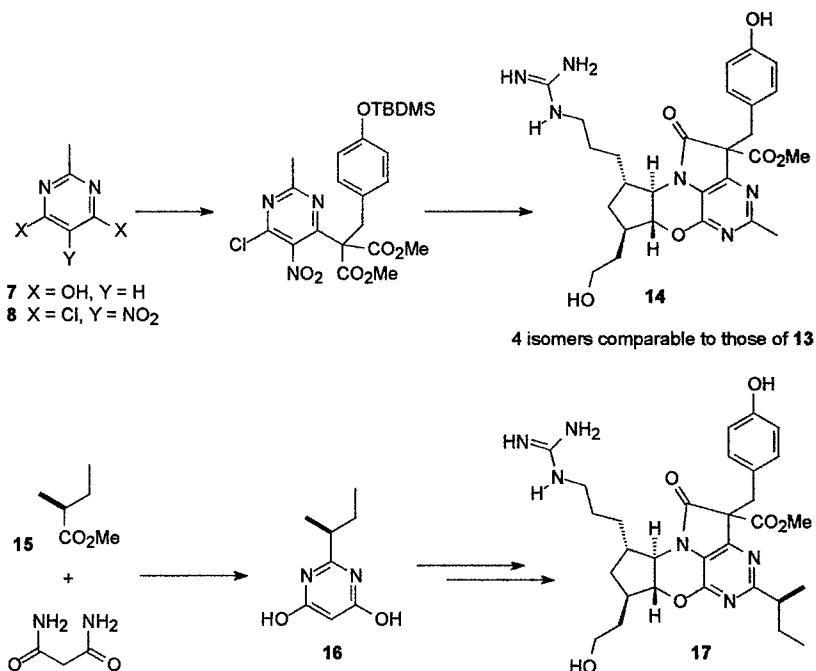
the first compounds to be tested in the Starkey laboratory.

With the synthesis of compound **12**, the route to a variety of materials based on the **candidate 16** structure is secure. Our plan is to generate a variety of closely related compounds in quick succession so as to produce a body of testing data. These data will then be evaluated to provide insights into structure-activity relationships and guide our future synthetic efforts to even more active compounds.



Our plan calls for completion of the synthesis of **13** from **12**. This transformation will lead to four compounds, since **12** is actually a mixture of 2 diastereomers, each of which is a mixture of enantiomers. The diastereomers of **12** are easily separable by simple column chromatography, so each isomer is individually taken on to tetracycle **12**.

A key compound is **14**, in which the phenyl ring will be replaced by a phenol to afford a true tyrosine-type functionality. Compound **17** possesses the complete side chain array of **candidate 16**, but in addition has the lactam and ester. We have not attempted to remove these extraneous groups since the lactam brings two additional benefits to the design of **candidate 16**; namely, the extra rigidity in the tyrosine side chain and the presence of an additional chiral center. The ability to separate the diastereomers generated by this new chiral center will allow for a fine-tuning of the phenol position in a way that is not possible with the more mobile ethylene chain of **candidate 16**.



Assumptions

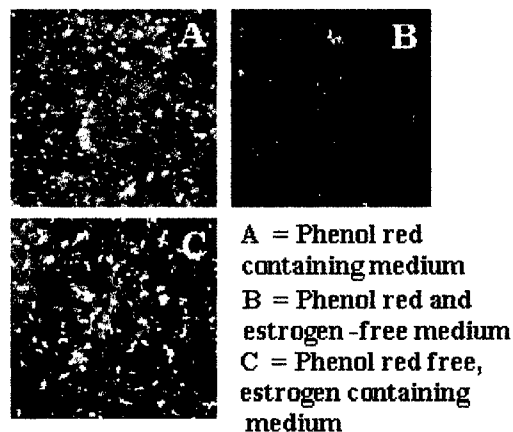
None

Results

Although a successful synthetic design was produced for Candidate 16, its poor efficiency precluded further work on this compound. We plan to complete synthesis of a simpler compound in the future. As soon as we have sufficient material, this mimetic will go into preclinical testing against human breast cancer cell lines in the Starkey lab. This will include anti-invasive and anti-metastatic testing in SCID mice. The process of patenting the design methodology, Candidate 16, and related compounds has been initiated. This is required for any further drug development.

Discussion and Recommendations

Computer *de novo* design of mimetic structures has one major uncertainty. That is: ranking the ease of synthesis of a very large number of candidate compounds. In fact, this is the most active area of research for the INVENTON program at this time. Candidate 16 looked readily accessible, but proved to be a major challenge. However, it should be noted that our very small collaborative group was attempting to synthesize a mimetic compound where a drug company would have used many more people working in parallel. It is significant that we succeeded in synthesizing our target compound at a fraction of the cost which traditional approaches would have incurred. It took us much longer than we anticipated, and almost all of the preclinical testing will happen after the DOD support for this project has expired. Nevertheless, as an idea project, we are happy with the outcome: a successful design for the synthesis of Candidate 16 was found and a number of structurally related compounds were synthesized.



Progress on Technical Objective 3, task 9.

Experimental Methods and Procedures

Anti-invasive activity of peptide 11 and its analogs/mimetics is tested using an in vitro two-chamber "Transwell" assay. An 8 μ m pore size polycarbonate filter separating the upper and lower chambers of a 6.5 mm Transwell (Costar) is impregnated with a 1:20 dilution of Matrigel. 5×10^4 tumor cells are added to the upper well in 0.2 ml complete medium, and 0.8 ml complete medium was added to

Figure 9. Confocal micrograph of shed LBP, bound to matrix laminin-1 and visualized using avidin-FITC. Laminin-1 substrate was exposed to 24 hour conditioned medium from CHO cells.

the lower well, and medium was changed daily. Incubation of the "Transwells" is continued for 3 days and cells invading into the bottom well are quantitated using the colored MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) metabolite. The lung colony assay is used to evaluate anti-metastatic activity. Tumor cells are harvested from subconfluent cultures with minimal trypsin/EDTA exposure. Prior to injection, mice are warmed at 37°C for 30 minutes. A set number of monodispersed tumor cells are injected per mouse in 0.2 ml via the lateral tail vein. Where the experiment requires co-injection of tumor cells with peptide, cells are prepared as indicated above. One mg peptide dissolved in the injection buffer is first loaded in 0.1 ml into the syringe, then the aliquot of tumor cells added in a further 0.1 ml. The contents of the syringe are gently mixed by inversion and injected as described above. The mice are killed several weeks later and autopsied. All tissues with suspect tumor colonies are rinsed in PBS and fixed in Bouin's fixative for gross and histological examination. The number of superficial nodules in the Bouin's-fixed tissues are determined using a dissecting microscope.

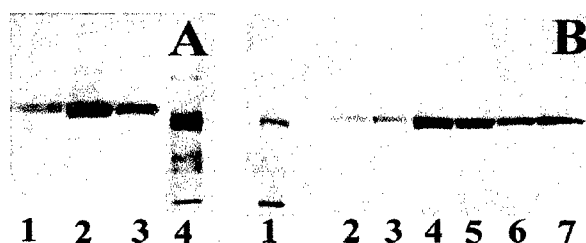


Figure 10. Silver stained SDS-PAGE gel of shed LBP affinity isolated from 3ml MCF-7 (A) and OVCAR (B) cell conditioned medium. Lanes 1A,3 B= 10- 7M, lanes 2A,4 B= 10-8M and lanes 3A,5B = 10-9 M 17 β estradiol.

While we have conducted most of our preliminary work with standard rodent test cell lines, we are now working exclusively with human breast cancer cell lines. For the MDA-MD-453 and MDA-MD-231 cell lines, *SCID* mice are employed for the experimental metastasis assays. We selected variants of both the MDA-MD-453 and MDA-MD-231 cells by serial lung colony development in *SCID* mice. The selected variants reproducibly develop high numbers of lung metastases after intravenous injection. Both of these lines are estrogen receptor (ER) negative. We have also subjected the ER +ve human breast cancer cell line, T47D, to selection for a more aggressive phenotype by a combination of *in vivo* passage and selection for the ability to invade through Matrigel basement membrane matrix *in vitro*. Success of selection for a more invasive phenotype *in vitro* was obvious from the time to first observation of invaded cells in the transwell assay. For serially selected T47D cells, this time shorted from 10 days to 4 days to 2 days for the three rounds of selection. For T47D cells serially passaged three times in the mammary fat pad of *SCID* mice, invaded cells were first seen at 2 days, and their frequency was higher than the cells only selected *in vitro*. The ER status of the selected cells is determined. So long as the cells are still ER +ve, they should provide useful variants for the preclinical studies. Since we have determined that shedding of the LBP molecule is estrogen responsive (Figs 9,10), it is important to test mimetics against both ER +ve and ER -ve breast cancer cell lines.

Assumptions

The general, and unavoidable, assumption made with the animal experiments is that human breast

cancer cells will behave in *SCID* mice in the same way as they do in the human patient. There is also an assumption made that the "Transwell" *in vitro* invasion assay will reasonably predict anti-metastatic activity. After many years of experience with both assays, we are confident that the two assays give roughly parallel results for the type of work being done on this project. Certainly, the *in vitro* assay is a useful screen before going into animal experiments.

Results

The NMR experiments, carried out under our NIH award, to determine the active conformation of peptide 11 utilized Tr-NOESY (Transferred Nuclear Overhauser Effect Spectroscopy) experiments where the peptide interacted with purified LBP. Dithiothreitol is used to prevent dimerization of free peptide 11, but was not used in the presence of the LBP which has an internal disulfide bond. Detailed examination of Tr-NOESY spectra revealed that the bound conformation of peptide 11 was dominated by a structure which could not be distinguished from synthetic peptide 11 disulfide dimer. Since peptide 11 can spontaneously dimerize under the conditions used in these NMR experiments, the presence of peptide 11 dimer was initially viewed simply as an unwanted complication.

N-acetyl-peptide 11 was found to dimerize very much more slowly than non acetylated peptide 11 in the absence of dithiothreitol (7-10 days compared with 6-7 hours for peptide 11 at room temperature at pH = 5.0). Surprisingly, N-acetyl-peptide 11 dimerized rapidly in the presence of the 67 kDa LBP. Rapid dimerization occurred when the N-acetyl-peptide 11 was present in 32 fold excess of concentration over the LBP, and the rate of dimerization was found to increase with increasing concentration of the LBP. Enzymatic activity of the 67 kDa LBP was suspected, and after appropriate controls were carried out, this was confirmed. Thus, the 67 kDa LBP appears to have a sulfhydryl oxidase activity. This activity was demonstrated for shed LBP isolated from conditioned tissue culture medium, for LBP isolated from EHS basement membrane matrix and for recombinant protein expressed in *E. Coli*. We have succeeded in demonstrating that LBP can facilitate the refolding of denatured RNAase A (Fig 11), but inhibits the reverse reaction of reducing and denaturing insulin. The sulfhydryl oxidase activity is most pronounced for the shed LBP.

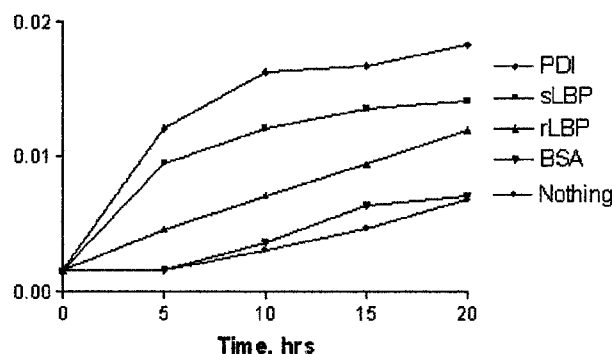


Figure 11. RNAase A refolding assay for sulfhydryl oxidase activity. RNAase activity assayed for 20 hours of refolding using 2':3'-cyclic cytidine monophosphate

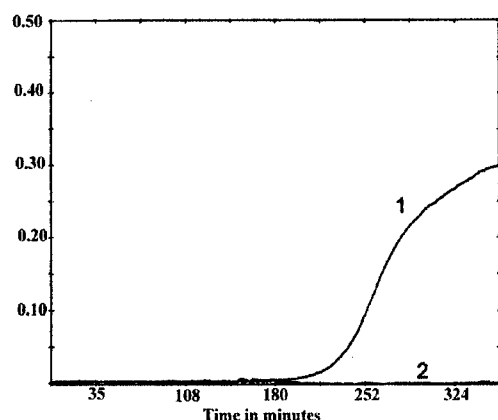


Figure 12. DTT driven Insulin reduction. 1 – no sLBP present. 2 – in the presence of 1.5 μ M sLBP

Since the two Cys residues in the LBP protein are separated by 13 residues, there is no canonical CXXC motif as would be expected for a classical protein disulphide isomerase. Also, as indicated above, we could not demonstrate any induced disulphide reduction attributable to the LBP protein. Therefore, LBP does not appear to be a protein disulphide isomerase. The mechanism of the sulhydryl oxidase activity is unclear. Therefore, we mutated the Cys residues in rLBP to Ala, both singly and together, and examined the effects on RNAase A refolding. No major differences in activity were found. All three mutant proteins were somewhat

less stable than the wild type. It may be that loss of the disulphide bond which is known to be present in the LBP molecule changes the conformation of the protein sufficiently to reduce the enzymatic activity.

Our current theory is that shed LBP could facilitate tumor cell invasion. Preliminary experiments, quantitating invasion of DG44CHO cells through basement membrane matrix *in vitro*, revealed a significant increase of invasion over three days for matrix pretreated with 67 kDa LBP (Figure 13.). This work has been expanded to ovarian cancer cell lines where similar results were found (Figure 14) Given this preliminary data indicating that dimerization of peptide 11 might be involved in the bioactivities of the peptide, we compared the bioactivities of peptide 11, a non-oxidizable monomer of peptide 11 containing an Ac-m protected cysteine residue and peptide 11 dimer. The disulfide dimer was slightly more active *in vitro* (anti-invasion) and *in vivo* (anti-metastatic) compared to peptide 11, but significantly more active than the Ac-m protected peptide 11 monomer (Table 1., Table 2.). The Ac-m protected monomer was the least active in each case.

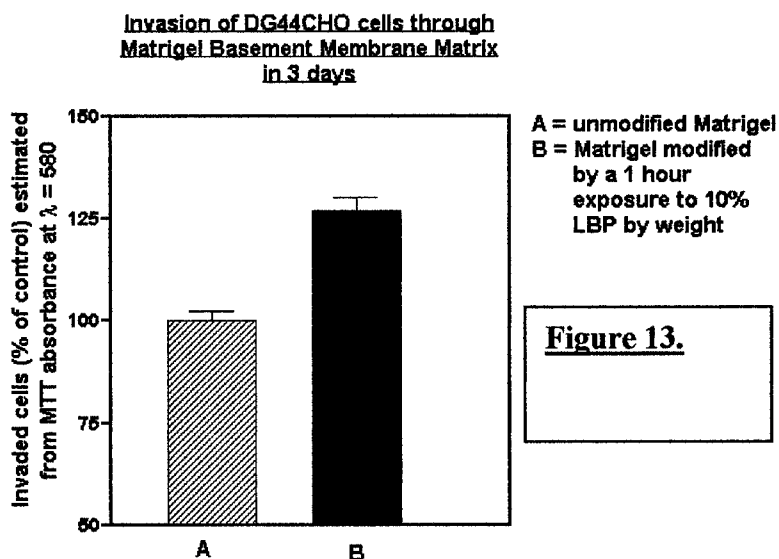


Figure 13.

**Invasion of Matrigel Basement Membrane by
NIHOVCAR Cells in one week**

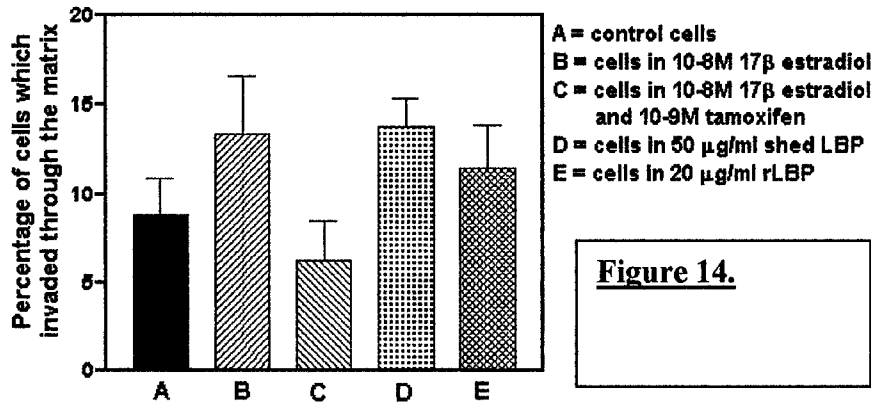


Figure 14.

Table 1. - Effect of Monomeric and Dimeric Peptide 11 on CHODG44 Cell Invasion of Matrigel Basement Membrane Assayed using the In Vitro Transwell Assay

Peptide ¹	% Inhibition of Invasion
Peptide 11	51.2 ± 3.6
Acm-peptide 11	11.4 ± 10.1
Peptide 11 dimer	75.7 ± 4.1

¹100μg peptide per ml medium.

²Each data point represents 6 replicates.

Table 2. - Effect of Monomeric and Dimeric Peptide 11 on Experimental Metastasis Formation by B16BL6 Cells

Peptide ¹	% Inhibition of metastasis	p value ²
None	None	-
Peptide 11	45	0.017
Acm-peptide 11	20	0.154
Peptide 11 dimer	51	0.007

¹1mg peptide used per mouse.

²Mann-Whitney U 2-tailed test. ³n=7 mice.

Discussion and Recommendations

While any experimental data shedding light on the possible mechanisms of action of the 67 kDa LBP in facilitating tumor cell invasion and metastasis is very welcome, the apparent disulfide isomerase enzymatic activity of the protein does complicate the structural studies being carried out on the NIH award. Fortunately, the dimer is symmetrical, so all our previous work is valid. Since the minimal active sequence of peptide 11, YIGSR, is just as active as peptide 11 itself, YIGSR is still viewed as the most appropriate template for drug design. We expect to be able to elucidate why this short sequence is so active when we have determined the NMR structure of the LBP ligand binding domain. Most likely, the longer peptide requires interactions with the LBP at the N-terminal Cys residue as well as docking via the Tyr and Arg sidechains. The smaller YIGSR peptide may be able to fit into the binding pocket without the Cys interaction.

Progress on Technical Objective 3, task 10.

Work on this task will commence with the receipt of a suitable mimetic by the Starkey lab. *The University of California, Santa Cruz has started the process of patenting candidate 16 and related compounds so that further development, which would require the involvement of a drug company, can go forward. UCSC does not usually initiate patenting without financial input from an interested commercial concern, so the action is considered an unusually aggressive move on the part of the university.*

Conclusions

Several advances have been made in the past year which encompass the work aimed at elucidating the structure of the ligand binding domain of the 67 kDa LBP. Although difficulties with our isolation procedures for ligand derivatized LBP slowed down the studies on identifying the contact residues for peptide 11 in the ligand binding domain, the alternative approach of using phage display studies to pinpoint regions of interacting sequence worked very well. The phage display studies indicated that three separate sequence domains in the C-terminal domain of the LBP could interact with peptide 11. The phage display studies make a good start to mapping which LBP residues take part in peptide 11 binding.

In order to undertake our NMR studies of the structure of the ligand binding domain of the LBP at reasonable cost, the binding domain needed to be expressed in a recombinant bacterial system. We have accomplished this both for the full length protein and a number of fragments some of which include the ligand binding domain. In most cases, the *E. coli* strain expresses large amounts of poly-His tagged protein which is readily purified over a Ni affinity column. The poly-His tag is easily removed using thrombin cleavage, and the molecular weight of the expression products were confirmed by mass spectrometry. As judged by circular dichroism studies, the recombinant proteins refolded well. While the full length and fragments encompassing the N-terminal 2/3 of the molecule are well folded, the fragment encompassing the C-terminal 2/3 is not. This precludes using it to determine the structure of the full ligand binding domain by NMR techniques. Although the full length rLBP is well folded, it is only stable

as a monomer in solution for 24-36 hours. Thus, the full length rLBP is also impractical for NMR structure determination. On the other hand, we have succeeded in making sufficient well behaved full length rLBP for X-ray crystallization trials. The relatively short time during which the monomer is stable in solution is only a minor nuisance for X-ray structure determination. We have submitted a grant to the State of Montana Competitive Research Grant Program to fund the Crystallization trials. If we obtain quality crystals, we will submit an application to the NIH to complete the structure.

The synthesis of candidate 16, which was designed by one of the early runs of the artificially intelligent INVENTON program, has been completed, but the yield was too small for us to undertake bioactivity testing. Dr. Konopelski has withdrawn from the collaboration due to the additional work associated with his appointment as department head. Therefore, in collaboration with members of the MSU Chemistry Department, we are planning to synthesize a simpler mimetic whose chirality can be forced. We will use this alternate mimetic for bioactivity testing. The results of these tests are very important, as they will indicate if the designed mimics are close to a potential drug lead, or if major modification to the INVENTON pharmacophore hypothesis will be needed. Refinement of the INVENTON input would also come from our X-ray studies on the structure of the ligand binding domain of the LBP. Future success with generating an X-ray structure would allow us to infiltrate the crystal with YIGSR in order to ascertain how YIGSR in fact binds to the LBP. Since we have shown the preferred solution structures for YIGSR and the inactive YIGSK are exceedingly close, there must be induced conformational changes on binding.

Finally, because some of our initial experiments indicated that the 67 kDa LBP had a protein disulfide isomerase activity and could facilitate dimerization of peptide 11 analogs, we compared the anti-invasive and anti-metastatic activity of peptide 11, a non oxidizable AcM protected analog of peptide 11 and peptide 11 dimer. The most active species was the dimer, with the AcM protected monomer being the least active in each case. Interaction with the LBP via the N-terminal Cys residue is likely to be important to ligand binding of the longer peptide 11, while it appears not to be so for the short, very active, YIGSR peptide. Any structure which we derive for the "binding pocket" of the LBP will need to be compatible with these results. Along with requirements for disulfide isomerase activity, this consideration could provide some very useful constraints when we come to evaluate our future NMR derived structural data for the "binding pocket".

The LBP is well known to be a very difficult protein to work with. It has lived up to this reputation, and we have frequently been forced to change our approaches. However, the IDEA award has allowed us to do this and we are now very confident that the original goals of the project can be achieved albeit using largely alternate technologies.

Key Research Accomplishments

- **Successfully identified regions in the LBP protein which interact with peptide 11 using phage display and limited proteolysis experiments.**

- **Successfully expressed full length LBP, the ligand binding domain, and GB1 fusion domains of the LBP in E. Coli.**
- **Successfully labelled rLBP with heavy isotopes for NMR spectroscopy.**
- **Determined which recombinant LBP domains give well dispersed preliminary NMR spectra, and so can be used for structure determination.**
- **Determined that structure determination of rLBP by X-ray crystallography is practical, and initiated this work.**
- **Determined that there must be an induced conformational change in YIGSR on binding to the LBP**
- **Determined a synthetic approach for Candidate 16.**
- **Synthesized a number of close structural relatives to Candidate 16 which will be useful for structure:activity studies to determine the optimal geometry for bioactivity.**
- **Selected variants of the MDA-MB-435 and MDA-MB-231 ER-ve human breast cancer cell lines which reproducibly produce good numbers of experimental lung colonies in *SCID* mice.**
- **Selected more invasive variants of the ER+ve T47D human breast cancer cell line to facilitate preclinical testing of mimetics.**
- **Determined that cell shedding of the LBP is sensitive to estrogen levels in ER+ve breast and ovarian cancer cell lines, and that this may be related to the ability of shed LBP to facilitate invasion *in vitro*.**
- **Determined that the LBP has a sulfhydryl oxidase activity, and that dimerization of peptide 11 may be related to its bioactivity.**
- **Initiated the process of patenting candidate 16.**

Reportable Outcomes:

1. Three manuscripts have been published and a fourth is *in preparation* at this time.

Kazmin, D.A., Hoyt, T.R., Taubner, L., Teintze, M., and Starkey, J.R. Phage Display Mapping for Peptide 11 -Sensitive Sequences Binding to Laminin-1. *J. Mol. Biol.* 298:431-445, 2000.

Starkey, J.R., Uthayakumar, S., Berglund, D.L. Cell surface and substrate distribution of the 67-kDa laminin binding protein determined by using a ligand photoaffinity probe. *Cytometry* 35:37-47, 1999.

Starkey, J.R., Dai, S., Dratz, E.A. Sidechain and backbone requirements for anti-invasive activity of laminin peptide 11. *Biochim. Biophys. Acta* 1429:187-207, 1998.

2. One presentation was given at the 98 AACR annual meeting, a presentation was given at the 2000 "Era of Hope" meeting, and two invited seminars were given (Chemistry Department, University of California, Santa Cruz; NIH, NIAID Hamilton, MT).
3. A new grant, based on work started under this award, has been obtained by Dr. Copié from the American Cancer Society. One other research grant application is pending.

Title = NMR Structure Determination of the Ligand-binding Domain of the 67 kDa Laminin Binding Protein
Agency = ACS
2 year award which activated Jan 1, 2000
Total award = \$380,000

4. One of the predoctoral students working on this project has received a \$30,000 dissertation award from the Komen Foundation.
5. Three predoctoral students, and one postdoctoral fellow, have worked on this project.

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DEPARTMENT OF THE ARMY
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REPLY TO
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21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information
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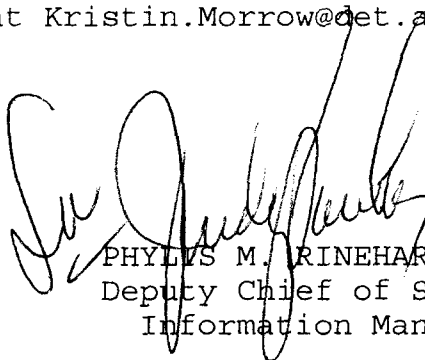
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