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13. ABSTRACT (Maximum 200 Words)

We have synthesized a nine amino acid peptide, AFPep, that inhibits the growth of ER+ human breast. Understanding the process by which AFPep 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. We have developed and optimized an affinity chromatography procedure to identify the receptor for AFPep by using the peptide as "bait" to isolate proteins from solublized cells which have an affinity for the peptide. A protein of approximately 70 kDa which shows specificity for the linear form of the peptide, AFPep, has been isolated and identified by mass spectroscopy as Hsp72 which together with Hsp90 and other components forms a heterocomplex that binds to the ER and mediates ligand binding and transcriptional activities. The specificity of the interaction between AFPep and Hsp72 and the relevance to the anti-breast cancer activity of AFPep are currently being evaluated. In addition, an in vitro cell culture method which shows consistent inhibition by AFPep of estrogen-stimulated growth of human breast cancer cells has been developed and will be used to evaluate the mechanism of action of the peptide.

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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, AFPep (previously referred to as 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) Although the anti-breast cancer activity of AFPep 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 AFPep 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 AFPep will elucidate the first step in the mechanism of anti-oncotic action of this peptide.

During the first year of this training grant, peptides have been synthesized for use in the affinity chromatography (AC) procedure to isolate proteins that have an affinity for the AFP-derived peptides. These peptides have been linked to an affinity column matrix, and the procedure for isolation of breast cancer cell-associated proteins has been optimized. A 70 kDa protein was retained by columns derivatized with both active and inactive peptides, including those with a disrupted pharmacophore, indicating putative non-specificity in the strategy designed to isolate the AFPep receptor. The second year investigations studied this 70 kDa protein and the isolation strategy in more depth. In addition, work to determine the pharmacophore of the peptide has progressed and has culminated in a publication in the *Journal of Peptide Research* (10).

2. Body:

Research Accomplishments for the Second Year:

A. Two Dimensional (2D) Electrophoresis of Affinity Chromatography Eluents from Peptide Columns: Identification of a Non-Specific Cellular Binding Protein, Mortalin: To ascertain if the 70 kDa band seen previously represented one or more proteins and to identify these protein(s), the eluents from the affinity columns were separated using 2D electrophoresis. Multiple spots with an approximate MW of 70 kDa were seen, as well as numerous lower molecular weight species, but none of these spots were specific. The most prominent spot was sequenced by mass spectrometry (MS) by the Keck Laboratory (Univ. of Virginia). Twenty-one unique peptide fragments were sequenced and compared to the known protein database. The data indicate that the prominent spot is mortalin, a member of the heat shock protein (Hsp) 70 family. Mortalin has two isoforms, mot1 and mot2, which differ by only two amino acids in the C-terminal region (11). The sequencing data obtained is unable to differentiate between these two isoforms. Mot 1 was originally identified as a cytosolic protein in mortal mouse embryonic fibroblasts but lacking from immortal fibroblasts (12). Disrupting the activity of Mot-1 by micro-injection of an antibody to this protein resulted in transient stimulation of senescent mortal fibroblasts to divide (12). Mot-2 has a contrasting function. It has a perinuclear localization and has been isolated from immortal fibroblasts (13). It has been shown to interact with wild-type p53 in transformed cell types where it impairs the transcriptional activation of the latter (11).

B. One Dimensional (1D) Electrophoresis of AC Eluents: Identification of a Specific Cellular Binding Protein, Heat Shock Protein (Hsp) 70. Because multiple spots were seen during 2D electrophoresis, 1D electrophoresis was performed using these same samples but at a higher

concentration than that allowed for by 2D electrophoresis and a lower gel porosity to allow for better separation of multiple bands at approximate MW 70 kDa. When the bands were visualized by Coomassie staining, it was apparent that there were bands that were present in the eluent from the AFPep column that were not present in the eluent from the Scrambled control peptide column (Figure 1, page 8). The band that was missing (noted by an arrow in Figure 1) was submitted to the Keck Institute for identification by MS analysis. Twenty-one unique sequences were used for identification by comparison to the known protein databases. The band was identified as Hsp72 (inducible Hsp70), a member of the Hsp70 family (but is not mortalin). This protein is a required member of a heterocomplex which includes Hsp90, and p60 (Hop, Hsp-organizing protein) (14). This heterocomplex binds to steroid hormone receptors, including the estrogen receptor and is essential for maintaining the receptor in a conformation that allows for ligand (estradiol) binding and is therefore important in potentiating the transcriptional activity of the ER (15). The heterocomplex has also been found to interact with a number of signal transduction molecules many of which have been demonstrated to be involved in estradiol-induced signaling or cross-talk such as c-Src, Raf-family kinases, IGFR, MEK, Akt, PDK-1, and FAK (14). The specificity of the interaction between AFPep and Hsp72 is currently being investigated using affinity chromatography procedures and commercially obtained antibodies to Hsp72. Once this interaction is confirmed, its role in the anti-breast cancer activity of AFPep will be examined.

AFPep has also been labeled with the fluorescent dye Alexa 488 and the biological activity of the labeled peptide evaluated. The labeled molecule was found to have activity comparable to that of the unlabeled compound using the Inhibition of Estrogen-dependent Growth of Immature Mouse Uterus Assay (1;2). This fluorescent peptide can be used to confirm the interaction between AFPep and Hsp72 by evaluating the fluorescence intensity of immunoprecipitated Hsp72 from whole cell lysate treated with the labeled peptide.

AFP receptor protein (AFPR) was obtained from Advanced Immunochemicals and an antibody to this protein was also purchased. MCF-7 solublized cells, AC eluents from these cells, solublized tumor tissue, and AC eluents from the tumor tissue were immunoblotted for the presence of AFPR, but no immunoreactive bands were found. AFPR protein was used as a positive control in these experiments and reacted with the antibody as expected. These studies indicated that the proteins retained by the AFPep affinity column were not AFPR. Also, control studies showed that the AFPep column does not retain the (commercially-obtained) AFPR protein.

C. Development and Optimization of an *in vitro* Method for Evaluating the Activity of AFPep. *In vivo* methods to assay the antiestrogenic activity of AFPep (human breast cancer xenograft in immune-deficient mice (2) and Inhibition of Estrogen-stimulation of Immature Mouse Uterus (1:2)) have yielded consistent and reproducible results. However, *in vitro* cell culture assays performed on plastic cellware have not produced such consistent results. To adequately study the mechanism of action of the AFPep, it was necessary to optimize the *in vitro* assay conditions so that these could be utilized to examine cell-signaling, cell cycling, and for use in blocking studies using antibodies to isolated cellular binding proteins for AFPep. Since one of the most apparent differences between the *in vivo* assays and those performed in cell culture using plastic cellware was the absence of components of the extracellular matrix (ECM) in the latter, we chose to evaluate the cellular proliferative response to treatment with estradiol alone and in combination with AFPep. T47D human, ER+ breast cancer cells were cultured on 24-well plates coated with the ECM components fibronectin (Fn), collagen IV (Col IV), collagen I (Col I), or laminin, or with the non-specific attachment factor polylysine (Poly K). Uncoated (plastic) plates were also used as a control. As shown in Figure 2 (page 8), significant inhibition of estrogen-stimulation of proliferation of these cells by AFPep was observed when they were cultured on Col I, Col IV, or Fn, but not when cultured on laminin, Poly K, or plastic (uncoated). Dose response curves for AFPep were subsequently performed on Col I, IV and Fn and maximal inhibition of estrogen-stimulated growth was seen at 1 – 0.1 μM. No significant anti-proliferative response was seen when cells were treated with an inactive peptide and estrogen. The data collected from these experiments indicates that

cells cultured on extracellular matrix components have a more consistent, reproducible response to AFPep than cells cultured on plastic. These conditions can be used for cell signaling experiments (see below).

D. Phospho-protein Screening of T47D Human Breast Cancer Cells Cultured on Fibronectin-coated Plates and Treated with AFPep and Estradiol. Once it had been established that plating human breast cancer cells on ECM components resulted in more consistent inhibition of estrogen-stimulated growth of the cells, these conditions were used to study differential phosphorylation of a number of signaling molecules that could be involved with the activity of the peptide. The cells were treated with estradiol at 0.1 nM, peptide at 1 μM, or peptide followed 35 minutes later by estradiol. A fourth control flask received no treatment. Cells were harvested 30 minutes after the estradiol treatment, lysed, and the total protein content determined using the Lowry method (Bio-Rad reagent). Lysates were diluted to 1 mg/mL in Lammelli buffer and submitted for phospho-protein screening by Kinexus, Inc. (Vancouver, BC, Canada). Data for 34 phosphorylation sites on 22 signaling proteins was returned. Estrogen treatment showed increased phosphorylation with a concurrent reduction in phosphorylation when treated with both estrogen and AFPep for two serines on FAK and the combined activation site (Y239/Y240) on Shc. Other molecules that showed a significant effect of peptide when combined with estrogen compared to estrogen treatment alone included p38α MAPK, Akt1, SAPK/JNK, c-Kit, MEK1, and the Y577 site of FAK. This data has been used as a foundation for additional studies of signal transduction induced by AFPep. These include Akt, SAPK/JNK, Shc, p38 MAPK, c-Src, FAK (multiple sites), and p53, as well as ERα (multiple sites) and Erk 1 /2. In addition, the amounts of total protein (phophorylated and unphosphorylated) will be quantitated, and tubulin and histone H1 will be used as markers of cytoplasmic and nuclear fractions respectively.

Future Work:

Evaluation of interactions between Hsp72 and AFPep: The optimized affinity chromatography procedure will be repeated with freshly-prepared AFPep and control peptides and repeated. The eluent containing highly-retained proteins will be separated by SDS-PAGE, transferred to a membrane and immunoblotted with an antibody to Hsp72 to confirm the specificity of the interaction between Hsp72 and AFPep. Furthermore, an attempt will be made to treat cell lysates with fluorescently-labeled peptide and then isolate the Hsp72/Hsp90/Hop complex by immunoprecipitation using an antibody to Hsp72 and/or Hsp90. This immunoprecipitate will then be evaluated for the presence of labeled peptide. Inactive peptides which have been similarly labeled will be used concurrently to try to prove the specificity of the interaction.

Elucidation of the Signal transduction pathway(s) involved in AFPep inhibition of estrogen-dependent growth: Timecourse experiments to look at the signaling initiated by treatment with AFPep and estradiol will be run. T47D cells will be plated on Col IV and treated with estradiol, AFPep, or the combination. No treatment will be used as the control. Cells will be harvested at 8 timepoints over 24 hours and lysed. The lysates will be immunoblotted to look for differential phosphorylation pattern of various signaling molecules (See Body, Research, D.)

Microscopy to determine subcellular localization of AFPep using Alexa 488-labeled peptide: Labeled peptide will be used to look at the uptake, if any, of AFPep. Cells grown on ECM-coated coverslips will be treated with peptide alone and peptide with estrogen and placed at 4°C. The degree of uptake of the peptide and cellular localization will be studied using confocal microscopy as the cells are placed at 37°C to allow for endocytosis. This experiment will provide information on the means of internalization of the peptide.

Training Accomplishments for the Second Year

- Completed all of the requirements for her M.S. degree. Awarded at graduation in May 2003.

- A manuscript for publication entitled "Synthetic peptide derived from alpha-fetoprotein inhibits growth of human breast cancer: Investigation of the pharmacophore and synthesis optimization" has been submitted and accepted for publication by the *Journal of Peptide Research.*
- Attended the 94th Annual Meeting of the American Association for Cancer Research as an Inglenook Scholar-in-Training where she presented a poster highlighting analogs of the AFP-derived peptide.
- Completed four intramural presentations during this funding period including presentations at one thesis committee meeting, a departmental colloqium, and a poster at the annual Student poster day entitled "Inhibition by AFPep of Estrogen-dependent Growth of T47D Human Breast Cancer Cells is Facilitated by Adhesion to Specific Extracellular Matrix Components." She was awarded the Deans Prize for Extramural Research Activities at the Albany Medical College Awards Day. She also presented a poster at the Center for Immunology and Microbial Disease Annual Retreat which received the Grand Prize for her department.
- Participated in numerous meetings with Albany Medical College investigators to learn 2D electrophoresis, cell cycle analysis, immunoblotting, evaluation of mass spectrometric analysis, and techniques to search the protein databases.
- Presented journal articles pertinent to cancer research at two journal club meetings and attended these meetings weekly.
- Attended departmental colloquia and seminars weekly.
- Instrumental in the laboratory training of two undergraduates and one medical student.

3. Key Accomplishments

- Completed optimization of an affinity chromatography method to isolate cellular binding proteins for AFPep.
- Used 2D electrophoresis to evaluate the proteins that are highly retained by the AFPep and Scrambled (control) affinity columns. The most prominent spot (non-specific) was sequenced by Mass Spectrometry (MS) and identified as mortalin.
- Separated the highly-retained proteins from the AFPep and Scrambled affinity columns by 1D electrophoresis, isolated a protein which appears to bind specifically to AFPep, but not to the control column. This protein has been identified by MS as Heat shock protein 72.
- Determined that AFPR protein was not retained by AFPep affinity column.
- Developed and optimized an *in vitro* method for evaluating the activity of AFPep on cells cultured on extracellular matrix (ECM) components.
- Performed a phospho-protein screening of T47D human breast cancer cells cultured on ECM and treated with estradiol, peptide, and the combination.
- Labeled AFPep with Alexa 488 fluorescent dye and evaluated its biological activity.

4. Reportable Outcomes

- First author on a peer-reviewed publication. *J. Peptide Res.*, 2004. 63. 409-419 (10).
- Presented a poster 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." Received an Inglenook Scholar-in-Training Award to be utilized for travel expenses to attend this meeting.
- Received M.S. degree from Albany Medical College after successful completion of all requirements.
- Progress of this research project has been reported at a departmental colloquium and a Ph.D. thesis committee meeting.
- This research was presented as a poster at the Albany Medical College Student Research Day and was awarded the Dean's Prize for Extramural Research Activities at the Albany Medical College Student Awards Day.

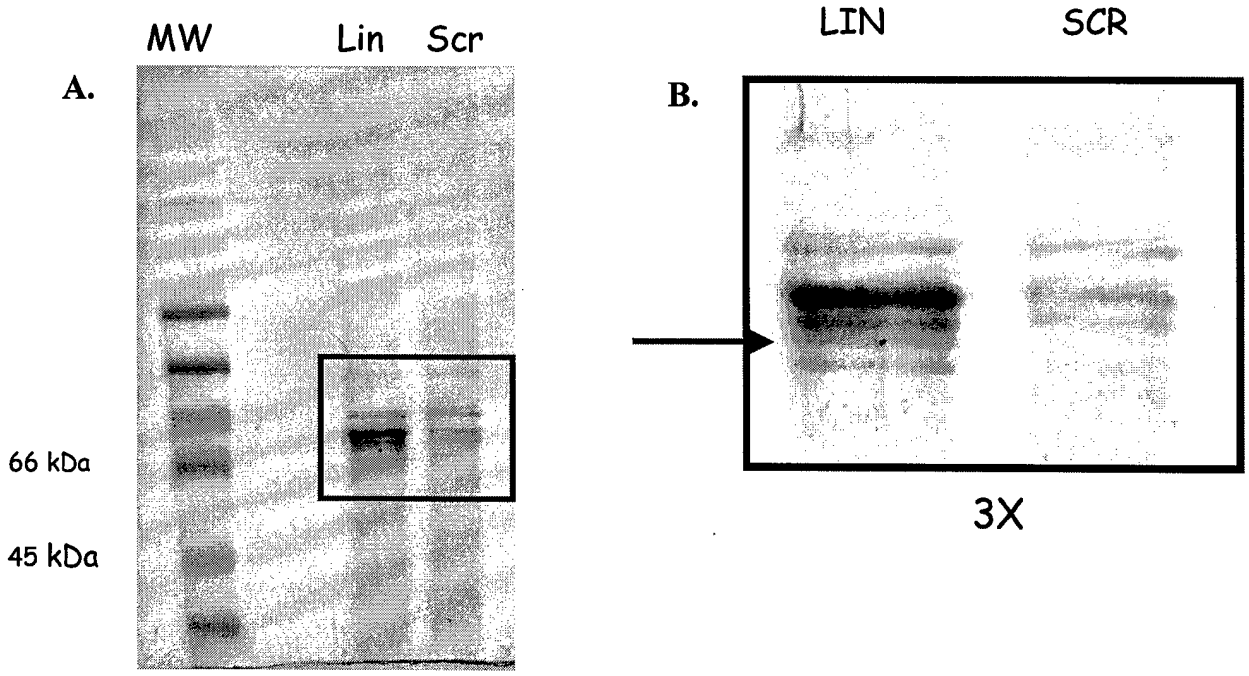
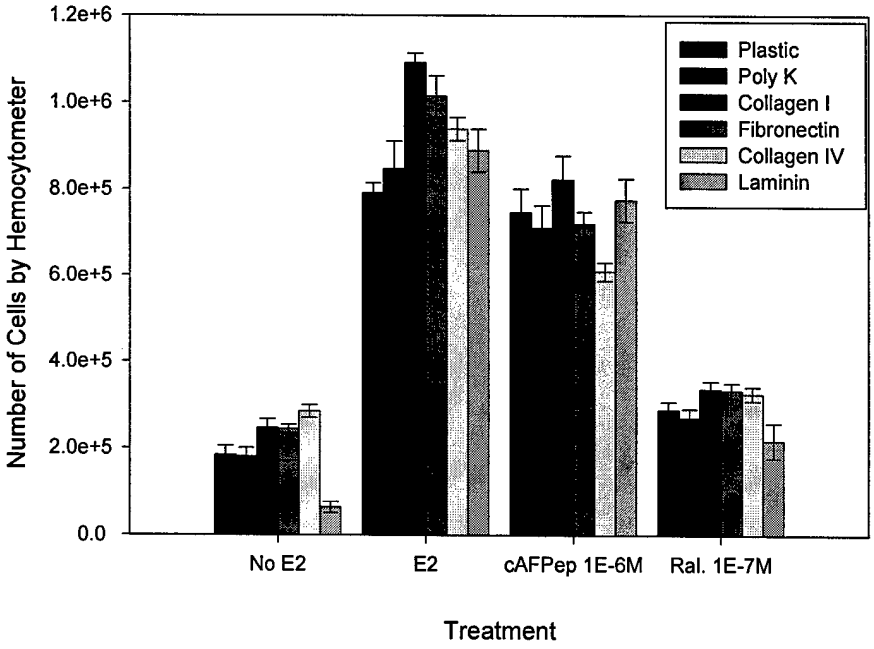


Figure 1: Eluents from AFPep(Lin) and Scrambled Control (Scr) columns separated on a 7.5% SDS-PA gel and visualized by Coomassie Blue staining. The specific band of approximate molecular weight 70 kDa is denoted by the arrow in B. This band has been identified by mass spectrometry as heat shock protein 72.



ECM	E_2	Inh	p
Plastic	4.3	7	0.46
Polylysine	4.7	21	0.13
Collagen I	4.4	32	<0.001
Fibronectin	4.2	38	<0.001
Collagen IV	3.3	50	<0.001
Laminin	14	14	0.14

Figure 2: Inhibition of estrogen-dependent growth of T47D human breast cancer cells cultured on various extracellular matrix (ECM) components. Cells were treated daily with AFPep at 1 uM followed 1 hour later by estradiol (E2) at 1 nM. Raloxifene (Ral) was used as a control. Significant inhibition as a result of peptide treatment was seen when cells were plated on Collagen I and IV and fibronectin but not on plastic, laminin, or polylysine (See table for fold stimulation by estradiol (E_2), percent inhibition by peptide (Inh), and p values by T-test (as compared to estradiol treatment alone,)

5. Conclusions: The work thus far indicates that cellular preparations from T47D cells contain a protein that specifically has an affinity for the AFP-derived peptide. In addition, a considerable amount of work has been undertaken to study the intracellular signal transduction that occurs as a result of treatment with the AFP-derived peptides with and without concurrent estrogen treatment. Overall, work is on track to identify the binding site for this peptide and to expand the understanding of the mechanism of action of these peptides. This will be a seminal advance in understanding how these peptides stop breast cancer growth.

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7. Appendix - *J. Peptide Res.* 2004, 63: 409-419.

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Synthetic peptide derived from α-fetoprotein inhibits growth of human breast cancer: investigation of the pharmacophore and synthesis optimization

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Key words: breast cancer; estrogen; α-fetoprotein; head-to-tail cyclization; synthetic peptide

Abstract: A synthetic peptide that inhibits the growth of estrogen receptor positive (ER+) human breast cancers, growing as xenografts in mice, has been reported. The cyclic 9-mer peptide, *cyclo*[EMTOVNOGQ], is derived from α-fetoprotein (AFP), a safe, naturally occurring human protein produced during pregnancy, which itself has anti-estrogenic and anti-breast cancer activity. To determine the pharmacophore of the peptide, a series of analogs was prepared using solid-phase peptide synthesis. Analogs were screened in a 1-day bioassay, which assessed their ability to inhibit the estrogen-stimulated growth of uterus in immature mice. Deletion of glutamic acid, Glu1, abolished activity of the peptide, but glutamine (Gln) or asparagine (Asn) could be substituted for Glu1 without loss of activity. Methionine (Met2) was replaced with lysine (Lys) or tyrosine (Tyr) with retention of activity. Substitution of Lys for Met2 in the cyclic molecule resulted in a compound with activity comparable with the Met2-containing cyclic molecule, but with a greater than twofold increase in purity and corresponding increase in yield. This Lys analog demonstrated anti-breast cancer activity equivalent to that of the original Met-containing peptide. Therefore, Met2 is not essential for biologic activity and substitution of Lys is synthetically advantageous. Threonine (Thr3) is a nonessential site, and can be substituted with serine (Ser), valine (Val), or alanine (Ala) without significant loss of activity. Hydroxyproline (Hyp), substituted in place of the naturally occurring prolines (Pro4, Pro7), allowed retention of activity and increased stability of the peptide during storage. Replacement of the first Pro (Pro4) with Ser maintains the activity of the peptide, but substitution of Ser for the second Pro (Pro7) abolishes the activity of the peptide. This suggests that the imino acid at residue 7 is important for conformation of the peptide, and the backbone atoms are part of the pharmacophore, but Pro4 is not essential.

Valine (Val5) can be substituted only with branched-chain amino acids (isoleucine, leucine or Thr); replacement by D-valine or Ala resulted in loss of biologic activity. Thus, for this site, the bulky branched side chain is essential. Asparagine (Asn6) is essential for activity. Substitution with Gln or aspartic acid (Asp), resulted in reduction of biologic activity. Removal of glycine (Gly8) resulted in a loss of activity but nonconservative substitutions can be made at this site without a loss of activity indicating that it is not part of the pharmacophore. Cyclization of the peptide is facilitated by addition of Gln9, but this residue does not occur in AFP nor is it necessary for activity. Gln9 can be replaced with Asn, resulting in a molecule with similar activity. These data indicate that the pharmacophore of the peptide includes side chains of Val5 and Asn6 and backbone atoms contributed by Thr3, Val5, Asn6, Hyp7 and Gly8. Met2 and Gln9 can be modified or replaced. Glu1 can be replaced with charged amino acids, and is not likely to be part of the binding site of the peptide. The results of this study provide information that will be helpful in the rational modification of *cyclo*[EMTOVNOGQ] to yield peptide analogs and peptidomimetics with advantages in synthesis, pharmacologic properties, and biologic activity.

Abbreviations: AFP, α-fetoprotein; E_2, 17β-estradiol; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, 1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high-pressure liquid chromatography.

Introduction

Small molecule analogs of proteins, intended for pharmaceutical or other uses, are usually intended to mimic a binding site or active site of the protein, and to provide some or all of the function of the parent molecule. After identifying molecules small enough to be synthetically economical and yet large enough to contain information adequate to provide the desired activity, it is often necessary to optimize the newly developed small molecule in terms of several additional parameters, perhaps including conformational flexibility, potency, storage stability or stability in vivo, or even other parameters that arise during the development process, such as targeting to one of several available receptors. Simultaneous optimization of several of these parameters might be achieved through library screening or combinatorial synthesis approaches. Alternatively, and perhaps especially for situations in which unexpected observations contribute importantly to the developmental process, rational design approaches offer unique opportunities to optimize the structure and function of the small molecule protein analog. Furthermore, explorations of the structure–activity relationships during rational or iterative design and synthesis activities would be expected to contribute to the understanding of the biology and mechanism by which the novel analogs function.

A peptide that inhibits the growth of estrogen receptor positive (ER+) human breast cancers, growing as xenografts in immune-deficient, mice has been synthesized (1). *cyclo*[EMTOVNOGQ] was derived from α-fetoprotein (AFP), a safe, naturally occurring human protein produced during pregnancy, which itself has anti-estrogenic and anti-breast cancer activity (2). The peptide was identified by systematically parsing the AFP molecule until it was demonstrated that the oncostatic activity was localized to an eight amino acid sequence (amino acids 472–479) in domain IIIB of the AFP molecule (1,3,4). Mesfin *et al.* synthesized the linear form of the peptide, EMTPVNPG, and showed that it was the minimal sequence necessary to maintain oncostatic activity against breast cancer cells growing as a monolayer in culture or as a xenograft in immune-deficient mice (4). Substitution of hydroxyproline (Hyp, O) for both prolines resulted in a molecule that showed comparable activity and increased storage stability (1). Head-to-tail cyclization of the molecule to yield *cyclo*[EMTOVNOGQ] was facilitated by addition of Gln to the C-terminus of the octapeptide, and it was demonstrated that this cyclic molecule had an active dose range broader than the linear form of the molecule, and peptide stability and shelf life were not compromised (1). The use of a 'head-to-tail' peptide bond, rather than the more usual disulfide bond, may be advantageous for purposes of shelf life, retention of structure and activity in vitro, and elimination of dimers, trimers, and higher-order aggregates that can sometimes develop when disulfide-bonded peptides are stored or used in conditions that do not carefully control the redox state. The method of cyclization that was used was developed by Kates *et al.* (5,6) and is straightforward and expedient.

Although the anti-breast cancer activity of the linear and cyclic AFP-derived peptides is well-documented, the pharmacophore of the peptide has not been elucidated. Consequently, we designed and synthesized a number of analogs of the linear and cyclized peptides to ascertain which atoms are crucial for activity. Because of the relatively few amino acids to be studied and the availability of interspecies homology data, a rational, rather than a combinatorial, approach to substitution was utilized. Conservative and nonconservative substitutions were made for each amino acid as appropriate. Biologic activity data from the analogs were used to determine the

pharmacophore and auxiliary portions of the molecule. Simultaneously, comparisons of synthetic outcomes yielded information that led to improved approaches for synthesizing these peptides. Peptides with key substitutions were produced with high purity, synthetic yield, and biologic activity. The lead analog, *cyclo*[EKTOVNOGN], was demonstrated to inhibit the estrogen-stimulated growth of human breast cancer cells growing as xenografts in immune-deficient mice.

Experimental Procedures

Materials

9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were obtained from Calbiochem-Novabiochem (San Diego, CA, USA). The amino acids used for synthesis included Fmoc-Glu(OtBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Hyp(tBu)-OH, Fmoc-L-Val-OH, Fmoc-D-Val-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gly-OH, Fmoc-Met-OH, Fmoc-Ala-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Tyr(tBu)-OH, and the carboxyl-protected amino acids Fmoc-Glu(OAll), and Fmoc-Asp(OAll). Reagents for peptide synthesis including 1,1, 3,3-tetramethyluronium hexafluorophosphate (HATU), N-hydroxybenzotriazole (HOBt), Fmoc-PAL-PEG-PS resin, 20% piperidine in dimethylformamide (DMF), and diisopropylethylamine (DIPEA) were obtained from PerSeptive Biosystems, Inc. (Framingham, MA, USA). Trifluoroacetic acid (TFA), 2-propanol, anisole, ethane dithiol (EDT), tetrakis triphenylphosphine paladium(0) [$Pd(PPh_3)$], 4-methylmorpholine, DMF, and 17β-estradiol (E_2) were purchased from Sigma-Aldrich (St Louis, MO, USA). Diethyl ether, ethylacetate, acetic acid, chloroform, and sodium tetra ethylenediaminetetraacetic acid (EDTA) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Dulbecco's modified eagle medium (DMEM), crystalline bovine insulin, MEM nonessential amino acids, penicillin/streptomycin, L-glutamine, and trypsin were obtained from GIBCO/BRL (Gaithersburg, MD, USA) and Cosmic Calf serum was purchased from Hyclone (Logan, UT, USA).

Peptide synthesis

Peptides were prepared using Fmoc solid-phase synthesis as previously described (1,4). A Pioneer Peptide Synthesis system (PerSeptive Biosystems, Inc.) was used to assemble the growing peptide chain on Fmoc-PAL-PEG-PS resin beginning with the C-terminus using N^{α}-protected amino acids. Activation of the C-terminus of incoming amino acids was accomplished by treatment with HATU and DIPEA. Following synthesis, linear peptide was washed with isopropanol and cleaved from the resin by incubation for approximately 4 h in 9.5 mL of TFA : anisole : EDT (90 : 2 : 3) per 0.1 mmol of peptide (0.5 g resin) and side-chain protective groups were removed concurrently. Peptide was precipitated by addition of cold (−20 °C) diethyl ether and washed by repeated extraction with diethyl ether and then with ethylacetate/diethyl ether (1.5 : 1). The peptide was dissolved in a volume of deionized water to achieve a concentration of 5–10 mg/mL and lyophilized.

Cyclization of peptides

Cyclization of peptides was accomplished using the method of Kates *et al.* (5,6). An additional residue, Fmoc-Glu(OAll) or Fmoc-Asp(OAll), was included at the C-terminus during synthesis to facilitate this process. This amino acid was coupled to the resin through the γ-carboxylic acid leaving the α-carboxyl protected with the allyl group. N^{α}-Fmoc deprotection allowed for sequential coupling of the remaining amino acids to the N-terminus. Following synthesis, cyclization of the peptide occurred through a three-step process. First, removal of allyl group from the α-carbonyl of Glu9 was initiated by treatment with 15 mL of a catalyst mixture containing 3 equivalents of $Pd(PPh_3)$ in chloroform/acetic acid/4-methylmorpholine (37 : 2 : 1) per gram of resin for 2 h in the dark. The resin was washed three times with 15 mL of DMF. Secondly, removal of the base-labile N^{α}-Fmoc was accomplished by treatment with piperidine (20% in DMF) followed by washing three times with 15 mL DMF. Finally, activation of the newly deprotected α-carboxyl of Glu9 by overnight treatment of the peptide-resin with HATU : HOBt : DIPEA (3 : 3 : 10 equivalents) in about 8 mL DMF per gram of resin, resulted in the formation of an amide bond between Glu9 and the deprotected-terminus. The peptide resin was washed three times with 15 mL of DMF. After each of the three steps, the peptide resin was evaluated for the presence of free amine groups using the Kaiser test (7). Subsequent cleavage of the cyclized peptide from the resin using the method outlined above resulted in the formation of γ-carboxyamido-derivative of Glu9 or Asp9, that is, Gln9 or Asn9, respectively.

Purification of peptides

Linear peptides were purified using a reverse-phase C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA). Briefly, a sample containing peptide of unknown purity was loaded onto a pre-washed cartridge and the sample was sequentially eluted with water, 10, 30, and 60% acetonitrile in water. The fraction containing peptide was then lyophilized. Purity of cyclic peptides was determined after separation of the main component from impurities on a Waters gradient semi-preparative reversed-phase liquid chromatographic system. The fraction containing the main peak was collected and lyophilized. Peptides used for structure–activity relationship analysis were purified prior to use in biologic assays, and those used in biologic assays were at 95% or greater purity. Peptides were evaluated by amino acid analysis and mass spectrometry.

Immature mouse uterine growth assay

The anti-estrotrophic activity of peptide was determined using the immature mouse uterine growth assay as described by Bennett *et al.* (2,3). Administration of 0.5 μg of 17β-estradiol (E_2) i.p. to each mouse has been demonstrated to double the uterine weight in 24 h with a corresponding increase in mitotic figures (2,3,8). Swiss/Webster female mice (13–15-day-old, 6–8 g body weight; Taconic Farms, Germantown, NY, USA) were weighed and distributed into treatment groups typically of five mice each such that groups contained mice of comparable weight ranges. Each group received two sequential i.p. injections spaced 1 h apart. The first injection contained test substance or vehicle control. A dose of 1 μg peptide per mouse was used; this is the dose which has been demonstrated to give the maximal inhibitory response for the linear and cyclic hydroxyproline-containing peptides. The second injection contained 0.5 μg E_2 or vehicle. Twenty-two hours after the second injection, mice were weighed and uteri dissected, trimmed free of mesenteries, and immediately weighed. Uterine weights were normalized to body weight (mg uterine per g of body weight) to compensate for differences between body weights of littermates. The inhibition of estrogen-stimulated uterine growth was calculated from the average values for each group using the following equation:

$$\% \text{ Growth inhibition} = 100 \times (\text{Full } E_2 \text{ stimulation} - E_2 \text{ stimulation in test group}) / (\text{Full } E_2 \text{ stimulation} - \text{No } E_2 \text{ stimulation})$$

Human breast cancer xenograft assay

An in vivo assay for anti-breast cancer activity was performed using the methodology of Bennett *et al.* (2,9). Confluent MCF-7 human breast cancer cells growing in monolayer in DMEM without phenol red supplemented with 5% bovine calf serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acids, and 10 ng/mL bovine insulin were released from the flask using trypsin/EDTA (0.25%/0.25%) and 20 million cells were pelleted by centrifugation at 200 **g** and then solidified into a fibrin clot by treatment with 10 μL fibrinogen (50 mg/mL) and 10 μL thrombin (50 units/mL). The solid tumor mass was then cut into pieces of approximately 1.5 mm diameter. Each tumor segment was implanted under the kidney capsule of an Institute for Cancer Research (ICR)-severe combined immunodeficient (SCID) male mouse (Taconic Farms) which weighed about 25 g. Estrogen supplementation was accomplished by s.c. placement of a 2 mm silastic tubing implant containing solid E_2 inserted on the day of tumor implantation. Peptide was injected i.p. once daily at a dose of 10 μg/mouse. This is the dose that has been demonstrated to give the maximal inhibitory response for the cyclic hydroxyproline-containing peptide in this assay. Tumor growth was monitored during survival laparotomy at 14 and 20 days after implantation by measurement of the short (d) and long (D) axes of the tumor by using a dissecting microscope equipped with an ocular micrometer. Tumor volumes were calculated assuming the tumor shape to be an ellipsoid of revolution about the long axis (D) using the formula $(\pi/6)d^2D$. Mean tumor volume ± SE was calculated for display in growth curves. All animal care procedures were approved by the Albany Medical College Animal Care and Use Committee.

Homology data

Sequence information was obtained by comparison of the homologous region of AFP for the six species studied using sequence data published in the protein data base of the National Center for Biotechnology Information provided by the National Library of Medicine.

Statistics

Significance of results for the immature mouse uterine growth assay was determined by Dunnett's multivariate

comparison using the 8-amino acid, hydroxyproline-containing linear molecule as the control. There were five to 10 replicates per group except for the Met2Lys and control peptide (8-mer Hyp) where there were 28 and 332 replicates, respectively. Results were considered significant at $P < 0.05$. Error is shown as ±standard error (SE) of measurement. Significance of differences between groups for the human breast cancer xenograft assay was tested using the one-sided Wilcoxon sum of ranks test. There were five to six mice in each treatment group.

Results

Part I: Identification of the active site of the peptide

To determine which residues were essential to the bioactivity of the 8-amino acid, linear peptide (sequence Glu1-Met2-Thr3-Pro4-Val5-Asn6-Pro7-Gly8), a series of linear analogs was prepared and evaluated using the immature mouse uterine growth assay. It has been documented that there is excellent correlation between the uterine growth assay and the human breast cancer xenograft assay with regard to peptide inhibition of estrogen-stimulated growth (1,3). The uterine growth inhibition assay provided the advantages of faster assay time (1 day vs. 20 days) and substantially lower cost. Hence the xenograft assay was used only for the lead analog. Modifications of peptides included conservative substitutions for each of the amino acids, deletions of the N- and C-termini, and for some amino acids, nonconservative substitutions as well.

Glu1

Replacing the highly conserved Glu1 with Gln or Asn maintained the inhibitory activity of the linear peptide (Fig. 1). Substitution of this residue with Lys or Asp reduced the activity of the molecule, but did not abolish it. However, the nonconservative substitution of Glu1 with Ser resulted in an almost complete loss of the inhibitory activity of the peptide. Deletion of Glu1 ablated the inhibitory activity of the molecule.

Met2

Met2 is not highly conserved across species in AFP (Table 1) and can be replaced with Thr (chimpanzee), Glu (horse), or Ala (mouse, rat). A linear octapeptide was synthesized with a substitution of Lys for Met2. The peptide retained full inhibitory activity (Table 2). Substitution of Tyr for Met2 also maintained the inhibitory activity.

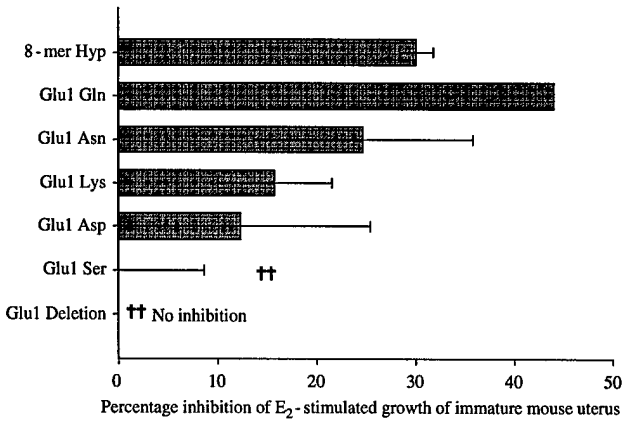

Figure 1. Anti-estrotrophic effect of substitutions for glutamic acid. Glu1 was substituted as shown in 8-amino acid, linear AFP-derived peptide containing substitutions of Hyp (O) for both Pro, EMTOVNOG (8-mer Hyp). Inhibition was measured using the immature mouse uterine growth assay. Peptide at 1 μg per mouse or vehicle control was injected i.p. into immature Swiss mice. One hour later, the mice were injected i.p. with 0.5 μg E_2 or vehicle control. Twenty-two hours after the second injection, mice were killed, the uteri dissected and trimmed free of mesenteries, and weighed. Percent inhibition is calculated as described in Experimental Procedures. 8-mer Hyp showed inhibition of growth of approximately 33% in this assay. $^{\dagger\dagger}P < 0.005$ compared with 8-mer Hyp using Dunnett's multivariate comparison.

***Table 1.* Comparison of sequence of AFP and albumin from different species**

Residue number	1	2	3	4	5	6	7	8
Human	***E***	M	T	***P***	*V*	***N***	P	***G***
Gorilla	***E***	M	T	***P***	*V*	***N***	P	***G***
Chimpanzee	***E***	T	T	***P***	*V*	***N***	P	***G***
Horse	***E***	E	S	***P***	*I*	***N***	P	***G***
Rat	***E***	A	N	***P***	*V*	***N***	S	***G***
Mouse	***E***	A	S	***P***	*V*	***N***	S	***G***
Albumin (human)	***E***	K	T	***P***	*V*	***S***	D	***R***

Amino acids that are in bold italic (Residues 1, 4, 6 and 8) are strictly conserved between species and those in italics are semi-conserved. Residues that are in bold (Residues 2, 3 and 7) are not conserved in AFP across those species studied.

Thr3

Analogs of the peptide with Thr3Val and Thr3Ala substitutions in the 8-mer peptide were prepared and tested (Table 2). Both of these analogs retained the inhibitory activity of the parent molecule. Substitution of Ser for Thr3 resulted in some reduction in inhibitory activity that was not statistically significant.

Pro4,7

The conservation of Pro4 across species (Table 1) suggested that this residue was required for the biologic activity of the

Table 2. **Anti-estrotrophic effect of nonconservative substitutions in AFP-derived linear peptide**

Nonconservative substitutions: analogs of linear peptides		
Peptide/substitution	**Sequence**	**Relative activity**
8-mer Hyp	EMTOVNOG	++
Met2	EKTOVNOG	++
	EYTOVNOG	++
Thr3	EMVOVNOG	++
	EMAOVNOG	++
	EMSOVNOG	+
G8-deleted	EMTPVNP	+
Horse	EESPINPG	+
Horse P4O,P7O	EESOINOG	++
Albumin peptide	EKTPVSDR	–

Percent inhibition was measured using the immature mouse uterine growth assay. Amino acid substitutions of the 8-amino acid, linear peptide containing either Pro or Hyp are shown in bold. ++, 25–40% inhibition; +, 12–25% inhibition; –, less than 5% inhibition.

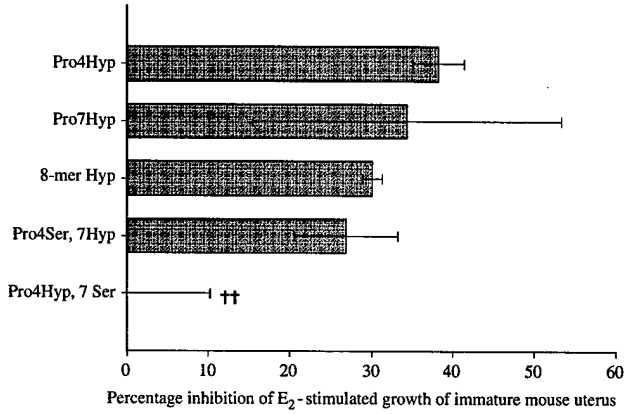

Figure 2. Anti-estrotrophic effect of substitutions for proline in linear AFP-derived peptide. Inhibition was measured using the immature mouse uterine growth assay. $^{\dagger\dagger}P < 0.005$ compared with 8-mer Hyp using Dunnett's multivariate comparison.

peptide; Pro7 is less strictly conserved. As shown in Fig. 2, analogs containing substitutions of hydroxyproline (Hyp) for either Pro4 or Pro7 resulted in retention of inhibitory activity. Similarly, an analog with Hyp substituted for both Pro4 and Pro7 retained inhibition. An analog containing the nonconservative substitution of Ser for Pro4 maintained inhibitory activity. However, substitution of Ser for Pro7 resulted in a complete loss of biologic activity (Fig. 2) although Ser replaces Pro7 in the AFP of rodents (Table 1).

Val5

Conservative substitutions for Val5 included Ala, Leu, and Ile, each of which has hydrophobic character. Substitution

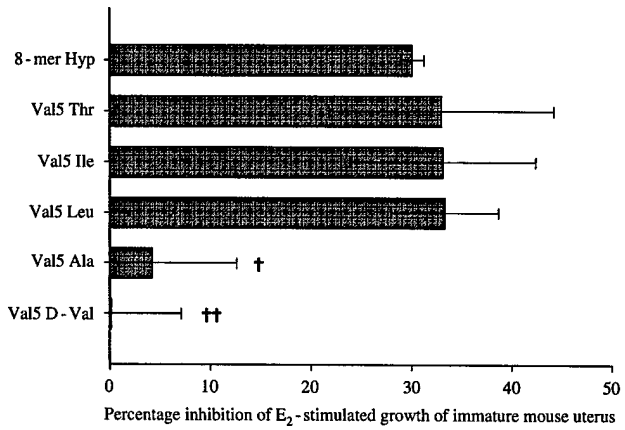

Figure 3. Anti-estrotrophic effect of substitutions for valine in linear AFP-derived peptide. Inhibition was measured using the immature mouse uterine growth assay. $^{\dagger}P < 0.01$; $^{\dagger\dagger}P < 0.005$.

of Val5 with Leu, Ile, or Thr maintained biologic activity (Fig. 3). However, replacement of Val5 with Ala resulted in a nearly complete loss of inhibitory activity, and replacement of Val5 with D-Val completely abrogated the activity of the analog.

Asn6

Peptide analogs were synthesized with conservative substitutions for Asn6 and analyzed for inhibitory activity using the immature mouse uterine growth assay. Replacement of Asn6 with Gln resulted in a significant loss of inhibitory activity (Fig. 4). Substitution with the carboxylic acid derivative of this residue, Asp, also resulted in a reduction in activity.

Gly8

Glycine has been noted as essential for the linear molecule because deletion resulted in a loss of inhibitory activity (4).

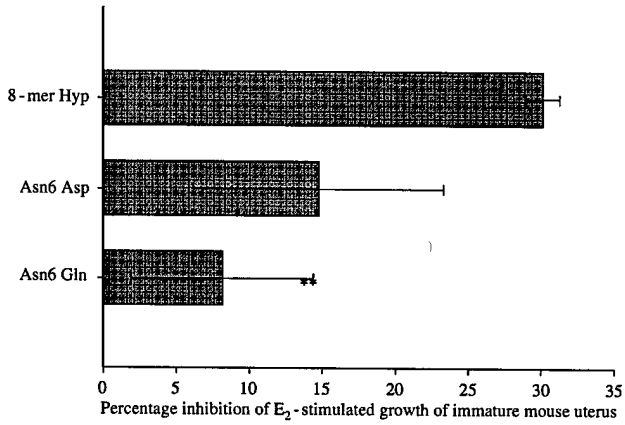

Figure 4. Anti-estrotrophic effect of substitutions for asparagine in linear AFP-derived peptide. Inhibition was measured using the immature mouse uterine growth assay. $^{**}P < 0.05$.

This is consistent with its conservation across species. However, replacing Gly8 with Asn resulted in a peptide that retained full activity (data not shown).

Albumin peptide

AFP is a member of the albumin family of proteins and has a 39% homology of primary structure with albumin (10). This similarity is greatest in the third domain where the AFP-derived peptides were identified. A synthetic peptide with the sequence of human albumin, EKTPVSDR (Table 1), was prepared and analyzed using the immature mouse uterine growth assay (Table 2). This molecule differs from the AFP-derived linear peptide by substitutions of Lys for Met2, Ser for Asn6, Asp for Pro7, and Arg for Gly8. The synthetic albumin peptide had no inhibitory activity.

Part II: Comparison of activity, yield, and purity of cyclic analogs of peptide

Mesfin *et al.* (1) had shown previously that the linear peptide could be cyclized and that the resultant molecule, *cyclo*[EMTOVNOGQ], was anti-estrotrophic and that cyclization of the molecule broadened the effective dose range. Cyclization of the molecule was facilitated by addition of a Gln to the C-terminus of the molecule, and, in the linear form, this addition did not alter biologic activity (1). As the data in Table 2 indicated that Lys could be substituted for Met2 without loss of biologic activity, we added either Gln or Asn to the C-terminus of the Met2Lys analog and cyclized it. These analogs had inhibitory activity comparable to that of the cyclized molecules containing methionine (Table 3). However, when the purity of the Lys-containing molecules was evaluated by HPLC, the chromatograms showed the presence of predominantly one peak. The cyclic Met-containing peptides chromatographed as multiple peaks and required further purification by semi-preparative HPLC. This additional step in processing of the peptides resulted in a significant loss of material. The cyclic peptide containing the Met2Lys substitution increased the purity of the preparation 2.4-fold from 39 to 94% for the Gln-containing analog and fourfold from 24 to 95% for the molecule cyclized using Asn (Table 3). As a consequence, the yield of the peptide was increased compared with the cyclic molecule containing Met.

The cyclized peptide containing the Met2Lys substitution was evaluated for its ability to inhibit the growth of ER+ human breast cancer cells growing as xenografts under the kidney capsule in immune-deficient mice. As shown in Fig. 5, growth of MCF-7 tumors was dependent on estrogen for growth: the presence of E_2 resulted in a threefold increase in tumor volume after 20 days in comparison with the control without E_2. Administration of 10 μg *cyclo*[EKTOVNOGN] daily to animals concurrently treated with E_2 prevented tumor growth over this same time period.

Table 3. **Activity, purity, and yield of cyclic analogs of AFP-derived peptide**

Analog name	Activity (% inhibition)	Purity (%)	Yield (%)
cyclo[EMTOVNOGQ]	29	39	56
cyclo[EMTOVNOGN]	23	24	62
cyclo[EKTOVNOGQ]	24	94	67
cyclo[EKTOVNOGN]	32	95	72

Cyclization of the 9-amino acid linear peptides was performed as described in Experimental Procedures. Biologic activity was measured in the immature mouse uterine growth assay after final purification of the peptides. Purity was determined as area percent by HPLC prior to purification. Yield was calculated as amount of peptide recovered after lyophilization without further purification by HPLC.

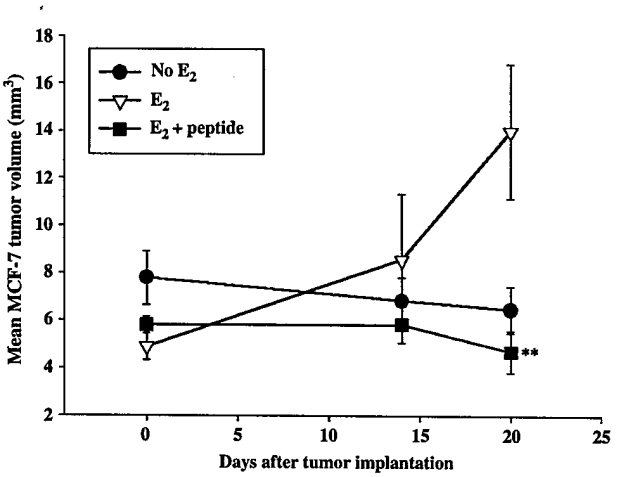

Figure 5. Anti-estrotrophic activity of *cyclo*[EKTOVNOGN] against MCF-7 human breast cancer xenografts. There were five to six mice per treatment group. The assay is described in Experimental Procedures. E_2 was provided by implantation of a silastic tube containing solid E_2 inserted subcutaneously on day 0. Peptide was given once daily at 10 μg/mouse. Peptide significantly inhibited the estrogen-dependent growth of the tumor. $^{**}P < 0.05$.

Discussion

Rationale for investigation of the pharmacophore of AFP-derived peptides

It had been demonstrated previously that both the linear and cyclic AFP-derived peptides were capable of inhibiting

the growth of estrogen-dependent breast cancers growing as xenografts in immune-deficient mice (1,3). Furthermore, it had been shown that these peptides inhibit ER+ tumors that had been made resistant to tamoxifen (3). Although the mechanism of the oncostatic action of the peptides has not been elucidated, it is known that, unlike tamoxifen or other selective estrogen receptor modulators, the peptides do not compete with estrogen for the ligand-binding domain of the estrogen receptor (3). Rather, it is likely that the peptides mediate their activity by interacting with a cell surface-binding protein, as does their parent molecule AFP, although this receptor has not been fully characterized (11–15). As the receptor for the peptides is unknown, we chose to evaluate the structure–activity relationship of the peptides employing rational design techniques to determine the key amino acids required for maximal biologic activity. Two important biologic endpoints, namely inhibition of estrogen-stimulated growth of uterus and of breast cancer were employed as measures of biologic activity. A combinatorial approach to analog development was not chosen because: (a) comparative sequence data for the oncostatic region of AFP were available for six mammalian species (Table 1) suggesting which residues were essential for activity based on conservation of those residues. Although the oncostatic potential of peptides derived from the AFPs of each of the six species shown in Table 1 was not tested, comparison of the sequences of these peptides served to guide our choices for individual amino acid substitutions when designing analogs; (b) relatively few amino acids (eight) comprise the minimal sequence necessary for anti-estrotrophic activity in AFP-derived peptides; and (c) data generated while determining the minimal sequence and optimizing the stability of the peptides (1,4) suggested key residues that were required for activity or stability in storage and other residues which could be replaced without loss of biologic activity. The unexpected observation that cyclic molecules provided a wider dose–response curve (1) suggested the importance of maintaining the residues necessary for the cyclization process. Thus, the rational approach seemed more appropriate for these studies.

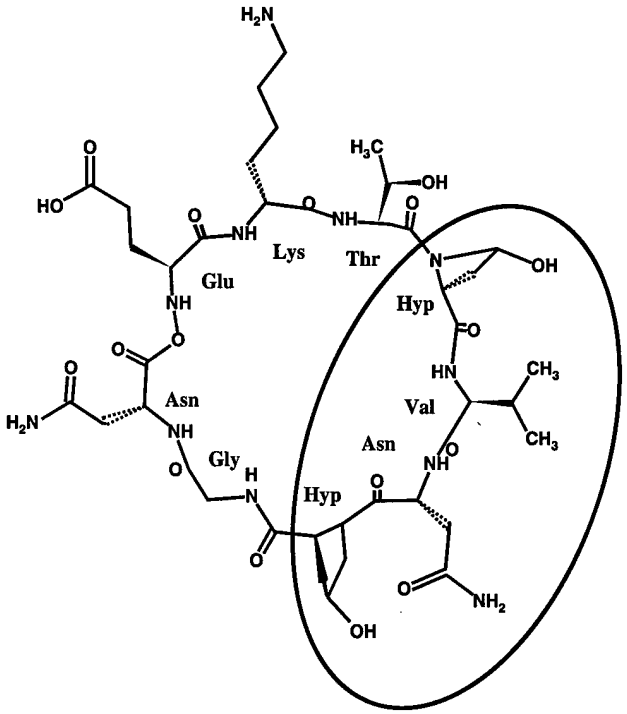

Figure 6. Sequence and structure of *cyclo*[EKTOVNOGN]. Structure of the cyclic nonapeptide. Amino acids are labeled adjacent to their side chains and the pharmacophore is circled.

Investigation of the pharmacophore

Cyclization of the peptide as previously described (1) constrains the molecule, causing the peptide to exist largely as a planar macrocycle (Fig. 6). The region of the cyclic molecule near the ring-closing peptide bond (the Asn to Glu link) is probably not the pharmacophore, as that peptide bond is artificial and does not exist in the parent protein. As such, it is logical to assume that the portion of the molecule diametrically across from that Asn to Glu peptide bond, that is, the 'middle' of the linear peptide, ought to be the pharmacophore. As a first approximation, the region may be postulated to consist of the amino acids between the two hydroxy proline residues (Fig. 6).

In the six species evaluated, Val5 is found at this site for all species except equine where the highly conserved Ile is found in place of Val5 (Table 1). Consistent with the presence of Ile in equine AFP, synthetic analogs with Val5 substitutions, which retained the hydrophobic and branched character, such as Ile and Leu, retained biologic activity. However, replacement of Val5 with the smaller, nonbranched Ala resulted in a significant loss of inhibitory activity, suggesting that the branched structure of the side chain at this site was required to maintain the activity of the peptide. To test this hypothesis, Thr was substituted for Val5, a substitution that conserves the branched nature, but is more hydrophilic than Val. This analog retained anti-estrotrophic activity suggesting that the branched nature of the residue at this position is necessary for maximal activity. Interestingly, substitution of Val5 with its enantiomer, D-Val, completely ablated the activity and suggested that the specific orientation of the Val side chain

or of the backbone participates in the binding of the pharmacophore to its target.

Asn6 is strictly conserved across those species evaluated and even conservative substitutions of Asp or Gln for Asn6 resulted in a loss of inhibitory activity. This strongly suggests that Asn6 is an essential residue of the active site of this molecule, which correlates with what was expected based on the interspecies homology.

As shown in Fig. 2, Hyp can be substituted for either or both proline residues without a loss of biologic activity and it has been demonstrated that Hyp increases the storage stability of the AFP-derived peptides (1). An interspecies comparison of Pro4 and Pro7 indicates that Pro4 is highly conserved, while Pro7 is substituted by Ser in rodents. To examine the requirement for proline at these sites, analogs were prepared which replaced Pro4 or Pro7 with Ser. Counterintuitive to the interspecies comparisons, the inhibitory activity of the molecule was retained when Pro4 was substituted, but completely abolished when Ser replaced Pro7. Thus, the imino acid at residue 7 is important for conformation of the peptide, and the backbone atoms at this site are part of the pharmacophore. The substitution of Ser for Pro4 suggests that Pro4 is not essential.

The pharmacophore of the peptide seems to lie between the two proline residues and includes the side chains of valine and asparagine, and the backbone atoms contributed by valine, asparagine, and Pro7. This region is highly conserved among mammalian species and conservative substitutions in this area led to diminution or loss of biologic activity.

Amino acids distal to the pharmacophore contribute to the conformational stability of the peptide

Glu1 is highly retained across species and this initially suggested that it may be important to the activity of the molecule. Conservative substitutions in the linear, 8-amino acid peptide of Gln or Asn showed no significant loss of activity (Fig. 1). Replacing this residue with Lys, which retains a charge at this site, or the highly conservative substitution of Asp showed some loss of activity, but it was not significant. However, replacing Glu1 with Ser causes a loss of biologic activity and deletion of Glu1 completely ablated the inhibitory activity of the molecule. Since substitutions in the linear molecule which affect both the charge at this site and the size of the side chain resulted in analogs which retained their biologic activity, it is unlikely that Glu1 is part of the binding site of this peptide. Rather it more likely plays a role in maintaining the conformational stability of the molecule.

Replacing Thr3 with Val, which is more hydrophobic but retains the branched nature of the side chain, maintained the inhibitory activity of the linear molecule (Table 2). The nonconservative substitution of Ala also maintained this activity. Although there was some loss of activity when Ser is substituted, this loss was not significant (data not shown), and considered with the nonconservative Val and Ala substitutions, it can be concluded that the side chain of this residue is not required for biologic activity.

The highly conserved nature of the Gly8 residue suggested that this residue may be required (Table 1). Deletion of Gly8 resulted in a reduction in the biologic activity of the molecule, but did not abolish it (Table 2). Furthermore, the nonconservative substitution of Asn for Gly8 resulted in a molecule that retained full activity. This indicates that Gly8 is not part of the active site of this peptide.

Met2 shows no interspecies homology for the six species evaluated (Table 1) and synthetic analogs in which Met2 was replaced by either tyrosine or lysine in the linear peptide showed no loss of biologic activity. Taken together, the data indicate that Glu1, Met2, Thr3, and Gly8 are not part of the pharmacophore, but it is likely that they contribute to the conformational stability of the molecule because they can be nonconservatively substituted without significant loss of activity. They probably function to maintain the linear molecule in a conformation that is conducive to binding to its receptor. The energy-minimized structure of the 8-amino acid Met-containing peptide has been previously published (4) and this molecule shows the potential to form a horseshoe shaped structure.

The substitution of Lys for Met2 was especially advantageous. Molecular modeling (not shown) indicated that the 8-amino acid molecule containing this substitution maintained the horseshoe structure seen previously with the methionine-containing molecule (4). Cyclization of the linear peptide containing the Met2Lys substitution (Fig. 6) produced a peptide that was far superior in synthetic outcomes with increased purity (Table 3). The optimized analog, *cyclo*[EKTOVNOGN] not only had anti-uterotrophic activity (Table 3) but also had oncostatic activity in that it inhibited the estrogen-stimulated growth of human breast cancer cells growing as xenografts in immune-deficient mice in a dose dependent manner consistent with that of *cyclo*[EMTOVNOGQ] (Fig. 5) (1).

The cyclic peptide is derived from AFP which itself has anti-estrogenic and anti-breast cancer activity. Sonnensch-

ein *et al.* (16) have demonstrated that the growth of an estrogen-dependent breast cancer is inhibited in rats bearing an AFP-secreting hepatoma. Tumor growth inhibition was not found when these investigators used an estrogen-independent breast cancer. Furthermore, Jacobson *et al.* (17) and Richardson *et al.* (18) have shown that elevated levels of AFP during pregnancy are associated with subsequent reduction in the lifetime risk for breast cancer. Jacobson and Janerich (19) have hypothesized that this reduction should be caused by a diminution in estrogen-dependent breast cancers. Bennett *et al.* (2,3) have shown that AFP purified from a human hepatoma culture and then injected into tumor-bearing immune-deficient mice stopped the growth of ER+, but not ER−, human breast cancer xenografts in these mice. Although AFP has demonstrated oncostatic activity (2), it has limited clinical usefulness. It has been shown to aggregate (20) and commercial preparations do not maintain biologic activity. The AFP used for the studies of Bennett *et al.* (2) utilized AFP isolated and purified from a hepatoma culture and the purification procedure is not trivial. Peptides produced using solid-phase Fmoc synthesis can be produced in high yield and purity in relatively large amounts, and the AFP-derived peptides have shown consistent activity over prolonged storage (1). Generation of a small, stable, biologically active peptide such as described here, and earlier (1,3,4), may suffice for investigations of the mechanism by which the peptide, and of AFP itself, stops the growth of tumor xenografts and exerts its anti-estrotrophic activity. Further, this peptide may be adequate for preclinical investigations as an oncostatic agent, especially if the peptide can be shown to have appropriate pharmacokinetics and low toxicity. At the very least, this cyclic peptide can serve as a model on which to develop peptidomimetics.

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