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PRINCIPAL INVESTIGATOR: Lori A. DeFreest
James A. Bennett, Ph.D.

CONTRACTING ORGANIZATION: Albany Medical College
Albany, New York 12208

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Lori A. DeFreest
James A. Bennett, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Albany Medical College
Albany, New York 12208

E-Mail: bennetj@mail.amc.edu

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We have synthesized a nine amino acid peptide (COP) that inhibits the growth of ER+ human breast. COP does not act like Tam or any other known agent currently used to treat ER+ breast cancer. Understanding the process by which COP mediates its inhibitory activity is extremely important for the development of this drug or other drugs which exploit the same biochemical pathways to arrest breast cancer growth. A logical first step in this process is to identify the receptor for COP. We have developed and optimized an affinity chromatography procedure to isolate cellular proteins which bind to COP by linking COP to an Affi Gel 10 column. After passing detergent-solubilized cells through the column, retained proteins are sequentially eluted, separated by SDS-PAGE, and visualized by Coomassie staining. To date, a 70 kDa protein has been consistently isolated in this manner, but has not been shown to be specific. Strategies have been identified to address this specificity issue. Binding proteins of interest including the 70 kDa protein described above will be identified and characterized by mass spectrometry, amino acid analysis, and sequence comparison to the known protein databases.

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1. Introduction:

We have synthesized a peptide that inhibits the growth of ER+ human breast cancers growing as xenografts in immune-deficient mice, including those that are resistant to tamoxifen (Tam) (1). This nine amino acid peptide, COP, is derived from alpha-fetoprotein (AFP) which itself has anti-estrotrophic and anti-breast cancer activity (2). It has been shown that the peptide does not act like Tam or any other known agent currently used to treat ER+ breast cancer (1;2) Although the anti-breast cancer activity of COP and its precursors is well-documented (1;3;4), neither the mechanism of its action nor that of AFP has been fully elucidated (1;2).

Both COP and its parent molecule AFP would not be expected to freely cross the plasma membrane because of their charge and size. Cell surface receptors have been isolated and characterized for peptide molecules such as oxytocin, vasopressin, and somatostatin and its analogs (5-7), and for proteins such as insulin and insulin-like growth factor (8). The receptors for these molecules act as mediators of signal transduction (5-7;9). Therefore it is reasonable to assume that this peptide interacts with (a) cell surface receptor(s) to mediate its anti-breast cancer activity. While it is possible that the AFP-derived peptide will interact with a receptor for AFP, this remains to be determined. Receptors for AFP have been studied in various undifferentiated cell lines, but have not been completely characterized and no sequence information is available. Isolation and characterization of the receptor for COP will elucidate the first step in the mechanism of anti-oncotic action of this peptide. The peptide has been shown to inhibit estrogen-dependent growth of ER+ human breast cancers and, in preliminary studies, to interfere with a phosphorylation step in the activation of the ER (Bennett and Lin, personal communication). Taken together this suggests that the peptide interferes with the signal transduction mechanism beginning at the cell surface that cooperates in ER activation in response to estrogen treatment but the intersection of these internal signals is not known.

During the first year of this training grant, we have synthesized the peptides for use in the affinity chromatography procedure to isolate proteins that have an affinity for the AFP-derived peptides, linked these peptides to an affinity column matrix, and optimized the procedure for isolation of these proteins. The peptides synthesized include COPK, a cyclized 9 amino acid peptide with a lysine incorporated to link the molecule to the affinity column matrix; AFPep, a synthetic peptide which is the minimal sequence of AFP that demonstrates anti-oncotic activity; Scrambled AFPep, 8 amino acid peptides comprised of the same amino acids as AFPep but in an order that disrupts what we believe to be the pharmacophore; AFPep and Scrambled AFPep with an N-terminal linker; and AFPep with D-valine, an analog of AFPep with a substitution of D-valine for L-valine which exhibits no biological activity. All of these peptides have been linked to the affinity column matrix and used to isolate protein(s) which have an affinity for them or were used concurrently as control columns. During the report period, the affinity chromatography procedure has been optimized to increase sensitivity and to stabilize the interaction between the binding protein(s) and their ligand. The electrophoretic conditions have also been optimized. In addition, the procedure to elute retained protein(s) has been modified to elute highly retained proteins by using sequential elution with high salt followed by a chaotrophic agent. Furthermore, the specificity of the proteins that have been isolated have been evaluated using blocking experiments and comparison to retention by control columns to which are coupled biologically-inactive peptides. In addition, work to determine the pharmacophore of the peptide has progressed and has culminated in an abstract and manuscript entitled "Synthetic peptide derived from alpha-fetoprotein inhibits growth of human breast cancer: Identification of the pharmacophore (working title)."

2. Body:

Training: During the first year of this training grant award, Lori DeFreest, the recipient of the award has completed a number of tasks that are required for attaining the Ph.D. degree. She has completed all required didactic coursework including Signal Transduction, Techniques in Molecular Biology, Tissue Remodeling and Cell Motility. She has successfully defended her Proposal in front of an Oral Examination Committee and therefore will be awarded her M.S. at graduation this May. Ms. DeFreest has first authorship on one abstract, co-authorship on two abstracts, and is preparing a manuscript for publication entitled "Synthetic peptide derived from alpha-fetoprotein inhibits growth of human breast cancer: Identification of the pharmacophore." She has received an Inglenook Scholar-in-Training Award to be utilized for traveling expenses to attend the 2003 Annual Meeting of the American Association for Cancer Research where she will present a poster highlighting analogs of the AFP-derived peptide. She is an Associate Member of this organization. In addition, Ms. DeFreest has been awarded a grant from the Susan G. Komen Breast Cancer Foundation in the amount of $30,000 over two years to provide funds for supplies and travel monies (no stipend or tuition support) for her project entitled "Identification of the Cellular Binding Sites for a Novel Human Anti-Breast Cancer Peptide."

Ms. DeFreest has completed four intramural presentations during this funding period. These included presentations at two thesis committee meetings, a departmental colloqium, and a poster at the annual Student poster day. Her poster entitled "Identification of Cellular Binding Sites for a Novel Human Anti-Breast Cancer Peptide" was selected as among the six best presented and consequently she was selected to present a talk on her research at the Albany Medical College Awards Day and was awarded the Dean's Certificate of Excellence in Research. In addition, she has presented journal articles pertinent to cancer research at two journal club meetings.

Ms. DeFreest has completed her second year as student representative to the Graduate Studies Operating Committee, a group that oversees admission of graduate students and graduate college policies. She has also completed her second year as a representative to the Honor Committee. This student-lead organization hears cases of unethical and unprofessional behavior by students and makes recommendations for remediation to the Deans Committee of Albany Medical College. During the past year, she has also been instrumental in the laboratory training of three undergraduates.

Research: Isolation of COP-specific Cell-Membrane Protein(s)

A. Prepare/Evaluate Peptides for Affinity Chromatography: During the past year Ms. DeFreest has spent a substantial amount of time synthesizing peptides to be utilized for this project. Peptides were prepared by Fmoc solid phase synthesis and were evaluated for biological activity in a screening assay of estrogen-stimulated growth of immature mouse uterus (1,2). Only those peptides which showed the expected activity were utilized to prepare affinity chromatography columns. Seven different peptides were used to prepare affinity chromatography columns during this period and this represents 11 separate peptide syntheses. In addition, three other batches were used in blocking and peptide elution experiments to evaluate binding specificity. Three AFPep analogs were also synthesized during this period as a continuation of work performed prior to the award period. This work was designed to elucidate the pharmacophore of the peptide. Determination of the pharmacophore of the AFP-derived peptides was essential so that they could be linked to the Affi-Gel 10 column matrix with this binding site exposed. The peptides synthesized for the affinity chromatography and pharmacophore experiments are shown in Table 1.

Peptides were linked to the Affi-Gel 10 column matrix using the procedure of the manufacturer (Bio-Rad). After coupling, any active sites on the matrix were blocked by treatment with 1M ethanolamine. Additional control columns were prepared which had matrix treated only with 1 M ethanolamine in excess (no peptide) to block all reactive sites, i.e. blank columns. To examine the coupling efficiency, COPK and blank columns were freshly prepared, but not blocked with

ethanolamine as had been done previously so that there would be no interference from this reagent in the subsequent amino acid or Kaiser analyses. The coupling procedure was examined by three methods.

Peptide	Sequence	No. of Batches	Use
COPK	*cyclo*[EKTOVNOGQ]	2	Affinity Column
COP-Orn	*cyclo*[E-Orn-TOVNOGQ]	1	Affinity Column
AFPep	EMTOVNOG	1	Affinity Column
Scrambled AFPep	OTNEVGMO	2	Affinity Control Column
AFPep with Linker	KA_4EMTOVNOG	3	Affinity Column
Scrambled AFPep with Linker	KA_4OTNEVGMO	1	Affinity Control Column
AFPep with D-valine	EMTO(d-V)NOG	1	Affinity Control Column
Scrambled AFPep	OMGVENTO	1	Blocking
AFPep	EMTOVNOG	1	Blocking
AFPep	EMTOVNOG	1	Elution
AFPep with A for T	EMAOVNOG	1	Pharmacophore
AFPep with V for T	EMVOVNOG	1	Pharmacophore
AFPep with S for T	EMSOVNOG	1	Pharmacophore

Table 1: Peptides synthesized during award period.

First, comparison of the HPLC chromatograms of the solutions before and after coupling indicated that the amount of peptide in the solution was reduced after the coupling reaction was at completion. Second, Kaiser analysis, a colorimetric determination of the relative amounts of free amine groups, was performed on aliquots of the peptide-coupled and blank Affi-Gel 10 which were dried and acid-hydrolyzed. The Kaiser test showed a more positive result for the peptide-coupled column than for the blank column which suggests that more free amine groups were present on the peptide column, that is, that amino acids were present. Third, amino acid analysis of the acid-hydrolyzed Affi-Gel10 column supports was performed to confirm the presence of peptide on the peptide-coupled column. Chromatograms that compare the amino acid composition of the peptide-coupled and blank columns are shown in Figure 1. The 7 amino acids that comprise COPK are all present in the hydrolyzed material from the peptide-coupled column, but are not found on that from the blank column. These tests confirmed the presence of COPK bound to the Affi-Gel.

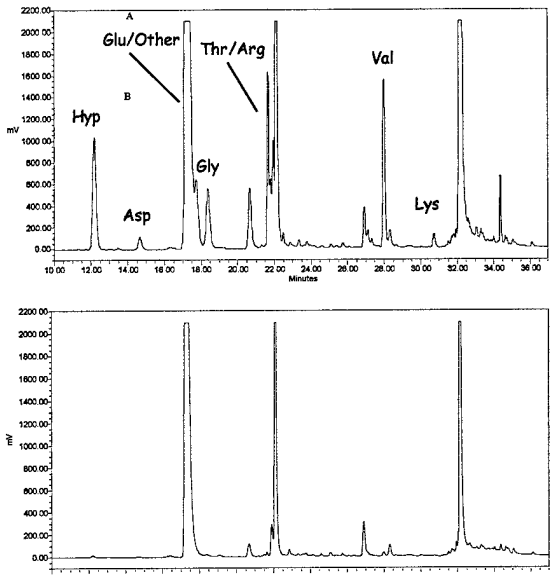

Figure 1: Comparison of chromatograms of the amino acid analysis of acid-hydrolyzed Affi-Gel 10 couple to COPK (A) or without peptide coupling (Blank) (B). Asparagine and glutamine are deaminated during hydrolysis and elute with their respective acids, Asp and Glu.

B. Solubilized Cellular Preparations, Isolation of Binding Proteins, Separation of Proteins by SDS-PAGE: The majority of Ms. DeFreest's time during the first year of this award has been devoted to optimizing a procedure to isolate cellular proteins that have an affinity for the cyclized AFP-derived peptide and its linear precursor, AFPep. MCF-7 human breast cancer cells were chosen as the cell line for these experiments because they are ER+ and have shown significant inhibition of estrogen-dependent growth in a xenograft assay when treated with AFP-derived peptides (1). The MCF-7 cells are cultured in medium containing 10% bovine calf serum (BCS) and grown to confluency (approximately 50 million cells per flask). The number of flasks of cells used for each experiment

depends on the number of affinity columns utilized. One day before the isolation procedure, the medium is removed from confluent monolayers of cells, the monolayers are washed three times with fresh serum-free medium, and the cells are then incubated overnight in fresh serum-free medium. This step is included to remove any serum albumin that is present in the 10% BCS medium. The following day, the serum-free medium is removed and replaced with a buffer containing the non-ionic detergent Triton X-100 and a protease inhibitor cocktail to inhibit degradation of the cellular proteins. The flasks are agitated for 2 hours at 4°C during which time the monolayers detach. This whole cell preparation is then homogenized, and centrifuged. Membrane and cytosolic proteins are in the supernatant. This supernatant is then passed repeatedly through the appropriate columns. The retained proteins were subsequently eluted using high salt (1 N NaCl) and the eluent was dialyzed to remove salt and concentrated against solid polyethylene glycol compound.

Initial experiments showed the presence of a prominent band at approximately 52 kDa, but subsequent work demonstrated that although the band was reproducible, it was not specific for the affinity column containing COPK and could be eluted from columns with no peptide coupled (blank) as well. An attempt was made to deplete the cell preparation of this band by use of a pre-column, but its presence was not reduced although it was shown that it was not an artifact of the procedure itself. The lack of specificity seen in the preceding experiments may have been influenced by a number of factors. Among these are inadequate stabilization of the protein/ligand (peptide) interaction during binding, incomplete elution of bound proteins using only high salt, the appropriateness of using MCF-7 cells as a source of binding proteins, and interference from protease inhibitors. Each of these issues was addressed in subsequent experiments. For the follitropin receptor, it had been shown that receptor/ligand interactions can be maintained by the addition of agents such as glycerol which aid in stabilization of the receptor protein in the presence of non-ionic detergent (10). Therefore, Triton X-100 concentration and 30% glycerol were maintained throughout the column washes. Sequential elution was performed using first 1 N sodium chloride (NaCl) followed by 3 M sodium thiocyanate (NaSCN), a chaotrophic agent which will more completely disrupt the interaction between the peptide and its binding protein(s) and allow elution of species which bind with high affinity. Eluents containing retained proteins were concentrated against solid polyethylene glycol (PEG) compound and then acetone precipitated to remove excess salt and detergent and to further concentrate the proteins. As a result of the additional concentration step, this procedure resulted in visualization of numerous bands that had not been seen previously as well as an increased amount of the non-specific 52 kDa band. However, there was no discernable difference between the eluents from the COPK and blank columns. The experiment was repeated using the serum-free supernatant removed from the MCF-7 cells after overnight incubation since previous studies had shown that cancer cells slough AFP binding proteins (11). SDS-PAGE of these eluents from the two columns produced a different banding pattern from that of the whole cell preparation, but again no bands that were specific for COPK were isolated.

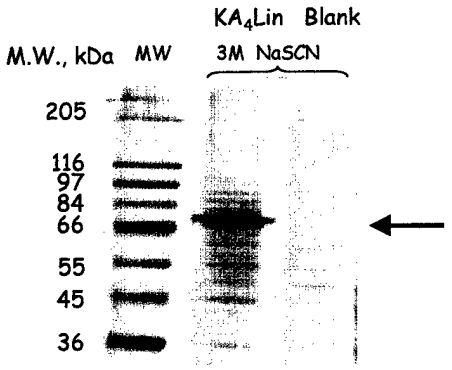

Figure 2: SDS-PAGE (10%) with 3 M NaSCN eluents from freshly-prepared KA_4AFPep and blank columns after dialysis, and concentration vs. PEG-compound, then acetone precipitation. Note the presence of a distinct band (approximately 70 kDa) that is present in the eluent from the peptide column, but not the blank column.

It is possible that the COPK column was binding to a specific protein, but that there was insufficient receptor present on the cells to be seen by SDS-PAGE against a background of non-specifically bound proteins. To ascertain if a receptor with a greater number of cellular sites could be targeted or if using a peptide with a less rigid conformation would allow for binding, a linear peptide was synthesized that contained a lysine at the N-terminus, a spacer of four alanines, and the 8 amino acids of the hydroxyproline-containing parent peptide molecule ($KA_4EMTOVNOG$, KA_4AFPep). This

molecule was linked to the Affi-Gel 10 matrix through either its N-terminus or the side chain of the terminal lysine residue using the standard procedure including ethanolamine blocking of unreacted sites on the affinity column matrix. Solubilized cell preparation was passed through the KA_4AFPep column and concurrently through a freshly-prepared blank column, the columns were washed with solubilization buffer and sequentially eluted with 1 N NaCl and 3M NaSCN as done previously. The results of this experiment are shown in Figure 2. As denoted by the arrow, the lane on the gel corresponding to 3 M NaSCN elution of the KA_4AFPep column shows the presence of a band at approximate MW of 70 kDa. Because it is retained by the column after the high salt wash and predominantly elutes with the chaotrophic agent, this suggests that it binds to the column with high affinity. This 70 kDa band is intriguing because it has not been seen as the primary band during elution previously and the MW corresponds to that of the AFP receptor isolated by Kanevsky from human breast cancer and embryonic tissues (12) and is similar to that seen by Suzuki (13) and Moro (14). The data also suggested that the binding is specific since no corresponding bands were seen in the 3 M NaSCN eluent from the blank column. This experiment was repeated with the addition of a control column which had a scrambled peptide with the KA_4 linker on the N-terminus. The 70 kDa band was again found in the 3M NaSCN eluent from the KA_4AFPep column, but was also present in the eluent from the scrambled peptide. To determine if the 70 kDa protein was being retained by the linker rather than the peptide, solubilized cellular preparations were pretreated with peptide with and without linker to block binding of the 70 kDa protein. When no pre-treatment was performed, the 70 kDa protein was evident in eluents from both the KA_4AFPep and KA_4Scrambled columns as was previously seen. When either AFPep or KA_4AFPep was used as a pre-treatment, they were able to block binding of the 70 kDa protein to the KA_4AFPep column. Pre-treatment with scrambled peptide without linker was also able to block binding of this protein to the KA_4Scrambled column. Blank columns did not retain the 70 kDa protein. These data indicate that the linker was not retaining the 70 kDa protein, but that it was instead retained by the peptide moities attached to the columns. To further evaluate the specificity of the 70 kDa protein for the peptide, an additional elution step using AFPep was performed. Columns with KA_4AFPep, KA_4Scrambled, and no peptide were sequentially eluted with first 1 N NaCl, then a buffer solution containing AFPep, and finally with 3M NaSCN. The 70 kDa protein was not eluted by the peptide solution, but was eluted by the NaSCN indicating that the protein is highly retained by the peptide columns. Blank columns did not retain any protein.

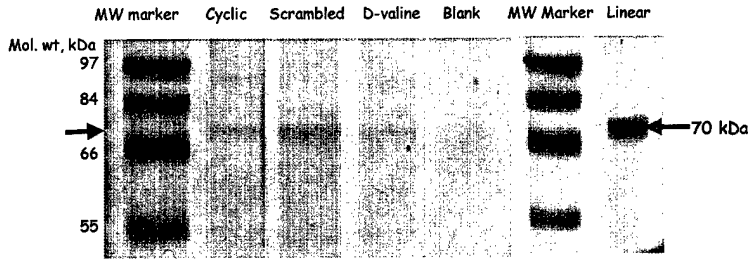

Figure 3: 70 kDa protein has been repeatedly isolated from whole cell preparations of MCF-7 human breast cancer cells. This protein binds is highly retained by columns with linear, cyclic, scrambled (OTNEVGMO), and D-valine (inactive) peptides, but not to a column to which no peptide is linked.

Finally, columns were prepared to which were linked AFPep, Scrambled AFPep, AFPep with D-valine (a peptide that shows no bioactivity), and COP-Orn (Note: the cyclized ornithine-containing peptide is functionally equivalent to COPK). A blank column was also prepared. Solubilized cellular preparations were made as previously described but 0.15N NaCl was added to reduce non-specific binding, and passed through the five columns. The retained proteins were eluted with 1 N NaCl and then with 3M NaSCN. The latter eluent was dialyzed and concentrated and the retained protein(s) separated a 10% polyacrylamide gel. This experiment was repeated with the same results shown in Figure 3. The 70 kDa band is highly retained by the AFPep, COP-Orn, Scrambled AFPep, and D-valine columns. This suggests that the 70 kDa protein is not specific for COP or AFPep because it can be retained by both active and inactive peptides including those which have a disrupted pharmacophore. Studies are underway to determine the identity of the 70 kDa protein by determining its sequence and comparing this to the known protein data bases.

Future Work

Radiolabeled peptides will be prepared for use in determining the binding affinity and capacity of the cellular binding protein(s) for the AFP-derived peptide. However, before the binding experiments will be undertaken, additional cell lines and tissues will be screened for an ability to bind to the peptide. These materials will include other ER+ human breast cancer cell lines, MCF-7 tumors grown as xenografts in SCID mice, and/or uterine tissue. Once an appropriate material has been determined, binding studies will be undertaken and the affinity chromatography procedure will be repeated. It has been suggested that adjustments to the solubilization buffer components such as use of a different non-ionic detergent, or homogenization only (without detergent) could be made which would increase the likelihood of a receptor/ligand interaction. Cellular fractionation to evaluate cytosolic and membrane preparations can be made to increase the sensitivity of the assay, and the pellet produced by the centrifugation step will be further evaluated to determine if a specific protein is present in the Triton X-100 insoluble fraction as would be the case if the receptor for the AFP-derived peptides is associated with lipid rafts. When a specific protein has been isolated, it will be sequenced by mass spectrometry and compared to the known protein databases for identification. Strategies to chemically cross-link a labeled form of the peptide to its cell surface receptor are being assessed. The peptide would be allowed to associate with whole target cells, chemically cross-linked, and then proteins would be isolated by SDS-PAGE after solublizing the cell membranes. Proteins will be visualized by autoradiography. A similar strategy has been used by Suzuki, et al. to isolate the AFP receptor (13).

3. Key Accomplishments

- Synthesized COPK and AFPep peptides with and without linker for use in affinity chromatography. Synthesized peptides for use in specificity (blocking) experiments and analogs for experiments to determine the pharmacophore.
- Designed and synthesized peptides to be used as controls for the affinity chromatography procedure. These include Scrambled AFPep and AFPep with D-valine.
- The biological activity of all peptides was evaluated using the estrogen-stimulated immature mouse uterine growth assay. Only peptides that had the anticipated biological activity were used in the affinity chromatography experiments.
- Peptides were linked to the affinity chromatography matrix using the manufacturer's procedure. Coupling was evaluated using HPLC, Kaiser test, and amino acid analysis.
- MCF-7 human breast cancer cells were cultured and the confluent monolayers solubilized in a buffer containing a non-ionic detergent. The solubilization buffer components were optimized to reduce degradation and promote stabilization of the binding protein/ligand interaction.
- The affinity chromatography procedure was further optimized to increase sensitivity and to allow for elution of highly retained proteins by the use of sequential elutions.
- Proteins retained by the affinity columns are isolated using SDS-PAGE with Coomassie Blue staining to visualize the proteins.
- Control peptides, blocking, and peptide elution experiments have been performed to evaluate the specificity of the binding proteins isolated.

4. Reportable Outcomes

- First author on an abstract for presentation at the 2003 Annual Meeting of the AACR entitled "Synthetic peptide derived from alpha-fetoprotein inhibits growth of human breast cancer: Identification of the pharmacophore."
- Co-author on two abstracts for presentation at the 2003 Annual Meeting of the AACR entitled "Molecular mechanisms of alphafetoprotein peptide-associated estrogen receptor activity and the role of mitogen activated protein kinase pathways" and "A peptide derived from the active site of alpha-fetoprotein prevents breast cancer in a rat model."

- Award of a Susan G. Komen Breast Cancer Foundation grant in the amount of $30,000 over two years to provide funds for supplies and travel monies (no stipend or tuition support) for the project entitled "Identification of Cellular Binding Sites for a Novel Human Anti-Breast Cancer Peptide."
- Received an Inglenook Scholar-in-Training Award to be utilized for travel expenses to attend the 2003 Annual Meeting of the AACR.
- A manuscript entitled "Synthetic peptide derived from alpha-fetoprotein inhibits growth of human breast cancer: Identification of the pharmacophore. (working title)" is being prepared for publication.
- Progress of this research project has been reported at a departmental colloquium and at two Ph.D. thesis committee meetings.
- This research was presented as a poster at the Albany Medical College Student Research Day. The poster entitled "Identification of Cellular Binding Sites for a Novel Human Anti-Breast Cancer Peptide" was selected as among the six best of the approximately 70 posters presented and consequently the research was presented as a talk at the Albany Medical College Awards Day and was awarded the Dean's Certificate of Excellence in Research.

5. Conclusions: The work thus far indicates that cellular preparations from MCF-7 cells contain proteins that have an affinity for the AFP-derived peptides and that the optimized affinity chromatography procedure is adequate for isolating these proteins. However, the primary protein that shows high affinity for the AFP-derived peptides does not appear to be specific in that it is retained by both active and inactive peptides including a scrambled peptide with the pharmacophore disrupted. We have identified strategies that address this specificity issue, and these strategies will be carried out during the second year of this project. Our knowledge of the topography of this anti-breast cancer peptide has increased so we know where it can be modified without loss of biological activity. In fact, some of these modifications have improved the synthetic yield of this peptide. Overall, work is on track to identify the binding site for this peptide which will be a seminal advance in understanding how it stops breast cancer growth.

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