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13. Abstract ***(Maximum 200 Words)*** AFP is a glycoprotein produced during pregnancy by fetal yolk sac and by fetal liver. A fetal physiological level of human AFP inhibits estrogen-stimulated growth of human breast cancer. This antiestrogenic activity of AFP is localized in a 34-mer peptide ($AFP_{447-480}$). Studies suggest that this 34-mer peptide tends to aggregate upon storage, concomitant with loss of activity. A series of parsing and amino acids substitutions in the $AFP_{447-480}$ sequence was intended to identify the shortest analog which retained antiestrogenic activity. The data suggested that an octapeptide ($AFP_{472-479}$) is the minimal sequence required for the antiestrogenic activity found with the full-length AFP. $AFP_{472-479}$ aggregated during storage to form inactive species. Thus, a number of octapeptide analogs were produced with intent of minimizing aggregation and enhancing structural stability. Two octapeptide $AFP_{472-479}$ analogs exhibited superior stability and retained biological activity during prolonged storage. The first analog, EMTOVNOG, where O is 4-hydroxyproline, is a hydrophilic linear peptide that was generated by substituting two prolines with 4-hydroxyproline. The second analog, *cyclo-(EMTOVNOGQ)* was a hydrophilic and structurally constrained cyclic analog. These analogs exhibited improvement in shelf life over the native octapeptide. Further, these analogs inhibited the estrogen-dependent growth of human breast cancer growing as xenografts in SCID mice, but did not affect the estrogen-independent growth of human breast cancer xenografts. These analogs may become novel agents for the management of breast cancer.

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1. INTRODUCTION

Alpha-fetoprotein (AFP) is produced by the fetal liver and ultimately incorporated into the maternal circulation. The Jacobson hypothesis states that maternal serum AFP (MSAFP) reduces a woman's risk for breast cancer (1). A meta-analysis of data from epidemiological studies was used to test this hypothesis and showed that parous women were at a lower risk for breast cancer than were nulliparous women (2, 3). Further, those who were mothers of twins (receiving a double dose of AFP) had further 40 % reduction in breast cancer risk compared to mothers who had borne only a singleton. This phenomenon has been consistent with a number of other epidemiology studies which showed that maternal conditions which resulted in elevated levels of AFP were inversely associated with breast cancer risk (4-11).

The findings that AFP inhibited estrogen-dependent tumor growth were further supported by the fact that AFP interferes with estrogen-dependent growth of human breast cancer as xenografts as well as estrogen-dependent growth of mouse uterus. Bennett et al (12) reported that AFP, purified from hepatoma cells growing in culture, inhibited the estrogen-stimulated growth of estrogen receptor-positive MCF-7 and T47 human breast cancers, but did not affect the growth of estrogen receptor-negative MDA-MB-231 and BT20 human breast cancers.

The structural instability of AFP placed a strong limitation on the development of full-length AFP as a therapeutic agent for breast cancer treatment. Thus, an effort has been directed by the AFP Research Group of Albany Medical College toward finding the active site of the AFP molecule that is responsible for its ability to inhibit the growth of estrogen-stimulated breast cancer. Molecular biology tools were used to parse the AFP molecule into its domain and subdomains and Subdomain IIIAB AFP was found to have activity similar to full-length natural AFP (13). Further, synthetic peptides from the active Subdomain IIIAB were produced using solid phase peptide synthesis, and a 34-mer peptide $AFP_{447-480}$ was found to be active (14). Subsequent studies showed that AFP-derived peptides tend to aggregate upon storage, accompanied by loss of activity. During this predoctoral/postdoctoral training, an attempt was made to overcome this loss-of activity by designing better peptide analogs.

2. BODY

2.1.TRAINING

During the past three years of this training grant award, Dr. Fassil Mesfin, the recipient of the training grant, completed a number of tasks that resulted in the award of the MS and PhD degrees, multiple publications, and multiple research presentations at national meetings. During the first year of this training grant, Dr. Mesfin completed the didactic courses and passed the written and oral qualifying examinations and published one first-authorship manuscript, two abstracts, and one co-authorship manuscript. He also performed six intramural and two extramural research presentations. During the second year of the training grant, Dr. Mesfin completed the research work required for the award of PhD degree, wrote, and defended his dissertation. During this period he

published one first-authorship manuscript, two abstracts, and one co-authorship manuscript. He performed five intramural and one extramural research presentations. During the third year of the training grant, Dr. Mesfin spent most of his time on the development of AFP-derived octapeptide analogs that were active in the human breast cancer xenografts assays. He published one co-authorship manuscript. Dr. Mesfin has received numerous awards and honors for his research performance during his training. For three consecutive years (1999 -2001), he was named an AACR Minority Scholar in Cancer Research. Additionally, he was the recipient of the 2001's Dean's Prize for Excellence in Research, and the 2001 Dean's Certificate and Dr. Frank C. Ferguson Jr. award for Excellence in the Quality of Science. Most recently, Dr. Mesfin was named the 2002 Bristol-Myers Squibb Academic Medicine Fellow by the National Medical Fellowship organization.

2.2. RESEARCH

2.2.1. METHODS

All peptides were synthesized by using Fmoc solid phase peptide synthesis method. The peptides were purified using reverse phase high pressure liquid chromatography. The amino acid composition of the peptides was confirmed by amino acid analysis. The molecular weight of the peptides was determined by mass spectrometry. Possible three-dimensional structure of the peptides was analyzed using ChemSketch program based on CHARMM parameterization. The aggregation state of the peptides was assessed by gel-filtration chromatography. The biological activity of the peptides were determined by estrogen-stimulated immature uterine growth assay, T47 human breast cancer cell proliferation assay, and MCF7, T47, and BT20 human breast cancer xenografts growth assay. For details of any of the experimental procedures, please refer to the attached published papers.

2.2.2. RESULTS

Note: For figures please refer to the attached published papers (references 14-18)

The 34-mer AFP-derived peptide ($AFP_{447-480}$) was synthesized. As shown in our published article (14), the activity of both full-length AFP and the 34-mer peptide was diminished during storage in the lyophilized state. Gel-filtration chromatography indicated that the 34-mer peptide exhibited a very complex concentration and age-dependent aggregation behaviors (15, Fig 1-Fig 7). These aggregation behaviors were directly associated with loss of biological activity. A secondary structure analysis of the 34-mer peptide resulted in identification of sequence regions in the peptide that may play a role in the aggregation. Based on possible secondary structure of the 34-mer peptide (16), a number of 34-mer analogs were synthesized in attempt to generate a structurally stable analog that retained antiestrotrophic activity during storage (14, Table 1). But, the biological activity of all analogs were diminished as function of time of storage (14, Fig 5). These analogs, however, were quite informative in elucidating the regions of the

peptide that were not required for biological activity and in suggesting that the biological activity might be retained in a shorter sequence derived from the 34-mer peptide.

Truncated forms of the 34-mer were synthesized to identify the active site of the 34-mer peptide (14, Table 2). The first group of truncated analogs was synthesized as non-overlapping sequences of the 34-mer peptide. This group consisted of two 10-mer peptides (N-terminal end and middle) and one 14-mer peptide (C-terminal). In the estrogen-dependent immature mouse uterine growth assay, the 14-mer peptide exhibited significant antiestrotrophic activity, while the two 10-mer peptides did not (14, Table 2). In the next group of smaller analogs, a number of truncated peptide analogs that contained an overlapping sequence of the 14-mer peptide were synthesized in order to identify the minimal sequence required for antiestrotrophic activity. An octapeptide $AFP_{472-479}$ derived from the C-terminus of the 14-mer was found to be the minimal sequence that retains the antiestrotrophic activity of the full-length AFP.

It was hypothesized that amino acid substitutions in the 34-mer peptide $AFP_{447-480}$, or its truncation to the minimal amino acid sequence required for full activity (octapeptide $AFP_{472-480}$), would increase the storage stability of these peptides. As shown in our published article (14), the activity of an octapeptide $AFP_{472-479}$ decays more slowly than that of the native 34-mer peptide $AFP_{447-480}$, but both were without activity after 4-5 weeks in storage (14, Fig 5). None of the substitutions in the 34-mer peptide solved this storability problem (14, Table 1). The failure to retain activity during storage is not due to any chemical modification as was determined by mass spectrometry. Gel-filtration chromatography analysis of octapeptide $AFP_{472-479}$ indicated that it aggregated during storage. In addition, urea treatment of aged octapeptide $AFP_{472-479}$ restored significant antiestrotrophic activity in the estrogen-dependent immature mouse uterine growth assay (17, Table 1). These results strongly indicated that this octapeptide aggregated during storage and that urea treatment was able to restore the antiestrotrophic activity by generating biologically active peptide species. Therefore, it was hypothesized that increasing the hydrophilicity of an octapeptide might the aggregation potential of this peptide.

In order to generate a more hydrophilic linear octapeptide, EMTOVNOG (where O is 4-hydroxyproline) was produced. Comparison of the energy-minimized structures of octapeptide EMTOVNOG and the native octapeptide EMTPVNPG indicated that the hydroxyproline substitution did not alter the horseshoe shape. Like the native octapeptide, this hydrophilic octapeptide exhibited dose dependent inhibition of estrogen-stimulated growth of immature mouse uterus with the maximal effect at a dose of 1 ug (17). This result showed that substituting prolines to 4-hydroxyproline did not affect biological activity. Of critical importance, this hydrophilic octapeptide exhibited indefinite shelf-life as measured in the immature mouse uterine growth assay (17). Further, octapeptide EMTOVNOG inhibited the estrogen-dependent growth of MCF-7 human breast cancer xenografts (18) indicating that peptide activity extended to human breast cancer tissue. In addition, the peptide inhibited the growth of estrogen-dependent/tamoxifen resistant MCF-7 human breast cancer xenografts (18, Fig 1). The peptide did not affect the estrogen-independent growth of BT-20 human breast cancer xenografts (18).

In an attempt to synthesize a structurally constrained form of the hydrophilic octapeptide EMTOVNOG, *cyclo*-(EMTOVNOGQ) was synthesized by head-to-tail cyclization of the precursor nonapeptide. This analog incorporates conformational constraint as well as hydrophilic substitution of amino acids. *Cyclo*-(EMTOVNOGQ) exhibited dose dependent inhibition of estrogen-stimulated growth of immature mouse uterus with the maximum inhibitory activity at a dose of 10 ug per mouse (17, Fig 7). Interestingly, this peptide retained significant antiestrotrophic activity at doses higher than 10 ug/mouse, leading to a rather broad active dose range. There was no evidence of toxicity to the mice, even at a dose of 1 mg/mouse. Furthermore, storage experiments indicated that *cyclo*-(EMTOVNOGQ) had indefinite shelf-life (17, Fig 6). *Cyclo*-(EMTOVNOGQ) significantly inhibited the estrogen-dependent growth of MCF-7 human breast cancer xenografts (18, Fig 1)

The mechanism by which AFP-derived peptide was mediating its biological activity is now under investigation. As a first step in the study of mechanism, it seemed reasonable to explore whether these peptides behaved like classical estrogen receptor antagonist which are currently in clinical use. As shown in Figure 4 (18) AFP-derived peptide did not interfere with estradiol binding to its receptor over a broad peptide concentration range, whereas raloxifene completely inhibited this association at a concentration of 100 nM. Thus the mechanism of action of AFP-derived octapeptide is different from classical estrogen receptor antagonist and requires further investigation to uncover its biochemical target(s).

3. DISSCUSION

The data reported here reject the null hypothesis of my pre-doctoral training grant which stated that the native primary and secondary structures of the existing 34-mer peptide $AFP_{447-480}$ were necessary for the antiestrotrophic and anti-breast cancer activities associated with the peptide. The hypothesis was tested by completing three specific aims. The first specific aim was to investigate the conformational dynamics of 34-mer peptide in solution. The objective of this aim was to understand the physical characteristics of the peptide so as to determine which structural features contribute to loss of activity and to investigate the mechanism by which the antiestrotrophic activity was diminished during storage. The goal was to understand the physical and chemical characteristics of this peptide and to use this understanding in rational design approaches to generate analogs that would overcome the structural and/or chemical instability. The second specific aim was to design, synthesize, and characterize analogs of $AFP_{447-480}$. Design aspects included alterations of amino acid sequences intended to improve solubility, and avert aggregation by increasing hydrophilicity. The goal was to produce a structurally optimal analog of $AFP_{447-480}$ that does not lose antiestrotrophic activity upon storage. The third specific aim was to determine whether smaller segments of this 34-mer peptide retained biological activity and if the biological activity of the lead compound inhabited estrogen-dependent and estrogen-independent growth of human breast cancer xenografts. The ultimate goal was to determine the lead compound that exhibits anti-breast cancer activity *in vivo*.

During the past three years of this training grant, we have demonstrated that synthetic peptide corresponding to amino acid 472-479 of AFP had significant

antiestrotrophic activity. The shelf-life of this peptide was significantly prolonged by increasing its hydrophilicity, most especially with proline to 4-hydroxyproline substitutions. The data suggested that monomeric units of the peptide EMTPVNPG were aggregating during storage, leading to biologically inactive oligomers. By mass spectrometry analysis, there was no evidence of other possible changes such as asparagine deamidation, methionine oxidation, or pyroglutamate formation. In this octapeptide, the hydrocarbon side chains of proline and valine may have created a hydrophobic pocket that associated with like pockets on adjacent monomers resulting in aggregation during storage. Increasing the hydrophilicity by substituting proline to 4-hydroxyproline apparently diminished this hydrophobic interaction since the biological activity of 4-hydroxyproline containing peptide did not diminish as a function of time in storage. This aggregation behavior has been seen with full length AFP (19) and with subunits of AFP (13). Wu et al. (19) showed that AFP tends to form aggregates, which may contribute to its loss of activity during storage. Eisele et al. (15) reported that oligomers of various sizes formed during storage of a 34-mer (amino acids 447-480) peptide derived from AFP. Similar aggregation behavior has been seen with a number of other protein and peptide pharmaceuticals including human interferon gamma (20), human calcitonin (21), insulin (22), and synthetic beta-amyloid peptide (23-25). Hughes et al. (26) and Hilbich et al. (27) had shown the inhibition of amyloid peptide aggregation by increasing hydrophilicity either by substitution of hydrophobic phenylalanine to hydrophilic threonine or by adding poly-lysine at the carboxy-terminal of the peptide.

Aggregation has been and continues to be a problem in the development of protein- or peptide-based pharmaceuticals. One way of dealing with this problem has been with the use of excipients (28). Although the data are not shown here, we evaluated a variety of excipients as cryoprotectants and lyoprotectants of the native AFP-derived octapeptide. Mannitol as well as dodecylmaltoside significantly prolonged shelf-life of the peptide whereas sucrose was not beneficial in prolonging its shelf-life. However, there are complicating factors associated with the use of excipients. Their presence can confound studies of mechanism of action of the primary agent, which is more troublesome during peptide development than during clinical use. Therefore, peptide design approaches were utilized to improve bioactivity and to extend the shelf-life of the peptide.

It was thought that cyclization of this peptide would limit its flexibility and thereby add to its structural stability. It was also thought that increasing the hydrophilic nature of the peptide would be useful. The combination of these two approaches was remarkably effective. The cyclic, hydrophilic analog, *cyclo*-(EMTOVNOGQ), has optimal biological activity and indefinite shelf-life. What was even more beneficial about this analog was that it changed the shape of the dose/response curve. With linear peptide the shape of the curve was biphasic with 1.0 ug/mouse being the optimal inhibitory dose. At higher doses linear peptide showed reduced antiestrotrophic activity. "Biphasic" dose/response profiles are not unusual with growth regulatory agents. Certainly estrogen itself is biphasic with lower doses stimulating growth and higher doses actually retarding growth (29). Similarly, angiotensin II (30), glucagon-like peptide (31), D-globin-derived synthetic peptide (32), and other protein pharmaceuticals are biphasic in that the optimal biological response modifying dose is less than and yields more biological activity than the maximally tolerated dose. However, with cyclo-

(EMTOVNOGQ) the shape of the dose/response curve was hyperbolic, with 1.0 ug up to 1.0 mg per mouse providing similar antiestrotrophic activity. This greatly expands the active dose range. One explanation for this is to invoke the possibility that there is one high affinity receptor for cyclized peptide but two cointeractive receptors (high affinity and low affinity) for linear peptide. The high affinity receptor would load first leading to an antiestrotrophic response, but as the amount of peptide is increased and high affinity receptor becomes saturated, then low affinity receptor loads with linear peptide (but not with cyclic peptide) and counteracts the effect of the high affinity receptor. Although we have not yet studied the receptors for peptide, it has been reported that there are two receptors for the peptide's parent protein, AFP (33). Continuing this line of thinking, it is possible that the cyclized peptide is fixed in a conformation that reacted only with the high affinity receptor. Clearly the receptor(s) for these peptides need to be studied and that work is underway, but the current data support the conclusion that the cyclized peptide is preferable to the linear peptide because of its expanded effective dose. A toxic dose has not yet been reached with either peptide. This is not surprising since the maximum dose given is 1 mg/mouse and the fetal physiological blood level of AFP is in the range of 3 mg/ml (34).

AFP-derived octapeptide is a novel agent in that it is not cytotoxic like most standard cancer chemotherapeutic agents. It is an estrogen receptor antagonist like tamoxifen (30). It will not deplete estrogen levels like inhibitors of estrogen synthesis (anastrozole). It will not bind and sequester estrogen (24). It is likely that we have discovered an anti-breast cancer agent a unique mechanism. This will open up new therapeutic possibilities for not only this agent, but also other agents with may interfere with the putatively unique mechanistic pathway.

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5. APPENDIES

5.1.Research Accomplishments

- AFP-derived peptides aggregate during storage in lyophilized state.
- AFP-derived octapeptide is the minimal sequence required for the antiestrotrophic activity of full-length AFP.
- The octapeptide inhibited the growth of estrogen-dependent human breast cancer in cell culture and *in vivo*.
- Like AFP and AFP-derived agents, the activity of the octapeptide diminished during storage in lyophilized state.
- AFP-derived octapeptide loses the antiestrotrophic activity via aggregation during storage.
- Hydrophilic octapeptide analogs have indefinite shelf-life. The analogs inhibit estrogen-dependent growth of human breast cancer xenografts.
- Cyclic, hydrophilic octapeptide analog retains improved shelf-life and has an extended active dose range.

5.2.Reportable Outcomes

5.2.1. Manuscripts

1. Bennett JA, **Mesfin FB**, Andersen TT, Gierthy JF, Jacobson HI. A peptide derived from alpha-fetoprotein prevents the growth of estrogen-dependent human breast cancers sensitive and resistant to tamoxifen. Proc Natl Acad Sci U S A. 2002 Feb 19;99(4):2211-5.
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5.2.2. Abstracts and Presentations

1. **Mesfin FB**, Bennett JA, Zhu SJ, Jacobson HI, Andersen TT. Novel Analogs of an Anti-Breast Cancer Octapeptide. Proc. Am. Ass. For Cancer Research, 42:778-779, 2001

2. Bennett JA, **Mesfin FB**, Zhu SJ, Gierthy JF, Jacobson HI, Andersen TT. An Alpha-Fetoprotein-Derived Peptide Inhibits Estrogen-Receptor-Positive Breast Cancers, Sensitive and Resistant to Tamoxifen. Proc. Am. Ass. For Cancer Research, 42:238, 2001
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4. **Mesfin FB**, Bennett JA, Jacobson HI, Zhu S, Andersen TT. Alpha-fetoprotein-derived antiestrotrophic octapeptide. American Association for Cancer Research Special Conference on the Steroid Receptor Superfamily, Palm Spring, CA January 1999.

5.2.3. Degrees obtained that are supported by this award

1. Albany Medical College awarded Master of Science Degree in Biomedical Sciences to Fassil B. Mesfin in May 2000.
2. Albany Medical College awarded Doctor of Philosophy Degree in Biochemistry to Fassil B. Mesfin in May 2001.

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Alpha-fetoprotein-derived antiestrotrophic octapeptide

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Abstract

Alpha-fetoprotein (AFP) is a major serum protein produced during fetal development. Experimental findings suggest that AFP has antiestrotrophic activity and that it can be developed as a therapeutic agent to treat existing estrogen-dependent breast cancer or to prevent premalignant foci from developing into breast cancer. The antiestrotrophic activity of AFP was reported to be localized to a peptide consisting of amino acids 447–480, a 34-mer peptide termed P447. A series of parsings and substitutions of amino acids in the P447 sequence was intended to identify the shortest analog which retained antiestrotrophic activity. Peptides related to P447 were generated using solid phase peptide synthesis. Several shorter peptides, including an 8-mer called P472-2 (amino acids 472–479, peptide sequence EMTPVNPG), retained activity, whereas peptides shorter than eight amino acid residues were inactive. The dose-related antiestrotrophic activity of AFP-derived peptides was determined in an immature mouse uterine growth assay that measures their ability to inhibit estradiol-stimulated uterine growth. In this assay, the maximal inhibitory activities exhibited by peptide P472-2 (49%), by peptide P447 (45%), and by intact AFP (35–45%) were comparable. The octapeptide P472-2 was also active against estradiol-stimulated growth of T47D human breast cancer cells in culture. These data suggest that peptide P472-2 is the minimal sequence in AFP, which retains the antiestrotrophic activity found with the full-length molecule. The synthetic nature and defined structure of this 8-mer peptide suggest that it can be developed into a new drug which opposes the action of estrogen, perhaps including the promotional effects of estradiol in the development of human breast cancer.

Keywords: Alpha-fetoprotein; Synthetic octapeptide; Breast cancer

1. Introduction

Alpha-fetoprotein (AFP) is a glycoprotein produced during pregnancy by the fetal yolk sac and by fetal liver and is a major protein constituent of fetal plasma throughout gestation [1]. It has a molecular weight of approximately 69 kDa and has 39% primary structural homology to albumin [2]. It has been proposed that the tertiary structure of AFP is composed of three domains based on its disulfide bonding pattern [3]. In studies designed to assess the regulation of breast cancer growth by AFP [4–13], we have reported that full-length AFP, isolated from a variety of sources including a human hepato-

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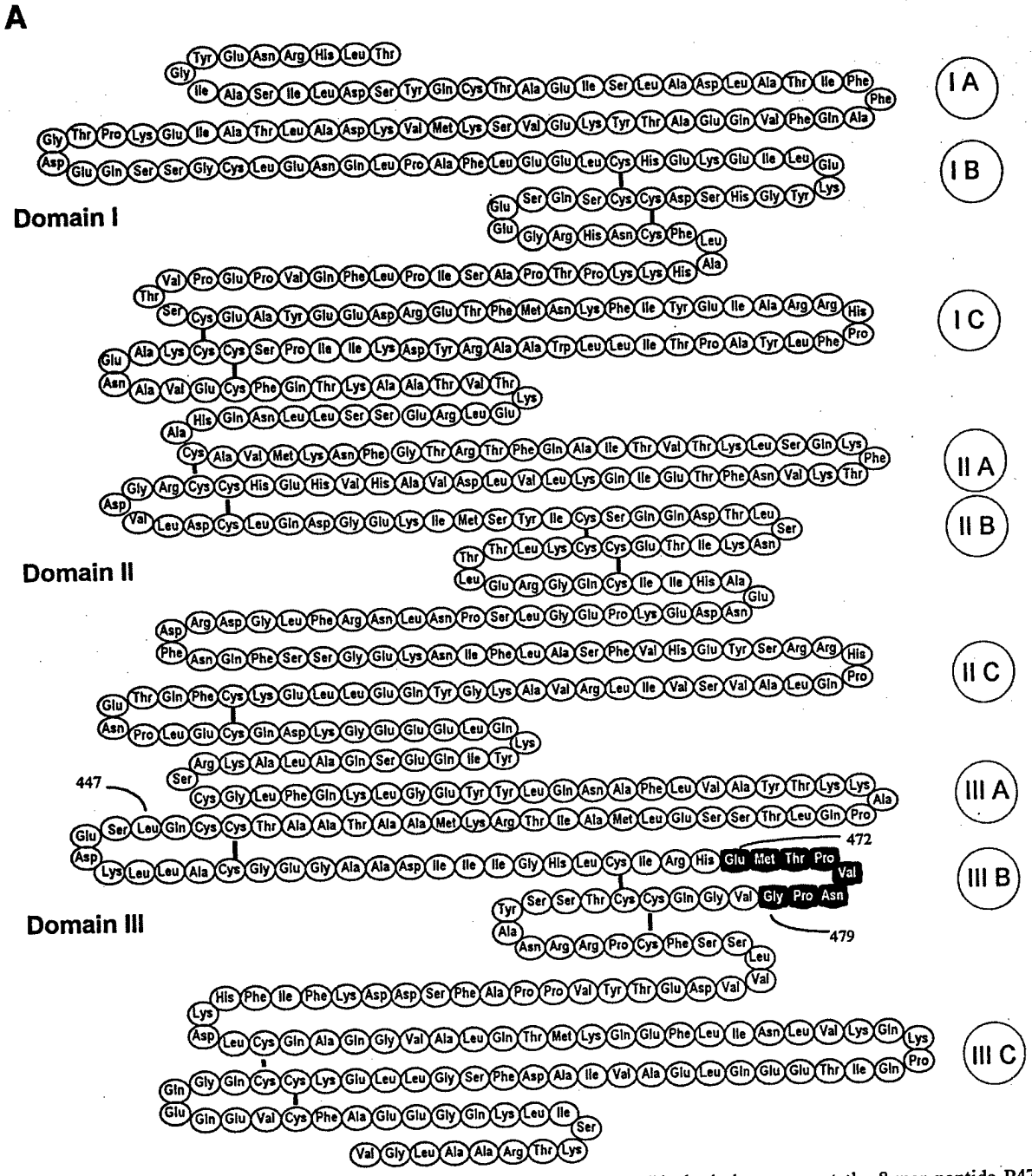

Fig. 1. (A) The primary structure, domain and subdomain designation of AFP. Black circles represent the 8-mer peptide P472-2. The 8-mer peptide is the minimal sequence required for antiestrotrophic activity. (B) Energy-minimized structure of peptide P472-2. Amino acids are labeled using three-letter code. The energy minimization calculations were done with ChemSketch program based on CHARMM parameterization.

ma cell line (HepG-2), stopped growth of estrogen-dependent breast cancers, but did not inhibit the growth of estrogen-independent breast cancers when these were growing as xenografts under the kidney capsule of immune-deficient (SCID) mice [4].

Part of our effort has been directed toward finding the active site of the AFP molecule that is responsi-

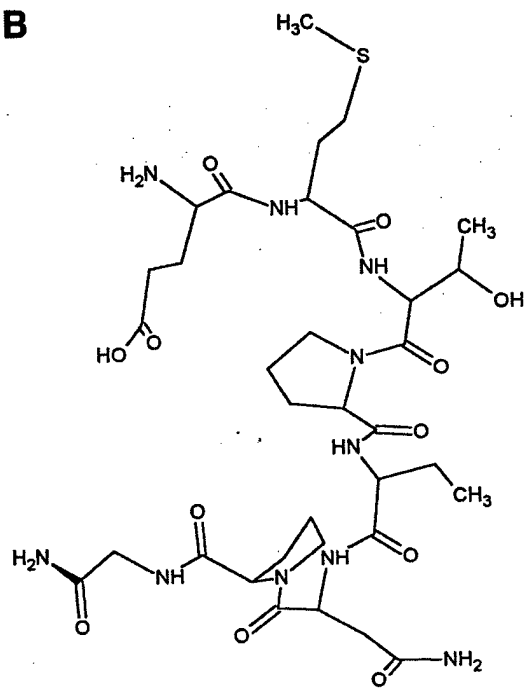

Fig. 1 (*continued*).

ble for its ability to inhibit the growth of estrogen-stimulated breast cancer. Molecular biology tools were used to parse the AFP molecule into its domains and subdomains (Fig. 1A). Domain III and subdomain IIIAB were found to be active [10,11]. Synthetic peptides from the active subdomain of domain III were generated using solid phase peptide synthesis, and a 34-mer peptide (amino acids 447–480) was found to be active [14]. In this paper, we report that an AFP-derived octapeptide comprised of amino acids 472–479 (Fig. 1) arising from the carboxy-terminus of the above 34-mer peptide (amino acids 447–480) is the minimal sequence required for antiestrotrophic activity (Fig. 1).

2. Materials and methods

2.1. Secondary structure prediction and homology search

Potential secondary structures of peptide P447 were predicted using Predictprotein, an internet-based program which reportedly predicts secondary structure of proteins and solvent accessibility at better than 70% accuracy [15]. In addition, a sequence homology assessment was performed using the protein data bank of Research Collaboratory for Structural Bioinformatics. Energy minimization computations were done with ChemSketch program. The program produces energy-minimized structure based on CHARMM parameterization [16]. Note that the program is not a full-scale molecular mechanics engine. Its design aims were to reliably reproduce reasonable conformations of 3D structure.

2.2. Peptide synthesis

Peptides were synthesized using Fmoc solid phase peptide synthesis on a Pioneer Peptide Synthesis System (PerSeptive Biosystem, Inc.). Briefly, peptides were assembled on a solid support (Fmoc–polyethylene–graft polystyrene support) from the C-terminus, reacting the deblocked N-terminus of support-bound amino acid with the activated C-terminus of the incoming amino acid to form an amide bond. Amino acids used in the synthesis had their N^{α}-amino group protected by the 9-fluorenylmethoxycarbonyl (Fmoc) group, which was removed by piperidine at the end of each cycle in the synthesis. Side-chain protecting groups of amino acids were Arg(Pbf), Asn(Trt), Asp(OtBu), Gln(Trt), Glu(OtBu), His(Trt), Lys-(tBoc), Ser(tBu), Thr(tBu), Cys(Trt), which were deprotected by trifluoroacetic acid (TFA) after peptide synthesis. The carboxyl group of the amino acid was activated with HATU [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] obtained from PerSeptive Biosystems Inc. The specific amino acid derivatives, supports, and reagents used in the synthesis were purchased from PerSeptive Biosystems Inc., Framingham, MA, USA.

Peptides were cleaved from the solid support (polyethylene–graft polystyrene) using TFA. After synthesis was completed, the resin was washed three times with 100% methanol and the cleavage reaction was initiated by incubating the resin in 10 ml TFA/thioanisole/anisole/1,2-ethanedithiol (90:5:2:3) per 0.5 g resin for 5 h (or other appropriate conditions). The cleavage reaction mixture was filtered using a sintered glass funnel to separate the solid resin from the peptide solution. Filtrate volume was reduced to 1 ml with a gentle stream of air and the peptides were precipitated by addition of 15 ml dry-

ice chilled ethyl ether. The peptides were allowed to settle for 5 min at −80°C, and the supernatant was aspirated. The peptides were then washed twice in similar manner with 15 ml of ethyl ether. After three further washes with 15 ml of 1.5:1 ethyl acetate: diethylether (room temperature), the peptides were dissolved in deionized water and lyophilized.

2.3. Purification of peptides

Lyophilized peptides were dissolved in deionized water and subjected to reversed-phase chromatography with C18 Sep-Pak Cartridges (Waters Inc.), or to gel filtration on Sephadex G-25. Subsequently, peptides were lyophilized, dissolved in deionized water and further purified using analytical or semi-preparative reversed phase HPLC.

2.4. Peptide characterization

Amino acid analysis of all peptides was performed using the Waters AccQ-Tag amino acid analysis system [17,18]. Additionally, peptides were analyzed by mass spectrometry using standard α-cyano-4-hydroxysinnipinic acid and sinnipinic acid matrices.

2.5. Immature mouse uterine growth assay

A bioassay for antiestrotrophic activity was performed according to Mizejewski et al. [19]. Swiss/Webster female mice, 6–8 g in body weight (13–15 days old), were obtained from Taconic Farms (Germantown, NY). Mice were weighed and distributed into treatment groups (typically five mice per group) such that each group contained the same range of body weight. In a typical experiment, each group received two sequential intraperitoneal injections 1 h apart. Test material or vehicle control for that material was contained in the first injectant. Estrogen or vehicle control for estrogen was contained in the second injectant. Twenty-two hours after the second injection, uteri were dissected, trimmed free of mesenteries, and immediately weighed. The uterine weights were normalized to mouse body weights (mg uterine weight/g of body weight) to compensate for differences in body weight among litters of the same age. Experiments employed a minimum of five mice per group, and the mean normalized uterine weights and standard error for each group were calculated. Percent growth inhibition in a test group was calculated from the normalized uterine wet weights according to Eq. 1. Groups were considered to be significantly different at $0.05 \geq P$, employing the non-parametric one- sided Wilcoxon sum of ranks test.

$$\text{Growth inhibition (\%)} = \frac{\text{Full } E_2 \text{ stimulation} - E_2 \text{ stimulation in test group}}{\text{Full } E_2 \text{ stimulation} - \text{No } E_2 \text{ stimulation}} \times 100 \quad (1)$$

2.6. T47 cell proliferation assay

A confluent culture of T47D human breast cancer cells growing in culture was released from monolayer by trypsinization (0.25% trypsin and 0.25% EDTA). Stock T47D cells were suspended in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dextran/charcoal-treated fetal bovine serum (DCFBS). Cells were then seeded into 6 well plastic tissue culture plates at a density of 1×10^5 cells/well in 4 ml of medium. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were depleted of steroids by replenishment of DCFBS-supplemented medium every other day for 5 days. After steroid depletion, estradiol (10 nM E_2), vehicle, or E_2 with various concentrations of test agents were added with medium change every other day for 9 days. At the end of each experiment, cells were released from monolayer by trypsinization, diluted in a 1:1 ratio of 0.1% trypan blue, and placed in a hemocytometer and counted. Wells were set up in triplicate for each group. Mean viable cell numbers and the standard error were calculated for each group.

3. Results

The 34-mer AFP-derived peptide described by Mizejewski et al. [14] was synthesized. It contained amino acids 447–480 of AFP (Fig. 1). Mass spectrometry analysis showed the expected molecular weight of the peptide and amino acid analysis re-

vealed that it had the expected amino acid composition. As shown in Fig. 2, it significantly inhibited estrogen-stimulated growth of immature mouse uterus, and its optimal dose was 1.0 μg per mouse.

Potential secondary structures of the 34-mer AFP-derived peptide (amino acids 447–480 in AFP) were assessed using Predictprotein [15], which suggested that the amino acids in sequence positions 461–471 of peptide P447 have potential to form extended β-sheet structure, while the amino- and the carboxy-termini have potential to form a random coil structure. Predictprotein predicted no potential for α-helix structure. Peptide P447 has two cysteine residues (C455, C468), which have the potential to form intra- or inter-peptide disulfide bonds. These observations led us to produce a number of P447 analogs with amino acid substitutions designed to alter or eliminate the potential β-sheet structures or to prevent disulfide formation (Table 1).

The specific rationales for designing analogs of P447 were:

1. To prevent disulfide bond formation. Analogs in this category included 34-mer peptides (P447-1, P447-2, P447-3), in which substitutions for the two cysteines were made (Table 1).
2. To reduce the β-sheet content. Analogs in this category include two 34-mer peptides (P447-2, P447-3), in which cysteine was substituted with aspartate or proline, respectively (Table 1). Aspartate substitution was expected to reduce potential β-sheet structure formation and to increase the solubility of the peptide, while proline substitution was expected to reduce potential β-sheet structure formation and increase random coil structure. These substitutions also eliminated the possibility of disulfide bond formation as did substitution with alanine to produce analog P447-1.

Biological activities of the peptides were evaluated using the immature mouse uterine growth bioassay as described in Section 2. AFP-derived 34-mer peptides P447, P447-1, P447-2, and P447-3 exhibited significant inhibitory activity (Table 1). However, their activities were reduced after storage for 3–4 weeks (data not shown). Importantly, P447-1 is active and contains no cystine or cysteine. Those residues are therefore not essential for antiestrotrophic activity, and disulfide bond formation is evidently not the cause of loss of that activity during storage. In addition, substitution that would disrupt possible β-sheet structure in peptides P447-2 and P447-3 did not abolish the biological activity, which suggests that β-sheet structure might not be essential for activity. Circular dichroism is not especially revealing for peptides as small as these, undoubtedly because they can access so many different conformations in solution. Similarly, secondary structure analyses of peptides this small are usually unrevealing for the same reason, and this is the case with these peptides. Further, any relationship between the solution conformation(s) of the peptides and the conformation they may adopt when bound to the receptor is unknown. Nevertheless, these results suggested that the antiestrotrophic activity might be retained by smaller analogs of the 34-mer peptides.

3. Truncated forms of the 34-mer were synthesized

Table 1
Antiestrotrophic activity and sequence of AFP-derived 34-mer peptides

Test agent	% Inhibition of E_2-stimulated growth of immature mouse uterus	Peptide sequence
Peptide P447	441*	LSEDK LLACG EGAAD IIIGH LCIRH EMTPV NPGV
Peptide P447-1	39*	LSEDK LLAAG EGAAD IIIGH LAIRH EMTPV NPGV
Peptide P447-2	45*	LSEDK LLADG EGAAD IIIGH LDIRH EMTPV NPGV
Peptide P447-3	30*	LSEDK LLAPG EGAAD IPIGH LPIRH EMTPV NPGV

Peptide 447 is an AFP-derived 34-mer peptide; P447-1 is an alanine-substituted 34-mer analog (C455A, C468A); P447-2 is an aspartate-substituted 34-mer analog (C455D, C468D); P447-3 is a proline-substituted 34-mer analog (C455P, I463P, C468P). The optimal inhibitory dose of peptide (1 μg) per mouse was given by the i.p. route. The optimal stimulatory dose of E_2 (0.5 μg per mouse) was given by the i.p. route 1 h after injection of test agent. Twenty-two hours later uteri were dissected, weighed, and compared to uterine weights from no E_2 (negative control) or E_2 alone (positive control) groups.

*Significant inhibition, $P < 0.05$; Wilcoxon sum of ranks test.

Table 2
Antiestrotrophic activity and sequence of AFP-derived small peptides

Test agent	% Inhibition of E_2-stimulated growth of immature mouse uterus	Peptide sequence
Peptide P447	44*	LSEDK LLACG EGAAD IIIGH LCIRH EMTPV NPGV
Peptide P447-4	10	LSEDK LLADG
Peptide P457	0	EGAAD IPIGH
Peptide P467-1	40*	LAIRH EMTPV NPGV
Peptide P471	38*	H EMTPV NPGV
Peptide 472-1	40*	EMTPV NPGV
Peptide P472-2	49*	EMTPV NPG
Peptide P472-3	20	EMTPV NP
Peptide P472-4	5	EMTPV
Peptide P473	0	MTPV NPG
Peptide P474	0	TPV NPGV

P447-4 is an aspartate-substituted 10-mer analog (C455D); P457 is a proline-substituted 10-mer analog (I463P); P467-1 is alanine substituted 14-mer analog (C468A); P471 is a 10-mer analog; P472-1 is a 9-mer analog; P472-2 is an 8-mer analog; P472-3 is a 7-mer analog; P472-4 is a 5-mer analog; P473 is a 7-mer analog missing N-terminal residue of the P472-2; P474 is a 7-mer analog missing two N-terminal residues of P472-2 and addition of C-terminal valine. The optimal inhibitory dose of peptide (1 μg) per mouse was given by the i.p. route. The optimal stimulatory dose of E_2 (0.5 μg per mouse) was given by the i.p. route 1 h after injection of test agent. Twenty-two hours later uteri were dissected, weighed, and compared to no E_2 (negative control) or E_2 alone (positive control). *Significant inhibition, $P < 0.05$; Wilcoxon sum of ranks test.

to identify the active site of peptide P447. Analogs in this category included two 10-mer peptides (P447-4, P457) and one 14-mer peptide (P467-1). The two 10-mer peptides P447-4 and P457 were derived from the amino-terminus and middle of 34-mer peptide P447, while a 14-mer peptide P467-1 was derived from the carboxy-terminus of 34-mer peptide P447. The 14-mer peptide P467-1 exhibited significant antiestrotrophic activity while 10-mer peptides P447-4 and P457 did not (Table 2). Therefore, the antiestrotrophic site was thought to reside within sequence position 467–480.

4. To identify the minimal sequence required for antiestrotrophic activity, truncated forms of the 14-mer 467-1 were synthesized. Analogs in this category included a 10-mer peptide (P471), a 9-mer peptide (P472-1), an 8-mer peptide (P472-2), three 7-mer peptides (P472-3, P473, P474), and a 5-mer peptide (P472-4). All the peptides in this category were derived from the carboxy-terminus of 14-mer peptide P467-1 (Table 2).

Peptide P472-2 is an 8-mer peptide from the C-terminus of the 14-mer, and this small peptide exhibited significant antiestrotrophic activity (Table 2). However, peptides smaller than the 8-mer, P472-3 (7-mer missing the 8-mer C-terminal residue), P473 (7-mer missing N-terminal residue of the 8-mer), P474 (7-mer missing two N-terminal residues of the 8-mer and having an additional C-terminal residue),

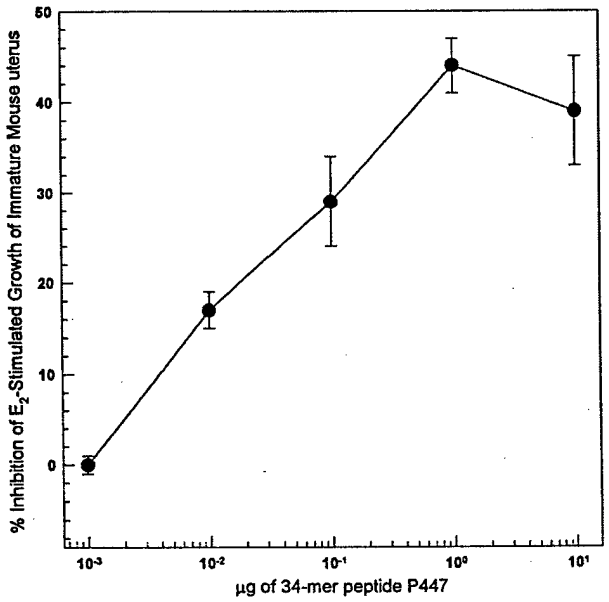

Fig. 2. Dose-dependent antiestrotrophic activity of AFP-derived 34-mer peptide P447. These results demonstrate that AFP-derived 34-mer peptide significantly inhibited estrogen-stimulated growth of immature mouse uterus, and its optimal dose was 1.0 μg per mouse.

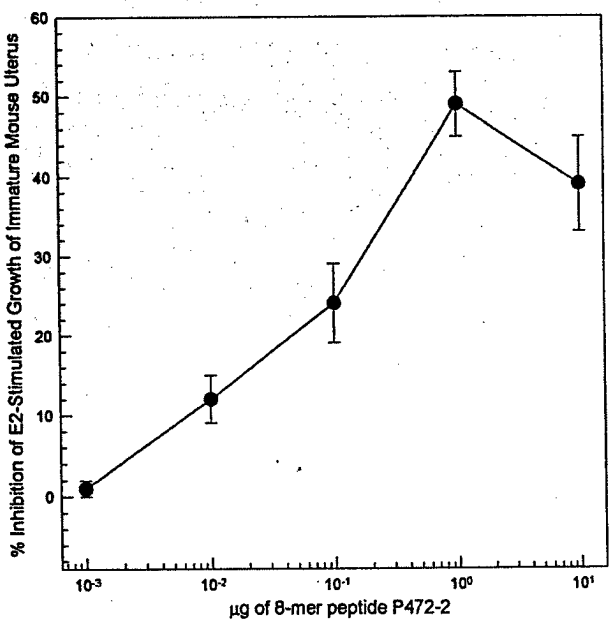

Fig. 3. Dose-dependent antiestrotrophic activity of AFP-derived 8-mer peptide P472-2. These results demonstrate that AFP-derived 8-mer peptide significantly inhibited estrogen-stimulated growth of immature mouse uterus, and its optimal dose was 1.0 μg per mouse.

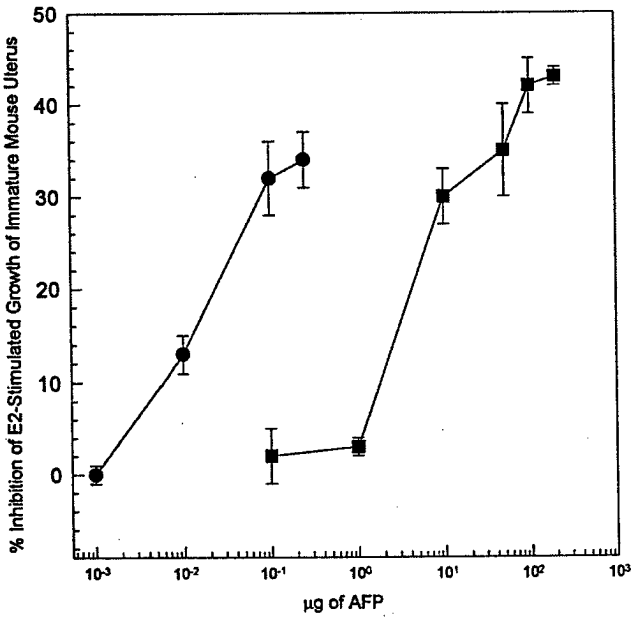

Fig. 4. Dose-dependent antiestrotrophic activity of intact AFP (■) and E_2-transformed AFP (●). These results demonstrate that intact AFP and AFP/E_2 (AFP preincubated with a molar excess estradiol) are antiestrotrophic. Transformed AFP shifts the dose–response curve two logs to the left and mimics the dose–response curve of the peptides.

and P472-1 (5-mer peptide missing three C-terminal residues of the 8-mer) were all without significant inhibitory activity (Table 2). These results indicated that the 8-mer peptide (amino acids 472–479 of AFP) is the minimal sequence from AFP that retains the antiestrotrophic activity of the full-length molecule. A dose–response curve of this peptide is shown in Fig. 3. It is virtually superimposable on the dose–response curve of the 34-mer peptide P447 (Fig. 2) suggesting no loss of activity as a result of truncating the larger peptide.

Interestingly, in comparing the dose–response curves of the two peptides (Figs. 2 and 3) to that of AFP (Fig. 4), it is apparent that the peptides are active at a dose 100-fold lower than AFP. However, as previously reported by our group [4,6,7], preincubation of AFP with a molar excess of estradiol shifts its dose–response curve two logs to the left (Fig. 4) which now mimics the dose–response curve of the peptides. We have speculated that the active site of AFP was more accessible to its receptor in the presence of estradiol, perhaps through an estrogen-mediated conformational change in AFP which alleviated some steric hindrance in placing its active site in apposition to the receptor [11–13]. It would appear

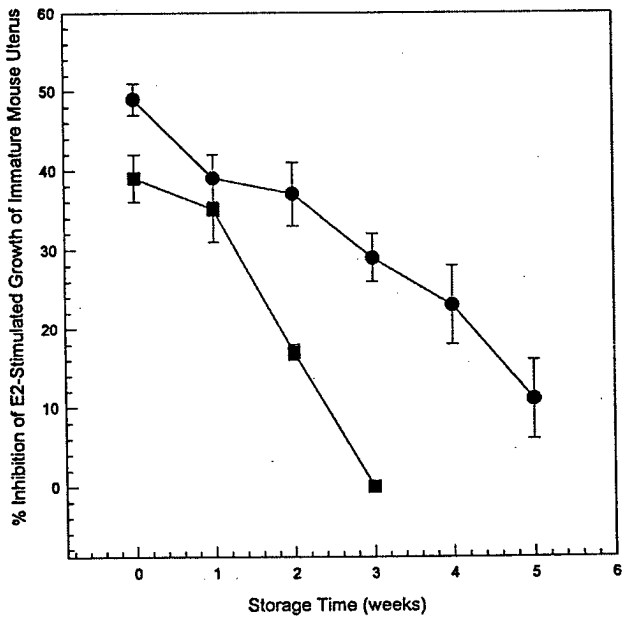

Fig. 5. The antiestrotrophic activity of octapeptide (●) and 34-mer peptide (■) following storage. Peptides were lyophilized and stored at 4°C. These results show that the antiestrotrophic activity of peptides was diminished upon prolonged storage.

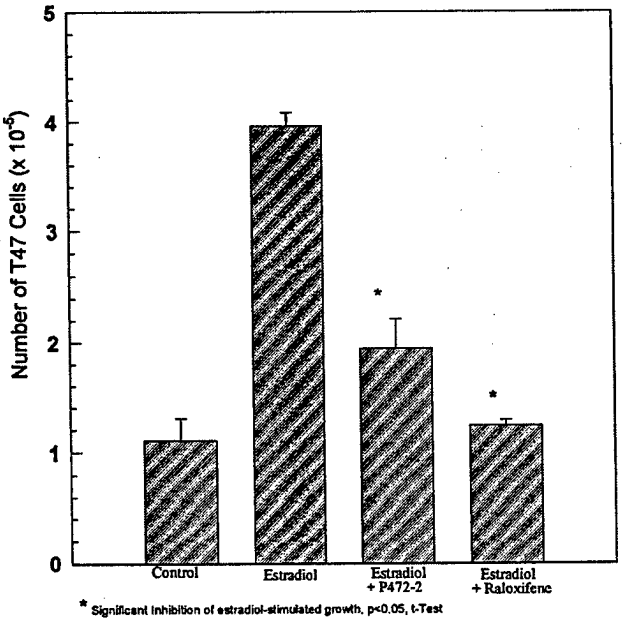

Fig. 6. Growth inhibitory activity of peptide P472-2 against T47D human breast cancer cells growing in culture. The concentration of estradiol (E_2), peptide, and raloxifene (LY156758) were 10 nM, 10 nM, and 100 nM respectively. For peptide and raloxifene these were their maximal inhibitory concentrations. These results show that AFP-derived octapeptide P472-2 inhibits estrogen-stimulated growth of T47D human breast cancer cells in culture.

that such hindrance is not present in either the 34-mer peptide or the 8-mer peptide, and in fact preincubation of these peptides with a molar excess of estradiol did not alter their antiestrotrophic activity (data not shown, [14]). On a molar basis it appears that the estradiol-transformed AFP is more active than the peptides. This may be due to differences in pharmacokinetics and/or active site stability.

It was hoped that the amino acid substitutions in the 34-mer and/or its truncation to the minimal amino acid sequence required for full activity (8-mer, P472-2) would increase the storage stability of these peptides. As shown in Fig. 5, activity of the 8-mer peptide decays more slowly than that of the native 34-mer peptide. None of the substitutions in the 34-mer peptide solved this storability problem. The failure to retain activity during storage is not due to any chemical modification as assessed by mass spectrometry, and is likely attributable to aggregation of these peptides even in their lyophilized state. This aspect of development of these peptides is still under investigation.

The anti-breast cancer activity of the octapeptide was assessed against estradiol-stimulated growth of T47D human breast cancer cells in culture. As shown in Fig. 6, AFP-derived octapeptide inhibited T47D growth by 65% (similar to 34-mer peptide P447, data not shown). Raloxifene (LY 156758) produced almost 100% growth inhibition of these cells in culture.

4. Discussion

The data reported herein demonstrate that an octapeptide, P472-2 (amino acids 472–479 of AFP), possessed the antiestrotrophic activity of full-length AFP. Festin et al. [10,11] had previously shown that this activity was contained in the third domain of AFP (amino acids 386–592), and Mizejewski et al. [14] showed the activity resided in a 34-mer peptide (amino acids 447–480) from domain III of AFP. This 8-mer peptide is a substantially truncated form of the 34-mer and yet retains significant activity. Smaller regions within this 8-mer were without activity. A homology search revealed a five-amino acid sequence (EMTPV, amino acids 472–476 of AFP) that was found in the tumor suppressor protein E-cadherin. This 5-mer peptide was therefore synthesized and tested, and it showed no significant antiestrotrophic activity. The AFP-appropriate amino acids were added to the carboxyl end of this peptide and no significant activity was obtained until the 8-mer (amino acids 472–479 of AFP) was reached. Thus the 8-mer is the minimal sequence required for antiestrotrophic activity, and this appears to be the active site of AFP that imparts this activity to the full-length protein.

Full-length AFP, domain III AFP and the AFP-derived peptides described herein lose activity during storage. Wu et al. [20] reported that AFP tends to form aggregates which may contribute to its loss of activity. Lai et al. reported that peptides are prone to chemical degradation pathways even upon storage in the solid state [21]. Possible chemical modifications of the 8-mer peptide P472-2 might include covalent modifications such as deamination of asparagine, oxidation of methionine, or N-terminal pyroglutamate formation, each of which could be detected by mass spectrometry analysis. Mass spectrometry analysis of the 8-mer peptide P472-2 when freshly prepared, or

after storage in the solid state for over 3 months, indicated the expected molecular weight of 842. This result suggested that the 8-mer peptide P472-2 was free of these chemical modifications and that chemical modification was not the cause of activity loss during storage. In addition to chemical instability, peptides may be prone to physical instability that alters their biological activity [22]. Preliminary studies suggest that the 8-mer peptide and the 34-mer AFP-derived peptides exhibited physical instability, through aggregation. It was noted from gel filtration chromatography profiles that the 34-mer peptide P447 (the parent peptide of P472-2) would readily form dimers, trimers, and large molecular weight aggregates (unpublished observation). This aggregation process of the 34-mer peptide in the lyophilized state resulted in a time-dependent loss of biological activity as assessed by the immature mouse uterine growth bioassay. Preliminary data suggest that the 8-mer, like the 34-mer, forms aggregates during storage in the lyophilized state. Further chemical modifications of the peptide may be necessary to prevent aggregation and concomitant loss of activity during storage.

The mechanism by which AFP and the peptides derived therefrom produce their antiestrotrophic effect is not known. Bennett et al. [4] suggested that sequestering of estrogen by AFP is unlikely because human AFP does not have a high-affinity binding site for estrogen. That the 8-mer retains antiestrotrophic activity further supports this concept because it is highly unlikely that such a small molecule could sequester significant amounts of estrogen. Moreover, the inhibitory mechanism is not like that of raloxifene or tamoxifen in that AFP does not block the binding of estrogen to the estrogen receptor; nor is it like that of letrozole because AFP does not reduce the serum estrogen levels [4]. It is certainly different from cytotoxic chemotherapy, because all evidence has shown AFP to be cytostatic and non-toxic [4]. Although the mechanism of both AFP and AFP-derived peptide is not yet known, when elucidated it is likely to be novel since the more obvious processes described above were not implicated.

AFP is found in the serum of pregnant women, and term pregnancy reduces a woman's future breast cancer risk. Epidemiological studies have been performed to test the hypothesis that AFP in maternal serum (MSAFP) is the agent that reduces risk. Several studies have examined surrogate variables that are known to be associated with elevated serum levels of AFP. It was conjectured that twin pregnancies, in which MSAFP is double that for singleton pregnancies, should generate a much greater reduction of breast cancer risk. Using data from the Cancer and Steroid Hormones Study [23], Jacobson et al. [24] examined the reproductive histories (parity and incidence of twins) of 3918 women who were newly diagnosed with breast cancer and of 4097 control women. As expected, parous women were found to be at lower risk for breast cancer than were nulliparous women. More noteworthy, those who were mothers of twins had a further 40% reduction in risk compared to mothers who had borne only a singleton. Elevated MSAFP is also a characteristic of women who are hypertensive during pregnancy or who are bearing a fetus with an open neural tube defect. Epidemiologic investigations have found that in both of these problem pregnancies, the woman's subsequent breast cancer risk was reduced below that which follows a normal pregnancy [25,26]. Recently, Richardson et al. [27] have reported that the concentration of AFP in cryogenically stored maternal sera was inversely correlated to the risk of breast cancer in these mothers 20–30 years after their pregnancies. These findings support the contention that the action of AFP in maternal serum is preventing appearance of many breast malignancies. In rats, pregnancy reduces the incidence of *N*-nitroso-*N*-methylurea-induced mammary tumor [28]. Sonnenschein et al. [29] have shown that hepatomas secreting AFP resulted in inhibition of estrogen-dependent growth of rat mammary tumors. This suggests that AFP-derived peptides may be developed as agents for breast cancer prevention in women. This speculation is supported by the fact that full-length AFP inhibited the estrogen-stimulated growth of estrogen receptor-positive MCF-7 and T47 human breast cancer xenografts, but did not affect the growth of estrogen receptor-negative MDA-MB-231 and BT20 human breast cancers [4]. It is noteworthy that the AFP preparations which completely inhibited estrogen-stimulated growth of MCF-7 human breast cancer xenografts had 35–45% growth inhibitory activity in the immature mouse uterine growth assay [4]. This suggests that the AFP-derived octapeptide

P472-2, which had antiuterotropic activity in the 40–50% range, will have a potency similar to that of AFP against human breast cancer xenografts. Studies to confirm this speculation are under way.

AFP-derived octapeptide should be more readily adapted for use in the clinic for the treatment of estrogen receptor-positive human breast cancer than full-length AFP. The primary structure of the 8-mer peptide is small relative to full-length AFP (8 vs. 590 amino acids). The peptide is easier to produce. Moreover, since it is a relatively small molecule it will be easier to design analogs. Furthermore, peptides or analogs can be produced in large quantities in forms that are stable on storage. Additionally, peptidomimetic analogs may be produced that would be effective in the treatment of human breast cancer. Since AFP is a natural product, therapeutic agents derived from it may be less toxic than some of the therapeutic agents currently used to treat breast cancer. Furthermore, since almost all breast cancers initially develop as estrogen receptor-positive cells and are estrogen-driven, AFP-derived peptides may be valuable for preventive as well as for therapeutic management of human breast cancers. Thus, further development of this 8-mer peptide appears to be indicated because of its compelling potential to be a novel agent for the management of breast cancer and for the reduction of breast cancer risk in women.

Acknowledgements

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Studies on analogs of a peptide derived from alpha-fetoprotein having antigrowth properties

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Abstract: A 34-amino acid portion of the third domain of alpha-fetoprotein possesses antigrowth and anticancer activities. Three analogs of this sequence were chemically synthesized, in which the two cysteines of the original sequence were replaced by alanines, glycines or serines. The original cysteine and alanine peptides formed trimers at 0.20 g/L in pH 7.4 phosphate buffer, and the glycine and serine peptides formed dimers. Trimer preparations were more potent in inhibiting estrogen-induced growth in the mouse uterine assays than the two dimeric oligomers. Of salient importance is that the alanine peptide retained its trimeric form in solution much longer than the cysteine peptide. Antigrowth assays were performed starting with stock solutions at a peptide concentration of 0.20 g/L, because at very high peptide concentration (8.0 g/L) the peptides aggregated extensively. All the peptides, although differing in biological activity, had almost identical secondary structures. Unlike alpha-fetoprotein, the three peptides have low amounts of α-helix. Trifluoroethanol has the ability to convert peptides into a helical conformation when they have a propensity for that structure. At trifluoroethanol concentrations of 20% and higher, the alanine and glycine peptides were changed into highly helical structures.

Alpha-fetoprotein is synthesized by the human fetus and can be found circulating from there throughout the serum of pregnant women. After birth, the concentration of the protein decreases to a low value that is maintained throughout life. Some tumors produce higher alpha-fetoprotein concentrations. Human alpha-fetoprotein consists in

a single polypeptide of ≈ 68 000 Da molecular mass and is glycosylated at a single site. The protein is composed of three domains. It binds various molecules in the performance of its functions (1–5). A number of conditions causes changes in the conformation of the protein (5–14).

The genesis of this work arises from the important observation that purified alpha-fetoprotein has anticancer activity that can be greatly augmented by treating this protein with estrogen in large molar excess prior to its use as an anticancer agent (15, 16). In the presence of high molar excesses of estrogen, alpha-fetoprotein and its third domain undergo conformational change (6). The estrogen-transformed alpha-fetoprotein and its third domain have greatly increased growth-inhibitory and anticancer properties (6,15–17). These properties of transformed alpha-fetoprotein may explain an exciting epidemiological proposal that pregnancy, when brought to term, offers some protection to women from breast cancer for many years after pregnancy (18–23).

A 34-amino acid peptide has been chemically synthesized by copying a sequence from the third domain of human alpha-fetoprotein. The peptide exhibits the antigrowth function of the transformed protein (24). Antibodies to the peptide recognize a site on the estrogen-transformed alpha-fetoprotein, but not on nontransformed protein (25). This discovery strongly suggested that the peptide is linked to the anticancer function of its parent protein.

The sequence of the peptide is LSEDKLLACGEGAADIIIGHLCIRHEMTPVNPGV (24). There are two cysteine residues, which in buffer at peptide concentrations of 0.10–0.20 g/L apparently slowly form disulfide bonds (26). The resulting peptide, containing disulfide bonds, loses its growth-inhibitory property in the mouse uterus growth assay (26). In addition to the apparent formation of the disulfide bonds, the peptide undergoes a change in oligomer status from trimer to dimer.

Analogs and fragments of this peptide have also been synthesized chemically (25,27). Certain of these fragments have appreciable antigrowth abilities (25,27). Mesfin *et al.* (27) examined the effects of shelf life on the biological activities found with certain of these peptides, and synthesized analogs with significantly prolonged activity.

It was of interest to chemically synthesize peptide analogs in which both the cysteines were replaced, so that there would be no possibility of losing biological function through disulfide bond formation, or in the change in oligomerization that accompanies the formation of the disulfide bonds (26). Three analogs were prepared in which the two cysteines were both replaced by alanines, glycines or serines. The alanine and glycine peptides were studied most extensively, and the serine peptide was examined only selectively.

Studying the behaviors of these peptides may lead to an improved understanding of how alpha-fetoprotein functions as a growth-inhibitory agent, and may also help develop pharmacological agents to fight cancer. The effects of amino acid substitution in the peptides on their biophysical and biological properties may yield a better insight into the development of more effective peptide analogs for the treatment of cancer. In addition, it is of interest to study a usually hidden sequence of a protein that, upon surface exposure through a conformational change, affects the function of the protein.

Experimental Procedures

The glycine and serine peptides were synthesized chemically at Albany Medical College and the alanine peptide was made at the Wadsworth Center; all were synthesized using Fmoc solid-state chemistry. The glycine and serine peptides were made using a Pioneer Peptide Synthesis System (PerSeptive Biosystem, Inc.) and the alanine peptide was made using the 431A peptide synthesizer from Applied Biosystems. A typical peptide synthesis protocol consisted of assembling a peptide on an Fmoc-polyethylene-graft polystyrene support from the C-terminus, and reacting the deblocked N-terminus of the support-bound amino acid with the activated C-terminus of the incoming amino acid to form the peptide bond. The Fmoc group was removed after each cycle by piperidine. Side-chain protecting groups were removed using trifluoroacetic acid after peptide synthesis. The carboxyl group of the amino acid activated with o-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium. After cleavage from the solid support, the three peptides were purified by preparative reverse-phase high-performance liquid chromatography.

Purification of the synthetic peptide from this crude preparation for this project was accomplished using a Delta-Pak C_{18} reverse phase column from Waters. The column used a bonded phase packing material, which was synthesized with a pore diameter of 300 Å. The column dimensions were 19 mm in diameter and 30 cm in length. A 650E liquid chromatography system (Waters) equipped with a 486 tunable absorbance detector and a 600E system controller was used. Elution was monitored at 230 nm. The flow rate was 7 mL/min. The column was operated from 0 to 4 min in 0.1% trifluoroacetic acid in water followed by a 45-min linear gradient formed by the addition of

a 0.1% trifluoroacetic acid in acetonitrile. The selected elution band was lyophilized, dissolved in water and lyophilized again. The resulting yields of purified peptide were in the range 30–40 mg per synthesis batch.

The molecular masses of the peptides were determined by mass spectrometry at the Biological Mass Spectrometry Core at the Wadsworth Center. The purified peptides had a single species of the correct molecular mass in these experiments. For example, the alanine peptide has an expected molecular mass of 3507.9 Da and the result obtained was 3507.3 Da. There were no other significant ions observed by mass spectrometry over a very flat baseline. Protocols for mass spectrometry were the same as those described previously (24). Amino acid sequencing supported the correct sequences for these peptides using protocols described previously (24).

Gel-filtration column chromatography was used to separate peptide aggregates and to obtain their molecular masses. A Waters 625 liquid chromatography system equipped with a 717 autosampler and a Waters Protein Pak 200 SW column with dimensions of 8.0×300 mm was employed. The flow rate was 0.8 mL/min. The column was operated isocratically using pH 7.4 buffer (0.1 M NaCl and 0.01 M $NaPO_4$) at room temperature, 24–25°C. This buffer was used in all experiments except those involving trifluoroethanol. Elution was monitored at 230 nm. The molecular masses used for calibration of the column were 2350, vitamin B12; 17 000, myoglobin; 44 000, ovalbumin; and 158 000, gamma globulin (Bio-Rad). The standards were run daily. The void volume was detected with Dextran Blue. The molecular masses of the peptide aggregates were determined from the elution times by use of a calibration curve obtained from the standards, and the aggregation state was obtained by dividing the molecular masses by the monomer molecular mass of each peptide.

Circular dichroism was performed using a JASCO J720 spectropolarimeter. Circular dichroism experiments were performed with solutions at 0.20 mg/mL. Usually a 0.02-cm light path was used, and experiments were at a controlled 25°C. Most experiments were performed in pH 7.4 phosphate buffer with saline, with the exception that no saline was present in the trifluoroethanol experiments. NaCl was not used in these experiments to allow circular dichroism spectra to be taken to near 180 nm. In addition, the presence or absence of NaCl allowed some study of the effect of ionic strength on these solutions. Secondary structure was calculated using the Selcon method (28).

The immature mouse uterine growth assay was based on the finding that the injection of 0.5 μg 17β-estradiol into 14–18-day-old female Swiss mice results in a 70% increase in uterine wet weight with parallel increases in the mitotic index in 22 h. This relatively quick assay is useful for assessing agents that would interfere with estrogen-stimulated growth. Immature female Swiss mice (15–18 days old, Taconic Farms, Germantown, NY, USA) were injected with various quantities of the alpha-fetoprotein-derived peptide, whereas mice in a control group were injected with buffer. The various peptides were dissolved in buffer shortly before injection into the mice. One hour after the first injection, test and positive control mice were injected with 0.5 μg 17β-estradiol, whereas negative control mice were given 0.1 mL buffer. Sacrifice occurred 22 h after the second injection; the uteri were dissected, trimmed free of mesenteries, and immediately weighed. Uterine weights were normalized to mouse body weights (mg uterine weight/g of body weight) to compensate for differences in body weight among litters of the same age. Each experiment was performed with five animals per group, and the mean normalized uterine weights ± SE for each group were calculated. Percent growth inhibition was calculated using the following relationship:

(positive control group – test group)/(positive control group – negative control) × 100.

Animals were sacrificed using protocols approved by the Institutional Animal Care and Use Committee of Albany Medical College. Statistical analysis utilized the Wilcoxon sum rank test.

Results and Discussion

Peptide solutions were prepared at room temperature at 0.20 mg/mL of peptide in pH 7.4 buffer from purified and lyophilized peptides. A gel-filtration column chromatography system was used to obtain their molecular masses (Fig. 1). When these values were divided by the corresponding molecular masses of the peptides, it was found that the alanine peptide was a trimer and the glycine and serine peptides were dimers. The oligomer findings were: for the alanine peptide 3.03 ± 0.16, for the serine peptide 1.98 ± 0.07 and for the glycine peptide 2.16 ± 0.26.

The original cysteine peptide had interesting behavior at 0.20 mg/mL in this same buffer. Early after dissolving the peptide in buffer, it was found to be a trimer. After several hours, the trimers gradually converted to dimers, which lacked antigrowth activity in the mouse uterus assay. The dimers were found to have disulfide bonds as indicated by

the effects of reducing agent on their stability (26). Because they lacked cysteines, the oligomers of the serine, glycine and alanine peptides remained stable in solution for at least 24 h (data not shown). This stability would be a benefit in the development of anticancer drugs.

At 8.0 mg/mL, the alanine and glycine peptides partially aggregated to a very large size (data not shown). This large aggregate was in the approximate molecular mass range of 530 000–550 000 Da for the alanine and glycine peptides. This lack of homogeneity at this concentration indicated that high-resolution NMR experiments to obtain the conformation of the peptides probably would not be successful. The large aggregates themselves appeared to change their molecular masses with time (data not shown). The oligomeric states of the small molecular mass bands were 3.52±0.03 for the alanine peptide and 2.35±0.36 for the glycine peptide, and these results might suggest that at the higher peptide concentration the trimers and dimers were partially aggregated into larger oligomers that eluted with them.

It is interesting that very conservative replacements of amino acids would result in changes in the peptide's oligomeric state. One possibility is that the different peptides have different secondary structures and this variation promotes a change in oligomers.

Using circular dichroism, the secondary structures of these peptides were determined (Fig. 2). The circular dichroism spectra of the three analogs, the alanine, glycine and serine peptides, were found to be almost identical. Analysis of the circular dichroism showed a small α-helix content (Table 1).

Hydrophobicity can be a key factor in peptide–peptide interaction. The order of hydrophobicities of these amino acids is Cys>Ala>Gly>Ser (29). The higher hydrophobicities for the Cys and Ala amino acids correlate with the occurrence of trimeric oligomers.

The peptides all show little α-helix in their secondary structures (Table 1). In contrast, alpha-fetoprotein is highly α-helical. Most recently, the secondary structure of alpha-fetoprotein was determined to be 65% α-helix and 0% β-sheet (7). There are two possibilities for this difference in structure, either these peptides are derived from an extremely atypical part of the protein, or the peptides

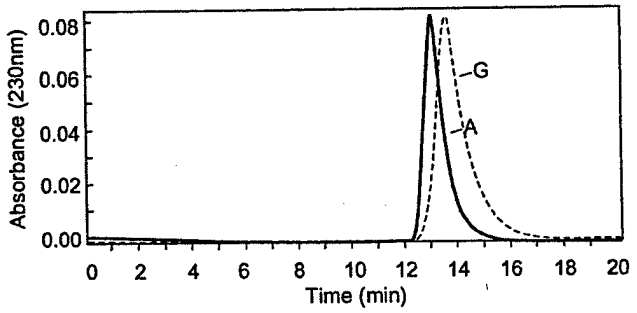

Figure 1. Gel-filtration column chromatography of the alanine and glycine peptides at 0.20 mg/mL. Samples were run at ambient temperatures, 25–26°C, in an isocratic solvent, pH 7.4 phosphate buffer, containing 0.1 M NaCl. The two peptides were run in the same column on the same day to insure the validity of this comparison.

Table 1. **Secondary structures of the alanine, glycine, and serine peptides in pH 7.4 buffer with saline**

Peptide	α-Helix (%)	β-Sheet (%)	Turn (%)	Other (%)
Alanine peptide	9.2	33.5	28.0	28.1
Glycine peptide	9.6	34.0	27.5	27.9
Serine peptide	10.3	34.1	27.3	29.1

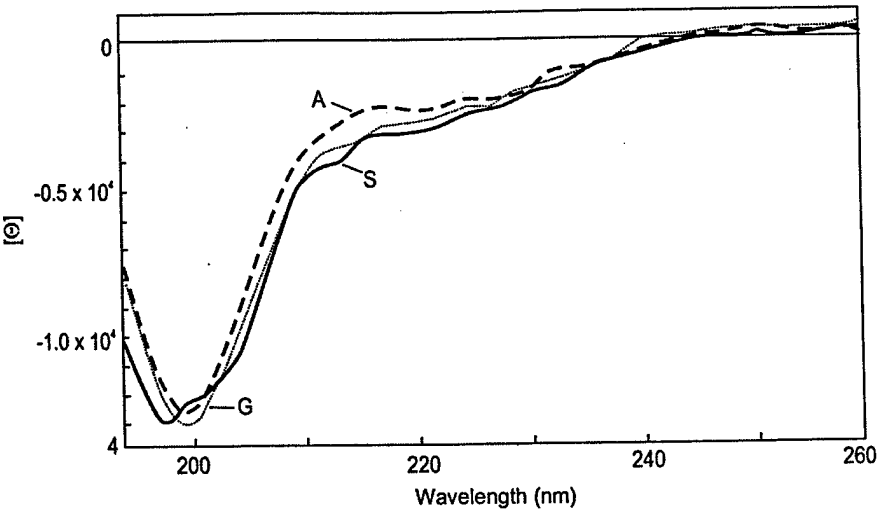

Figure 2. CD spectra of the alanine, glycine and serine peptides at 0.20 mg/mL. The temperature was 25°C, and the buffer was pH 7.4 phosphate(0.1 M NaCl).

become altered in secondary structure when they are not a part of the protein.

Trifluoroethanol has been used extensively in the study of a variety of peptides. Its action is to promote the formation of α-helical structure in a variety of peptides and proteins (e.g. 30–35). Often peptides have α-helical structure when part of an intact protein, but lose this structure as free peptide (31).

Because the peptides under study are derived from a highly α-helical protein, the use of trifluoroethanol is warranted. Circular dichroism studies were carried out on both the alanine and glycine peptides in trifluoroethanol (Figs 3 and 4). Analysis of the circular dichroism showed that both peptides adopted a high α-helical content in the alcohol (Table 2). This result suggested that the original peptide when existing as a sequence in alpha-fetoprotein might possibly be in a highly α-helical form.

The glycine analog was less affected by trifluoroethanol than the alanine analog (Fig. 4). Perhaps the unique ability of a glycine to exist in a great number of conformations, because of the lack of steric hindrance provided by its hydrogen atom side-chain, lessens the inclination of this residue to be placed in the highly ordered environment of an α-helix. Thermodynamic measurements have shown that alanine has the greatest inclination to be included in a helix, and glycine is one of the least favored for helix inclusion (36,37).

The largest change to helix comes at 20% trifluoroethanol, and this change is mostly produced by reduction in β-sheet (Table 2). Further conversions to helix can come from changes in all three structural elements. No isochroic points were observed.

Most experiments with these peptides were carried out in a pH 7.4 buffer containing 0.1 M NaCl (Fig. 1, Table 1). Circular dichroism spectra were also obtained at pH 7.4 in the absence of NaCl (Table 2). The secondary structures of the alanine and glycine peptides were essentially the same at both salt concentrations (Tables 1 and 2).

The alanine peptide had statistically significant antigrowth activity in the mouse uterus assay. In this assay, estrogen stimulates uterine growth, and the peptides are tested to measure whether they retard this estrogen effect. The glycine peptide had much less antigrowth activity than the alanine peptide (Table 3). The serine peptide had no observed growth inhibitory ability in the mouse uterus

Table 2. **The effect of trifluoroethanol on the secondary structures of the alanine and glycine peptides glycine peptide**

Trifluoroethanol (%)	α-Helix (%)	β-Sheet (%)	Turn (%)	Other (%)
Glycine peptide				
0	8.4	34.2	26.4	28.9
10	10.1	32.7	25.6	28.3
20	22.5	22.5	26.8	27.3
30	29.3	19.3	27.2	24.3
50	27.1	18.7	29.8	23.9
90	33.4	17.4	25.1	22.1
Alanine peptide				
0	11.7	31.6	29.5	28.9
10	18.0	26.6	27.6	28.2
20	32.8	14.8	27.3	24.8
30	36.1	15.7	27.0	20.6
50	38.4	13.7	28.8	19.3
90	43.4	15.0	19.8	20.7

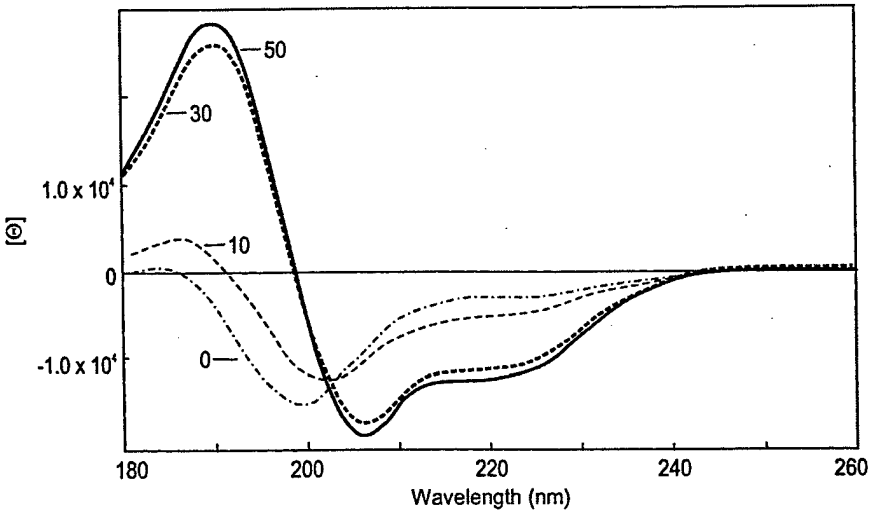

Figure 3. CD spectra of the alanine peptide in trifluoroethanol. The numbers on the curves are the percentages of trifluoroethanol. In addition to the alcohol, sodium phosphate buffer, pH 7.4 was present but there was no NaCl in these solutions. In all solutions, the peptide concentration was 0.20 mg/mL.

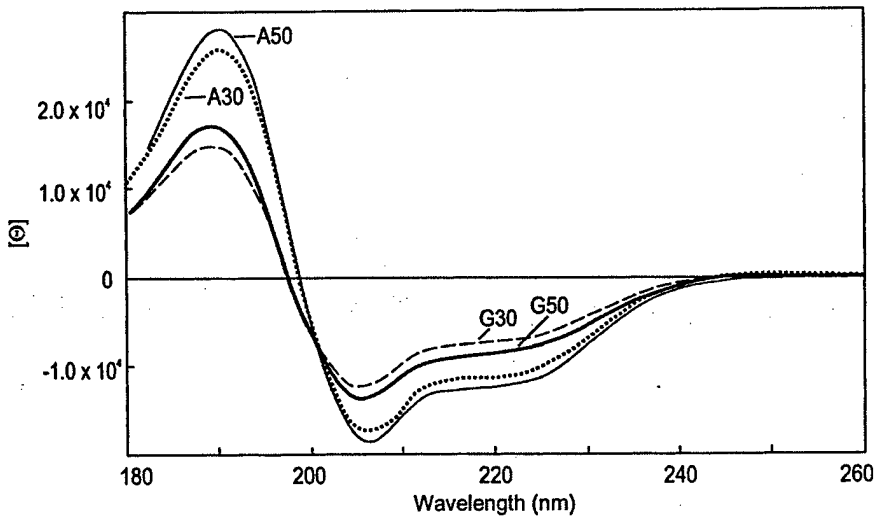

Figure 4. CD comparison of the effect of trifluoroethanol on the alanine and glycine peptides under the same conditions.

***Table 3.* Effect of various peptides on estrogen-stimulated growth of the immature mouse uterus**

Peptide[a]	Per cent inhibition of estrogen-stimulated growth of the immature mouse uterus[b] (mean ± SE)
Cysteine	34±5
Alanine analog	37±4
Glycine analog	17±2
Serine analog	0

a. Mice received 1 μg of peptide in a volume of 0.2 mL buffer.
b. Twenty-two hours after injection of 0.5 μg estrogen in a volume of 0.2 mL buffer, the uterine weights were evaluated and percent inhibitions were calculated as described in Experimental Procedures.

assay. It is important to note that the initial concentration of peptides used in these assays was 0.20 g/L, and these peptide solutions were greatly diluted when an optimal dose of 1 μg of peptide was injected into the mice. The optimal dose for the cysteine peptide was determined previously (24,25,27) and this result was used to determine dosage for these assays.

In the study of the cysteine peptide (26), it was not known whether the formation of disulfide bonds or the conversion of trimers to dimers was the direct cause of the loss in antigrowth ability. Dimers of the glycine and serine peptides both have low antigrowth properties, but they, of course, do not have any disulfide bonding. It is therefore possible that the cysteine peptide lost biological activity because it became a dimer. One very speculative possibility is that dissociation of the peptide into monomers is a key step in the expression of antigrowth activity, and trimers might have the ability to dissociate more readily than dimers.

It was of interest that the hydrophobicity of the amino acid replacements seemed to correlate well with the oligomerization. The change between dimers and trimers was not dependent on major changes in the properties of these residues, and it should be pointed out that the hydrophobicity differences among the four replacement amino acids were fairly modest. Oligomerization changes have been reported for other systems in which only very mild changes were made by a single amino acid substitution (e.g. Ref. 38). The two types of dimers, with or without accompanying disulfide bonds, are both less active than trimers in the mouse uterus assay, but these different types of dimers might vary in their activities in anticancer assays.

Most notable, the alanine peptide is very effective in inhibiting estrogen-induced growth in the mouse uterine assay, as well as being a more stable oligomer in solution over time than the cysteine peptide. Further study on such analogs is therefore indicated. Finally, it is observed that these peptides are derived from a human protein and have the chance of being well accepted by the immune system if used as anticancer agents.

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Studies on a growth-inhibitory peptide derived from alpha-fetoprotein and some analogs

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Abstract: A 34-amino acid synthetic peptide was derived from the third domain of human alpha-fetoprotein, and the peptide was shown to inhibit estrogen-stimulated growth. Under certain conditions, however, the peptide lost growth-inhibitory activity. A biophysical study of the peptide was undertaken with a goal of obtaining completely reliable preparations. The peptide was studied using gel-filtration column chromatography as a function of peptide concentration and age of solution, and was found to exhibit complex aggregation behaviors. During the early period (0–3 h) after dissolving lyophilized peptide into pH 7.4 buffer, solutions were composed mostly of trimers. At higher peptide concentrations (≥3.0 g/L), the trimers aggregated extensively to a large aggregate (minimum size ≈ 102 peptides). At 5.0–8.0 g/L, these large aggregates increased in size (up to ≈ 146 peptides) until trimers were largely exhausted from solution. During the later times (>3 h) after sample preparation, the trimeric oligomer of the peptide dissociated slowly to form dimers for samples at 0.10–3.0 g/L. After their build-up, a very small number of dimers associated to form hexamers. Disulfide bonds stabilized the dimers as indicated by the conversion of dimers to trimers upon the addition of a reducing agent, and the failure of dimers to form in the presence of reducing agent. Reducing agent did not affect trimer or large aggregate formation. Trimers were found to be active in an assay monitoring inhibition of estrogen-stimulated growth, whereas dimers and large aggregates were inactive. The two cysteines in the peptide were modified to either *S*-methylcysteine or *S*-(2-aminoethyl)cysteine, and both derivatives showed significant growth-inhibition activity. A serine analog in which both

cysteines were replaced had very different aggregation behavior than the cysteine peptide and lacked its growth inhibitory ability. Peptide aggregation is critically important in establishing the ability of the peptide to inhibit growth and have anticancer activity, but the state of its two cysteines is of little influence.

Alpha-fetoprotein is found in humans as a serum glycoprotein having 590 amino acids, divided into three domains, and the protein has 4% carbohydrate. The protein is found in fetal and maternal sera during pregnancy and the amount of protein decreases after birth to the relatively low levels found in healthy people. Elevated amounts of alpha-fetoprotein may also appear associated with tumors. Many of its properties have been investigated (1–19), including complex formation with unsaturated fatty acids, hormones, bilirubin, retinoic acid, metal ions, estradiol and many drugs, and in addition to transport processes alpha-fetoprotein has other physiological functions. Unsaturated fatty acids, but not saturated fatty acids, are tightly bound to alpha-fetoprotein and produce conformational changes in the protein, which delivers the unsaturated fatty acids to locations critical to fetal development (16–18). The functions of the protein may be linked to the sundry conformations that can be induced by the binding of various molecules. Complete removal of ligands from alpha-fetoprotein produces a large conformational change (20,21).

A novel function for the protein was discovered by the inclusion of high concentrations of estrogen into alpha-fetoprotein solutions (3,4,8), which also induces a conformational change in the protein (11). This conformational change generates a protein with a much greater growth-inhibitory effect than is found for untreated alpha-fetoprotein (3,4,8,11). These growth inhibitions are found for both breast cancers (4) and in a mouse uterus system (3,8), and these effects depend on reaction of the estrogen and protein prior to their introduction into the assays.

Smaller segments of alpha-fetoprotein, also, possess this growth-inhibitory activity including the third domain (11) and a chemically synthesized peptide derived from a small portion of the third domain (2). The peptide consisted of 34 amino acids from amino acid 447 to 480 of alpha-fetoprotein, and had a molecular mass of 3573 Da. The sequence of its amino acids is LSEDKLLACGEGAADIIIGHLCIR-HEMTPVNPGV. This synthetic peptide was found to retard growth (2), and provides a potential route for examination of the little-studied growth-retardation properties of alpha-fetoprotein. In cultures of human MCF-7 breast cancer cells, the peptide in low concentrations largely inhibited development of foci, which are mounds of cellular overgrowth (2). These results raised the possibility that the peptide possesses anticancer activity because these foci signify a loss of cell contact inhibition (22). Unlike the situation with alpha-fetoprotein, the peptide did not require treatment with estrogen prior to application to the biological system (2). It may be speculated that this peptide represents a hidden site, a neoepitope, on alpha-fetoprotein that is exposed via a conformational change brought about by the application of high concentrations of estrogen. The peptide also showed growth-inhibitory effects in frog metamorphosis (23). The mechanism by which the peptide inhibits growth is unknown. Studies of this peptide will contribute to a better understanding of one aspect of the function of alpha-fetoprotein and may lead to therapy for certain cancers.

As this peptide was studied, there were conditions in which its growth inhibition was reduced (unpublished results). In this study, the aggregation of the alpha-fetoprotein-derived peptide was studied as a function of peptide concentration and age of solution, and, in addition, the effects of covalently modifying both cysteines and replacing both cysteines in the peptide with serines were examined. The aggregation behavior was seen to be complex, and the aggregation state of the peptide was shown to correlate with its growth-inhibition activity.

Experimental Procedures

Peptides were synthesized and purified to homogeneity as described previously (2). The synthesis was performed in 1993. The crude peptide was lyophilized and quantities were purified as needed. Purification of the synthetic peptide from this crude preparation for this project was accomplished using a Delta-Pak C-18 reverse phase column from Waters. The column used a bonded phase packing material which was synthesized with a pore diameter of 300 Å. The column was 19 mm in diameter and 30 cm in length. A 650E liquid chromatography system (Waters) equipped with a 486 tunable absorbance detector and a 600E system controller were used. Elution was monitored at 230 nm. The flow rate

was 7 mL/min. Crude peptide, received in a lyophilized form, was dissolved in a solution of 0.1% trifluoroacetic acid (TFA; J.T. Baker) in water. The column was operated from 0 to 4 min in 0.1% TFA in water followed by a linear gradient formed by the addition of 0.1% TFA in acetonitrile (Mallinckrodt). The selected elution band was lyophilized, dissolved in water and lyophilized again. Alternatively, the serine peptide was taken directly from the cleavage reaction and was precipitated in anhydrous ethyl ether (Fischer). Peptide composition was assessed by amino acid analysis and mass spectroscopy (2). The high purity of the preparations was demonstrated by obtaining a single species by mass spectrometry having the correct molecular mass. Solutions were prepared by dissolving quantities of lyophilized peptide in pH 7.4 buffer (100 mM NaCl and 10 mM sodium phosphate).

Gel-filtration column chromatography was used to separate peptide aggregates and to obtain their molecular masses. A Waters 625 liquid chromatography system equipped with a 717 autosampler and a Waters Protein Pak200 SW column with dimensions of 8.0×300 mm was employed. The flow rate was 0.8 mL/min. The column was operated isocratically using pH 7.4 buffer at room temperature, 24–25°C. Elution was monitored at 230 nm. The molecular masses used for calibration of the column were 2350 Da, vitamin B12; 17 000 Da, myoglobin; 44 000 Da, ovalbumin; and 158 000 Da, gamma globulin (BioRad). Standards were run daily. The void volume was detected with blue dextran. The molecular masses of the peptide aggregates were determined from the elution times using a calibration curve obtained from the standards, and the aggregation state was obtained by dividing the molecular mass by the monomer molecular mass. When reducing agent was needed, the peptide was treated with dithiothreitol (Calbiochem) at 5.0 g/L in pH 7.4 buffer. When reducing agent was used as part of the elution buffer of the gel-filtration column, it was present at 1.0 g/L.

The cysteine residues of the peptide were modified chemically. Two reactions were employed to give modified cysteines (24). In one case, *S*-(2-aminoethyl)cysteine was prepared, and, in the other case, the product was *S*-methylcysteine. Mass spectroscopy was used to demonstrate that both the cysteines were modified. The mass spectroscopy protocols were the same as used to determine the mass of the peptide (2).

Absorption of fractions taken off the gel-filtration column was measured with a Beckman DU 640 spectrophotometer. The concentrations were determined from Beer's law, and the specific absorptivity was determined to be 1.0 at 230 nm in a 1-cm light path.

The immature mouse uterine growth bioassay was based on the finding that the injection of 0.5 μg 17β-estradiol into 14–18-day-old female Swiss mice results in a 70% increase in uterine wet weight with parallel increases in the mitotic index in 22 h. This relatively quick bioassay is useful for assessing agents that would interfere with estrogen-stimulated growth. Immature female Swiss mice (15–18 days old, Taconic Farms, Germantown, NY, USA) were injected with various quantities of the alpha-fetoprotein-derived peptide, whereas mice in a control group were injected with buffer. One hour after the first injection, test and positive control mice were injected with 0.5 μg 17β-estradiol, while negative control mice were given 0.1 mL of buffer. The mice were killed 22 h after the second injection; the uteri were dissected, trimmed free of mesenteries and immediately weighed. Uterine weights were normalized to mouse body weights (mg uterine weight per g of body weight) to compensate for differences in body weight among litters of the same age. Each experiment was performed with five mice per group, and the mean normalized uterine weights ± standard errors (SE) for each group were calculated. Per cent growth inhibition was calculated by the following relationship:

(positive control – test group)/(positive control–negative control) × 100.

Animals were killed using protocols approved by the Institutional Animal Care and Use Committee of Albany Medical College.

Results and Discussion

Early aggregation

Trimers and very large aggregates (composed of ≈ 102–146 peptides, as shown by gel-filtration column chromatography), dominated preparations of the peptide at early times after solution preparation. For all concentrations examined, the trimer was initially present in high amounts along with much smaller quantities of a large aggregate (Fig. 1). At high peptide concentrations (5.0–8.0 g/L), trimers aggregated extensively and rapidly (Figs 1 and 2). At 5.0 g/L, it is noted that only a small amount of trimers was still observed at 12 h (Fig. 1). The large aggregates were variable in size (Fig. 3) and attained sizes of ≈ 146 peptides. The chromatography data used to prepare Fig. 3 were similar in appearance

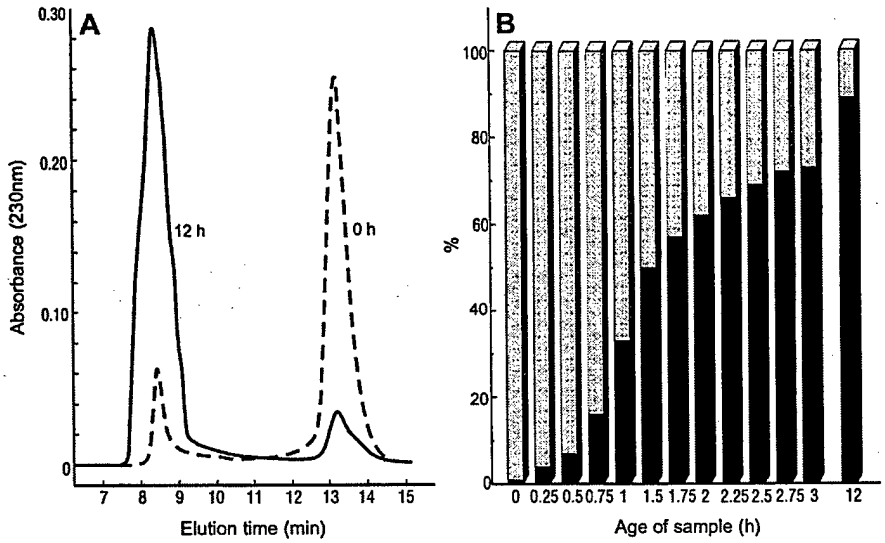

Figure 1. Gel-filtration column chromatography of the peptide at high concentrations and early times. (A) The 0 (broken line) and 12 h (solid line) chromatography profiles of a peptide solution at 5 g/L. The trimer appears on the right and the large aggregate on the left. (B) Gradual change with time of this solution over the early period. The dark portions of the bars represent the percentages of large aggregates, and the lighter portions show the trimer amounts.

to those shown above (Figs 1 and 2), and the shifting of the elution times for the large aggregate provided the molecular mass data (Fig. 3). The large aggregates continued to grow until trimers were substantially exhausted from solution. After attaining maximum size, they were later seen to be reduced in size. The concentration of trimers did not increase during this size reduction and therefore more large aggregates must have formed during this period, or there may have been a redistribution of molecular mass among large aggregates of different sizes. The large aggregate at high peptide concentrations may have become so large that it entered the void volume of the column. The 146-peptide size is therefore an estimate of the lower limit of the aggregate size. The lower and middle molecular mass range of the sizes of the large aggregate are accurate.

Peptide preparations at low concentration (0.10–0.20 g/L) behaved entirely differently. Trimers were found from the earliest time (Fig. 2), and their concentrations were unchanged during this early stage. A very small quantity of the large aggregate, consisting of ≈ 102 peptides, was always present. Its concentration and size were constant (Figs 2 and 3) at these low peptide concentrations.

Late aggregation/disaggregation

Preparations of peptide at low concentrations began to change in aggregation state at longer times after sample preparation (Figs 4 and 5). The trimers began to convert to dimers over the course of 24 h between 0.10 and 3.0 g/L.

The process of dimer formation from trimers could be reversed by treatment with a reducing agent (Fig. 6), and disulfide bonds therefore apparently stabilized the dimer. Peptide solutions aged at 3.0 g/L were treated in two ways. One portion was diluted with an equal volume of a 10 g/L solution of dithiothreitol, whereas the other was diluted with buffer. The nonreduced solution was a mixture of large

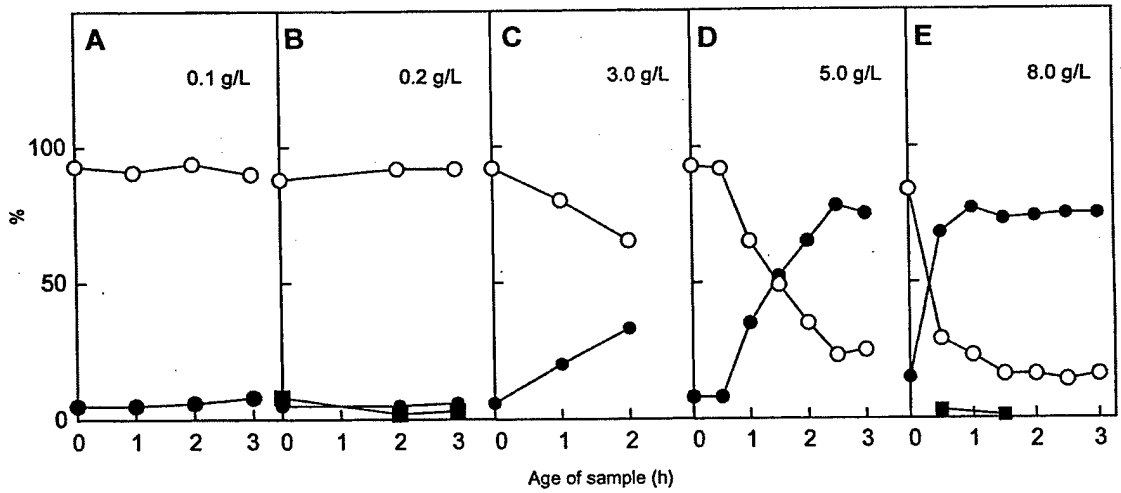

Figure 2. Gel-filtration column chromatography results for the peptide over a range of concentrations at early times. The open circles are the percentages of trimers; the closed circles are the percentages of large aggregates; and the squares are the percentages of hexamers.

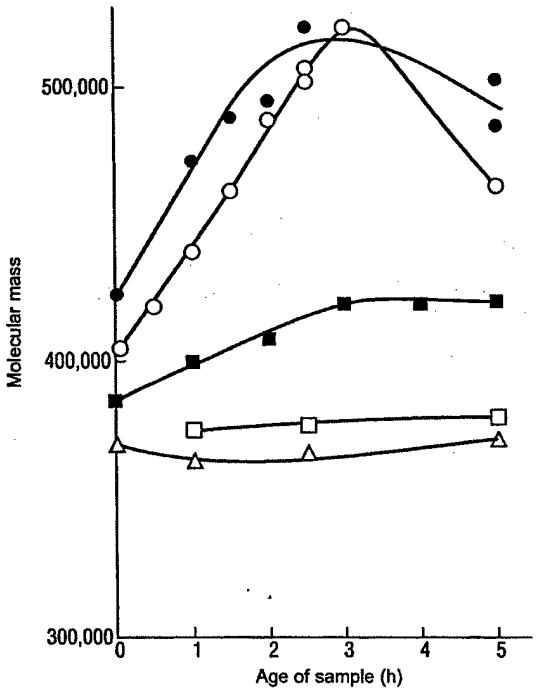

Figure 3. Molecular mass of the large aggregate over time. The solid circles, open circles, closed squares, open squares and triangles represent the molecular masses of peptide solutions of 8.0, 5.0, 3.0, 0.20 and 0.10 g/L, respectively.

aggregates, dimers and trimers (Fig. 6A). The solution that contained the reducing agent had the same amount of large aggregate, but the dimers totally converted to trimers within 30 min (Fig. 6B). The large aggregate was apparently unaffected by the reducing agent, and may therefore be stabilized by noncovalent forces. The presence of disulfide bonds in trimers could not be assessed using this protocol because trimers might have reformed rapidly on the chromatography column after the reducing agent separated from peptide.

To assess whether there were disulfide bonds in the trimer, peptide (0.20 or 5.0 g/L) was dissolved in buffer containing 5.0 g/L dithiothreitol at ambient temperature and injections onto the gel-filtration column began almost immediately. These injections continued for 24 h. The elution buffer for the gel-filtration column contained 1.0 g/L dithiothreitol, which should have prevented the formation of any disulfide bonds. At both peptide concentrations, the dithiothreitol had no effect on the formation and concentrations of trimer or large aggregate (data not shown). The gel-filtration results were the same as found in the absence of reducing agent (Figs 1 and 2) in regard to trimer and large aggregate. Dimers, which in the absence of reducing agent would be expected to appear at a peptide concentration of 0.20 g/L after several hours (Fig. 4), were not observed in these solutions. This inhibition of dimer formation by reducing agent supports the view that dimers of the alpha-fetoprotein-derived peptide are formed and stabilized by disulfide bonding. The appearance of trimers and the absence of monomers at the earliest observable times after treatment of dimers with reducing agent suggest that monomers very rapidly and completely associate to trimers in this buffer. Trimers are the stable oligomer in this buffer until disulfide bonds form. In the presence of disulfide bonding dimers are more stable. Disulfide bonding must be studied in more detail and the disulfide bonds may be either inter- or intrapeptide.

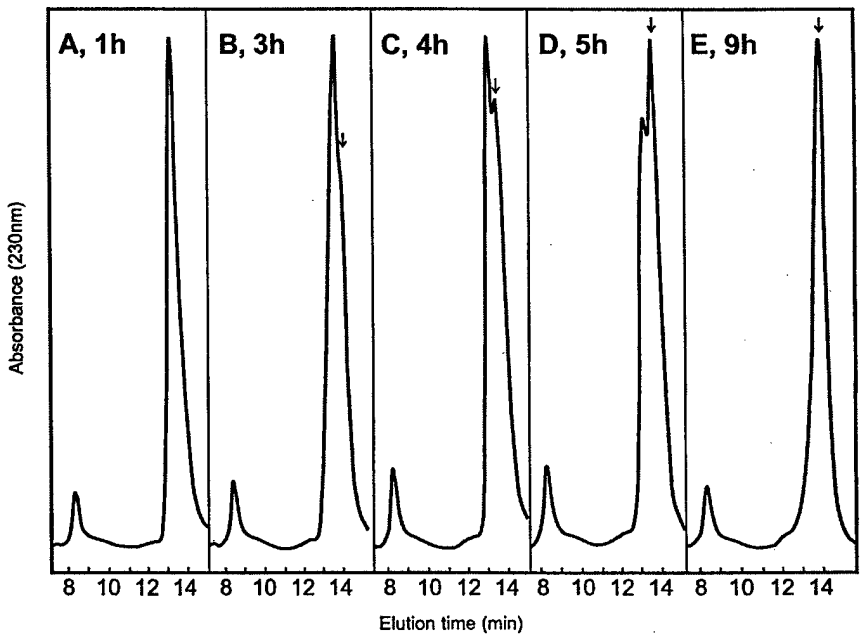

Figure 4. Conversion of peptide trimers to dimers studied by gel-filtration column chromatography. The arrows point to the dimers. Times after dissolving lyophilized peptide into buffer (A) 1 h, (B) 3 h, (C) 4 h, (D) 5 h, (E) 9 h. The peptide concentration was 0.20 g/L.

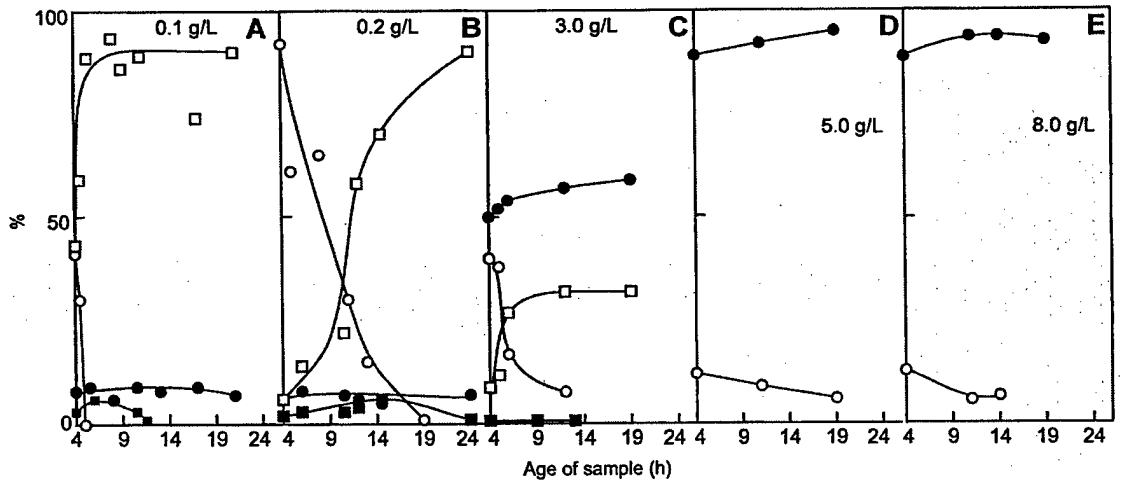

Figure 5. Gel-filtration column chromatography results in the pH 7.4 buffer for peptide at long times over a range of concentrations. The open circles, open squares, closed circles and closed squares are trimers, dimers, large aggregates and hexamers, respectively.

The peptide showed very different aggregation behaviors at low (0.10–0.20 g/L) and high (5.0–8.0 g/L) peptide concentrations. The aggregation at 3.0 g/L was unique and intermediate. Here the peptide formed large aggregates, similar to its behavior at high concentrations, and peptide also showed the trimer–dimer conversion observed at low concentrations (Fig. 5).

A question arises of why the trimers at high peptide concentrations did not form dimers. After a high percentage of large aggregates formed, low concentrations of trimers persisted (Fig. 5). Based on results at 0.10–3.0 g/L, these residual trimers would be expected to form dimers slowly, but they did not. One speculative answer might be that trimers are in rapid equilibrium with the large aggregates. Therefore, the trimers would be formed constantly from the large aggregates and would never age long enough in solution to undergo dissociation to dimers. It can be advanced tentatively that a conformational change occurs in the peptides of a trimer prior to disulfide bond formation, and this conformational change could result in disulfide bond formation either before or after trimer dissociation.

At late times after sample preparation, in samples containing large amounts of dimers, very small amounts of hexamers were observed (Fig. 7). Occasionally, hexamers were also observed to form in very small amounts early after sample preparation (Fig. 2). These observations of early hexamers were rare, and hexamers were usually present only after the development of high dimer concentrations. The early and late hexamers might differ in their structures or modes of association.

Identification of the aggregates was based on their elution time from the gel-filtration column, and the aggregation states for many measurements were: trimers, 2.95 ± 0.23;

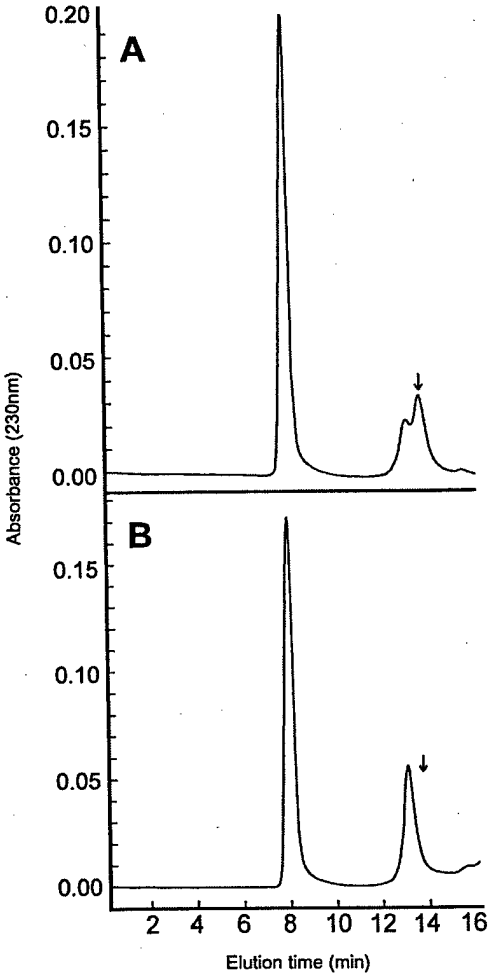

Figure 6. Treatment of peptide aggregates with reducing agent. The arrows point to the dimers, and in (B), from left to right, are the elution profiles of large aggregates and trimers. The solution was 1.5 g/L of peptide. Peptide was treated with dithiothreitol at 5.0 g/L. (A) Solution with no reducing agent, (B) solution with reducing agent.

dimers, 1.89±0.13; late hexamers, 5.86±0.62; early hexamers, 6.04±0.52; and trimers in the presence of reducing agent, 2.98±0.02. Early hexamers are those observed early after sample preparation in the absence of dimers, and late hexamers appear only after appreciable concentrations of dimers are present.

Biological activity

Homogeneous preparations of aggregates for the testing of biological activity were prepared by gel-filtration column chromatography. Oligomers were eluted from the column and their concentrations measured spectrophotometrically. The various aggregates were tested within 2 h after elution from the column in an estrogen-sensitive uterine growth assay in mice. Trimers were found to have growth-inhibitory activity, and their activity was similar to that found for peptide mostly in the trimer form, but not run through the column. The large aggregates and dimers were found to have no growth-inhibitory activity (Table 1). Because trimers were found in very high percentages at early times and low peptide concentrations, these conditions should be ideal for expression of the anti-estrogenic function of the peptide.

When trimers and the large aggregate were mixed together in equal specific amounts, no activity was observed (Table 1). There are two possible explanations for this effect. One is that the large aggregate might somehow interfere with the growth-inhibitory activity of the trimers. The other is that the large aggregate may grow in size by taking the trimers out of solution. This second possibility would correspond to the known properties of the large aggregates (Fig. 3).

Aggregation of the alpha-fetoprotein-derived peptide may be viewed as follows:

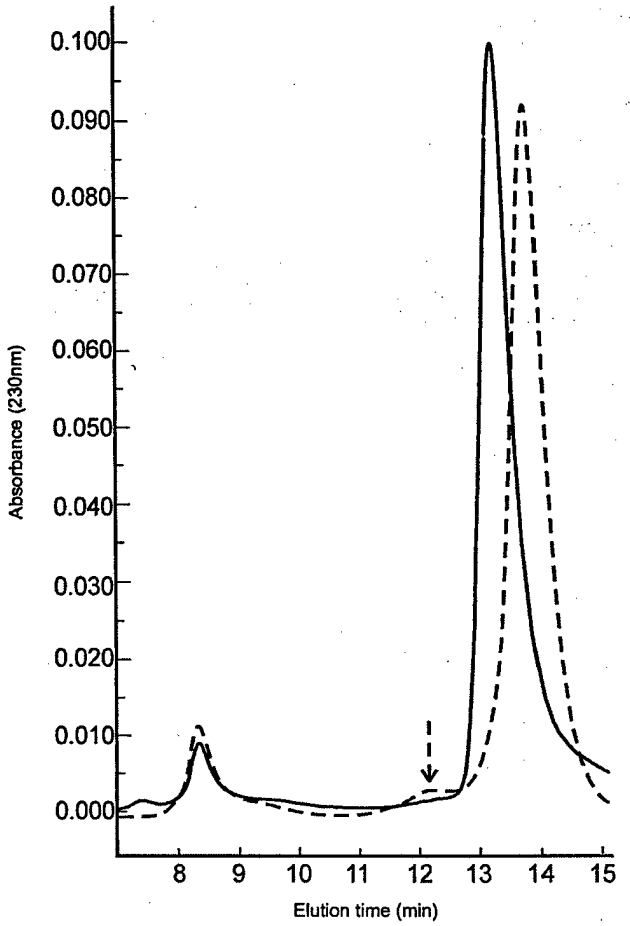

Figure 7. Formation of hexamers. The arrow points to the hexamer band. The solid line is the elution profile at early time showing the presence of trimers and a small amount of large aggregate, and the dashed line is the elution profile much later after the trimers had converted entirely to dimer. The trimer and dimer elution bands are on the far right and the large aggregate is on the far left. The peptide concentration was 0.20 g/L.

Early (0–3 h): $n\alpha_3 \rightarrow (\alpha_3)_n$

where n=34–49 depending on the peptide concentration.

Late (4–24 h): $2\alpha_3 \rightarrow 3\alpha_2$

$3\alpha_2 \rightarrow \alpha_6$.

Table 1. **Anti-estrogenic activity of alpha-fetoprotein-derived peptide**

Test agent	Percentage inhibition of estrogen-stimulated growth of immature mouse uteri: mean±SE
Peptide, freshly prepared, mostly trimers	40±4[a]
Peptide, large aggregate off column	0±4
Peptide, trimer off column	35±8[a]
Peptide, dimer off column	6±2
Peptide, mixture of large aggregate and trimer off column	0±3

a. Significant inhibition (P<0.05) compared with estrogen-stimulated uterine growth in mice not treated with peptide, based on the Wilcoxon sum of ranks test. In all cases, 1 µg of peptide was injected, which is within the optimal dose range (0.5–5 µg) of this peptide. In the mixing experiment, 1 µg each of large aggregate and trimer were injected together.

Trimer to dimer conversion occurs only at low and intermediate peptide concentrations (0.1–3.0 g/L). These reactions set the limits for the growth-inhibitory activity of the peptide. The zero time indication given above neglects the time from preparation of solutions from the lyophilized peptide to the various elution times off the column.

These experiments showed that the trimer of the alpha-fetoprotein-derived peptide has the key to antigrowth activity. Conditions were determined to maximize the presence of trimers and to show the range of their stability. High peptide concentrations must be avoided to minimize formation of the large aggregate, and, at low peptide concentrations, long times in solution should be avoided to eliminate the conversion of trimers to dimers. It is possible that these trimers dissociate to monomers in the biological assay and this dissociation is necessary for activity.

Cysteine residues

Inactivity of the dimer may be produced by a number of factors including: the active site may be sterically inaccessible in this molecule, the active-site peptide sequence may itself be locked into a conformation that is distorted and incapable of fitting a receptor surface, or the dimer may be unable to dissociate into monomers. Alternatively, it is possible that the cysteine residues are involved directly with the biological activity. To investigate this question, the cysteines were covalently modified to either *S*-(2-aminoethyl)cysteine or *S*-methylcysteine (24). Modified peptides were tested in the mouse-estrogen assay, and both derivatives were shown to have significant growth-inhibitory activity similar in magnitude to the original peptide. The results showed a 31 and 29% reduction in the growth-enhancing effect of the estrogen with the methyl and aminoethyl derivatives, respectively. The SE for both of these experiments was 3.0. The estrogen effect, causing growth enhancement, was measured for mice treated with buffer vs. mice treated with estrogen. These results show that unmodified cysteine is not required for growth inhibition, and the mechanisms discussed previously are more likely.

A peptide was also prepared in which both cysteines were replaced by serines. Cysteines were selected for replacement because of the ability of this amino acid to form disulfide bonds and because cysteines did not appear to be required for growth inhibition. Serine was selected for insertion because, of all the most common amino acids, it most resembles cysteine structurally. The serine peptide formed dimers at 0.20 and 8.0 g/L of peptide, and these dimers were stable for at least 24 h (data not shown). At 0.20 g/L, the oligomer was 2.03 ± 0.03, and at 8.0 g/L it was 2.31 ± 0.03. The slightly larger oligomer result at the higher concentration could be caused by a small amount of a larger aggregate under the band of the predominant dimer. The dimers were not active in the growth-inhibitory assay. Although not rigorously proven, it is currently assumed that it is the dimeric nature of the oligomer of the peptide and its serine analog that is responsible for the inactivity in the growth-inhibitory assay. The analog dimer might have stronger peptide–peptide interaction than the trimers of the cysteine peptide, and this stability might influence their activity in the growth inhibition assay. The original cysteine-containing peptide is the most interesting from the aspect of being a growth-inhibitory peptide in its trimer form, but in the future other analogs should be evaluated in a search for stable and biologically active peptides. These results suggest that analogs devoid of cysteines should be closely scrutinized.

Conclusion

In general, it is possible that peptides with potential promise as various types of drugs can be uncovered as partial sequences in a plethora of proteins. Discovering these biologically active fragments requires effort and ingenuity. Studies correlating biological with biophysical and biochemical properties, including assessing the aggregation state of the peptides in solution, would be of relevance.

After birth, alpha-fetoprotein levels decline and reach relatively low levels after ≈ 9 months (25). Epidemiological studies suggest that pregnancy may protect women very long-term against breast cancer (26,27). Alpha-fetoprotein, which is synthesized in the fetus and undergoes transplacental passage to the maternal serum and tissues, may be an important factor in this protection. Future investigations will determine if this particular peptide sequence is a part of this exciting line of inquiry, and, if it is, it can be speculated that the peptide may be useful as a preventive measure in the battle against breast cancer.

Previous results (3,4), showing that alpha-fetoprotein stops the growth of estrogen-supported human breast cancer xenografts and that the peptide-inhibited growth of cultures of human breast cancer cells, suggest that the peptide should indeed be investigated as a therapeutic agent for breast cancer. During pregnancy, alpha-fetoprotein promotes the growth and well-being of the fetus, and when tumor associated, the protein plays an autocrine

role in supporting the growth of the tumor hepatoma (28). It is, therefore, a reversal of normal function that part of this protein has growth-inhibitory properties, and it is ironic that a synthetic peptide derived from this protein is a candidate for an anticancer drug.

In summary, trimers of the peptide possess growth-inhibitory ability, which may have potential in the treatment of certain cancers. Dimers and large aggregates of the peptide lack the ability to be antigrowth factors in the assay used in this study. Peptides with covalently modified cysteines are also active as growth inhibitors, but dimers formed by peptides having cysteines replaced by serines are not. Aggregation is therefore saliently important in determining biological function.

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A peptide derived from α-fetoprotein prevents the growth of estrogen-dependent human breast cancers sensitive and resistant to tamoxifen

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An 8-mer peptide (EMTOVNOG) derived from α-fetoprotein was compared with tamoxifen for activity against growth of human breast cancer xenografts implanted in immune-deficient mice. Both peptide and tamoxifen prevented growth of estrogen-receptor-positive MCF-7 and T47D human breast cancer xenografts. A subline of MCF-7, made resistant to tamoxifen by a 6-month exposure to this drug in culture, was found to be resistant to tamoxifen *in vivo*. Peptide completely prevented the xenograft growth of this tamoxifen-resistant subline of MCF-7. Neither peptide nor tamoxifen was effective in slowing the xenograft growth of the estrogen-receptor-negative MDA-MB-231 human breast cancer. A worrisome side effect of tamoxifen is its hypertrophic effect on the uterus. In this study, tamoxifen was shown to stimulate the growth of the immature mouse uterus *in vivo*, and the peptide significantly inhibited tamoxifen's uterotrophic effect. The mechanism of action of peptide is different from that of tamoxifen in that the peptide does not interfere with the binding of [^{3}H]estradiol to the estrogen receptor. In conclusion, α-fetoprotein-derived peptide appears to be a novel agent that interferes with the growth of tamoxifen-sensitive as well as tamoxifen-resistant estrogen-receptor-positive human breast cancers; it inhibits the uterotrophic side effect of tamoxifen and, thus, it may be useful in combination with or in place of tamoxifen for treatment of estrogen-receptor-positive human breast cancers.

Several population studies as well as laboratory studies have indicated that α-fetoprotein (AFP) interferes with estrogen-dependent responses, including the growth-promoting effects of estrogen on breast cancer (1). For example, Couinaud *et al.* (2) have reported that women with AFP-secreting hepatomas develop amenorrhea, which self-corrects after removal of the hepatoma, and Mizejewski *et al.* (3) have shown that AFP inhibits the responsiveness of the uterus to estrogen. Jacobson *et al.* (4) and Richardson *et al.* (5) have shown that elevated levels of AFP during pregnancy are associated with a subsequent reduction in lifetime risk for breast cancer, and Jacobson has hypothesized that this should be caused by a diminution in estrogen-dependent breast cancers (6). Sonnenschein *et al.* (7) have shown in rats that an AFP-secreting hepatoma prevents the growth of an estrogen-dependent breast cancer in the same rat. Finally, we have shown that AFP purified from a human hepatoma culture and then injected into tumor-bearing, immune-deficient mice stopped the growth of estrogen-receptor-positive (ER+), but not estrogen-receptor-negative (ER−), human breast cancer xenografts in these mice and did so by a mechanism different from that of tamoxifen (1).

More recently, we have identified the active site of AFP responsible for its antiestrotrophic activity (8). It consists of amino acids 472–479 (EMTPVNPG), an 8-mer sequence in the 580-aa AFP molecule. We have synthesized this 8-mer peptide, modified it by substituting hydroxyproline (O) for proline (P) for the purpose of stabilization, and have shown that this new analog (EMTOVNOG) is stable during long-term storage and, like AFP, has the ability to inhibit estrogen-stimulated growth of breast cancer cells in culture and estrogen-stimulated growth of the uterus in immature mice (8, 9). Having the stable analog in hand, a purpose of the study described herein was to determine whether this peptide has anti-breast cancer activity *in vivo* like its parent protein, AFP. A second purpose of the study was to compare the activities of this peptide with those of tamoxifen. Currently, tamoxifen is the most widely used agent for the treatment of estrogen-responsive breast cancers and has provided significant benefits to women with this disease (10, 11). However, a vexatious problem connected with its clinical use is that not all ER+ breast cancers are sensitive to this drug. About one-third to one-sixth (depending on the lab cutoff for ER positivity) of the ER+, newly diagnosed breast cancers do not respond to tamoxifen (Elwood Jensen, personal communication; ref. 12). Moreover, it is not uncommon that women whose disease is being managed successfully by tamoxifen therapy in time will experience recurrence during treatment apparently because their tumor has acquired resistance to the drug. Because these two groups constitute a substantial number of women whose disease fails to respond to tamoxifen therapy, we considered it important to determine whether AFP-derived peptide would be active against ER+ breast cancer that had become resistant to tamoxifen. We therefore undertook a study of human breast cancers sensitive and resistant to tamoxifen that were grown as xenografts in immune-deficient mice and tested for sensitivity to AFP-derived peptide (EMTOVNOG), hereafter referred to as AFPep.

Materials and Methods

Cell Lines. T47D and MDA-MB-231 human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). Growth medium for T47D cells consisted of RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% (vol/vol) FBS (Life Technologies) and 8 μg/ml bovine insulin (Sigma). Growth medium for MDA-MB-231 consisted of DMEM (Life Technologies) supplemented with L-glutamine (2 mM), nonessential amino acids (1%; Life Technologies), and bovine insulin (1 μg/ml). The MCF-7 cell line was obtained from Alberto C. Baldi (Institute of Experimental Biology and Medicine, Buenos Aires), and was maintained as described by Gierthy *et al.* (13). This strain of MCF-7 demonstrated 17β-estradiol (E_2) sensitivity in regard to induction of tissue plasminogen activator, cell proliferation, and *in vivo* tumor growth and was sensitive to the suppression of these effects by tamoxifen (13–15). Continuous exposure of these cells to 1 μM tamoxifen citrate during routine culture conditions (1:10 sub-

Abbreviations: AFP, α-fetoprotein; ER, estrogen receptor; E_2, 17β-estradiol; AFPep, AFP-derived peptide.

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culture ratio once a week) resulted after 6 months in a strain that was resistant to the suppressive effects of tamoxifen *in vitro*.

Peptide Synthesis and Purification. AFPep (EMTOVNOG) was generated by using solid-phase peptide synthesis, as described (8, 9). Amino acids with their amino group protected by the 9-fluorenylmethoxycarbonyl (Fmoc) group were Fmoc-Asn(Trt), Fmoc-Glu(OtBu), Fmoc-Met, Fmoc-Pro, Fmoc-hydroxyproline(tBu), Fmoc-Thr(tBu), Fmoc-Gly, and Fmoc-Val. The carboxyl groups on incoming amino acids were activated by [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate], obtained from PerSeptive Biosystems (Framingham, MA). After synthesis, the resin was washed with propanol and partially dried, and peptides were cleaved from the solid support and deprotected simultaneously with 10 ml of trifluoroacetic acid/thioanisole/anisole/ethanedithiol (90:5:2:3) per 0.5 g of resin for 5 h. Peptide was recovered from the liquid phase after repeated extraction, first with ether and then with ethyl acetate/ether (1.5:1). The peptide was dissolved in water, purified by reverse-phase HPLC, and then lyophilized. Peptide quality was ascertained by amino acid analysis and mass spectroscopy.

Human Breast Cancer Xenograft Assay. A bioassay for anti-breast cancer activity was performed according to Bennett *et al.* (1, 16, 17). Confluent human breast cancer cells of several different cell lines were trypsinized into suspension and pelleted by centrifugation at 200 × *g*. The pellet of each cell line then was solidified into a fibrin clot by exposing it to 10 μl of fibrinogen (50 mg/ml) and 10 μl of thrombin (50 units/ml). The solid mass of tumor cells then was cut into segments 1.5 mm in diameter. A tumor segment was implanted under the kidney capsule of an immunodeficient Institute for Cancer Research (ICR)-severe combined immunodeficient male mouse (Taconic Farms) that weighed about 25 g. Estrogen supplementation was accomplished by s.c. implantation of a silastic tubing capsule containing solid E_2 inserted on the day of tumor implantation. Peptide was injected i.p. every 12 h at a dose of 1 μg per mouse. Tumor growth was monitored during survival laparotomy at 15-day intervals by measurement of the diameters of the short (d) and long axes (D) of each tumor by using a dissecting microscope equipped with an ocular micrometer. Tumor volumes were calculated by using the formula (Π/6)(d) (2)D, assuming the tumor shape to be an ellipsoid of revolution around its long axis (D). There were five to seven replicate mice included in each treatment group. Mean tumor volume ± SE in each group was calculated for display of growth curves. Significance of differences between groups was tested by using the one-sided Wilcoxon Sum of Ranks Test.

Immature Mouse Uterine Growth Assay. A bioassay for antiestrotrophic activity was performed by using an immature mouse uterine growth assay based on previous studies, which demonstrated that i.p. administration of 0.5 μg E_2 to these mice doubled their uterine weights with a corresponding increase in mitotic figures by 24 h after E_2 (3, 18). Swiss/Webster female mice, 6–8 g in body weight (13–15 days old), were obtained from Taconic Farms. Mice were weighed and distributed into treatment groups (typically five mice per group) such that each group contained the same range of body weights. In a typical experiment, each group received two sequential i.p. injections spaced 1 h apart. Test material or vehicle control for that material was contained in the first injectant. E_2 or vehicle control for E_2 was contained in the second injectant. Twenty-two hours after the second injection, uteri were dissected, trimmed free of mesenteries, and weighed immediately. The uterine weights were normalized to mouse body weights (mg uterine weight per g of body weight) to compensate for differences in body weight among litters of the same age. Experiments used a minimum of five mice per group, and the mean normalized uterine weight ± SE for each group was calculated. Significance of differences between groups was evaluated, employing the nonparametric Wilcoxon Sum of Ranks test.

Assessment of Estrogen Receptor Antagonism. Commercially obtained rabbit uteri (Pel-Freez Biologicals) were used as a source of estrogen receptor (ER). Uteri were pulverized in a stainless steel impact mortar under liquid nitrogen and homogenized (20% wt/vol) in assay buffer (10 mM Tris, pH 7.4/1.5 mM EDTA/10% glycerol/10 mM monothioglycerol/10 mM sodium molybdate) on ice. Centrifugation (50,000 × *g*) for 1 h yielded a supernatant containing cytosol, which was adjusted with assay buffer to 2.5 mg protein/ml. All incubations were carried out in triplicate, each containing 100 μl of cytosol, 20 μl of 10 mM 6,7-[^{3}H]estradiol [50 Ci/mmol (1 Ci = 37 GBq); NEN], and 80 μl of putative antagonist in assay buffer. Total count tubes received 20 μl of [^{3}H]estradiol and 180 μl of assay buffer. After incubation overnight at 4°C, all but the total count tubes received 300 μl of dextran-coated charcoal suspension; tubes were agitated for 15 min and then centrifuged (1,000 × *g*) for 15 min. Supernatants were decanted into counting vials, scintillant was added, and protein-bound tritium was determined by liquid scintillation counting.

Results

It was determined in a screening assay of the inhibition of E_2-stimulated growth of immature mouse uterus by AFPep that an effective antiestrotrophic dose of AFPep was 0.1–1.0 μg per mouse. Also, preliminary pharmacokinetic studies suggested that the biological half-life of this peptide in these mice was 2–3 h. Therefore, for the breast cancer xenograft studies, it was deemed reasonable to administer this peptide twice a day at a dose of 1.0 μg per i.p. injection into tumor-bearing severe combined immunodeficient mice. The ER+ MCF-7 human breast cancer was used as a first step in evaluating the effectiveness of AFPep against human breast cancer. As shown in Fig. 1*A*, MCF-7 xenografts were completely dependent on estrogen for growth in severe combined immunodeficient mice. They underwent an approximate 3-fold increase in tumor volume in the presence of a slow-release E_2 implant during the 30-day observation. Without E_2 supplementation, there was no tumor growth. When E_2-supplemented mice were given twice-daily injections of 1 μg of AFPep, tumor growth was prevented over the 30-day observation period. Similarly, when E_2-supplemented tumors were given once-daily injections of 50 μg of tamoxifen, there was no increase in tumor volume. When a subline of MCF-7 that had been made resistant to tamoxifen in cell culture was used, a rather provocative outcome was obtained; that is, AFPep completely prevented the *in vivo* xenograft growth of this tumor, whereas tamoxifen was only marginally effective in slowing the growth of this tumor (Fig. 1*B*). In fact, at day 30 after tumor implantation, the tumor volume in the E_2 plus tamoxifen group was not significantly different from that found in the group receiving E_2 only (Fig. 1*B*). In contrast, AFPep completely stopped the growth of this tamoxifen-resistant MCF-7 subline, and at day 30 after tumor implantation, tumor volumes in the E_2 plus peptide group were dramatically different from those found in the group receiving E_2 only. The peptide was also tested on ER+ T47D human breast cancer. Like the MCF-7, T47D xenografts were completely dependent on E_2 supplementation for growth (Fig. 2) and more than doubled in tumor volume over the 30-day observation period. Daily treatment with AFPep during this time interval also completely prevented the growth of this tumor (Fig. 2). An ER− human breast cancer, MDA-MB-231, then was tested for sensitivity to peptide. This tumor

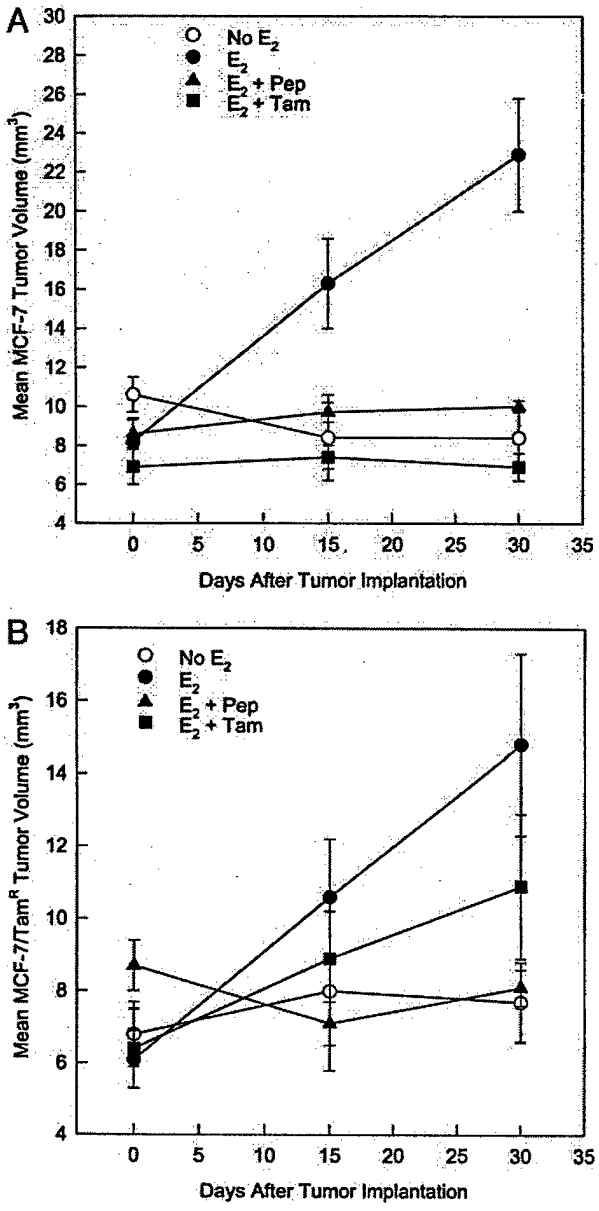

Fig. 1. Effect of AFP-derived peptide on growth of ER-positive MCF-7 and MCF-7/Tam human breast cancer xenografts. Tumors were implanted as described in *Materials and Methods*. Estrogen (solid symbols) was provided by means of a slow-release pellet of E_2 implanted s.c. ▲, Peptide (Pep) was given twice a day i.p. at a dose of 1 μg per injection. ■, Tamoxifen (Tam) was given once a day s.c. at a dose of 50 μg per mouse. Tumor volumes in each mouse were measured at the time of tumor implantation, again at day 15 after tumor implantation during survival laparotomy, and again at day 30 after tumor implantation during necropsy. There was a minimum of five mice per group. (*A*) MCF-7 tumors. At day 30 after tumor implantation, tumor volumes in the E_2 + Pep group and in the E_2 + Tam group were significantly different from tumor volumes in the E_2 alone group, $P < 0.05$. (*B*) MCF-7 subline made resistant to tamoxifen in culture. At day 30 after tumor implantation, tumor volumes in the E_2 + Pep group but not in the E_2 + Tam group were significantly different from tumor volumes in the E_2 alone group, $P < 0.05$.

grew independent of estrogen supplementation and demonstrated a rather aggressive growth rate during the second 2 weeks of the observation period (Fig. 3). Daily treatment with AFPep had no effect on the growth of this tumor at any time during the

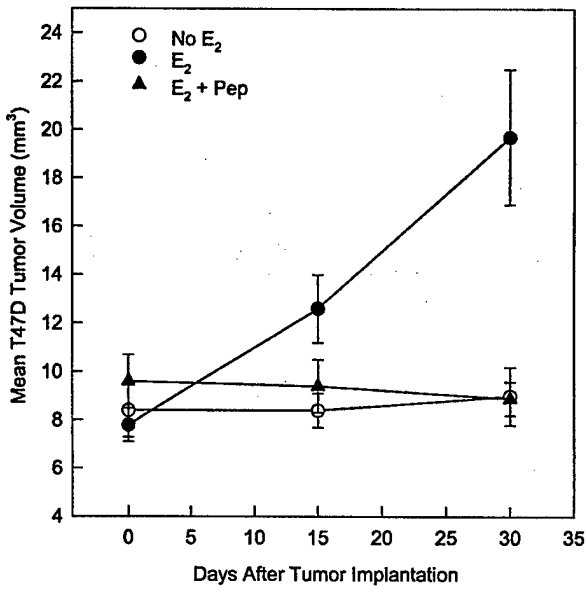

Fig. 2. Effect of AFP-derived peptide on growth of estrogen-receptor-positive T47D human breast cancer xenografts. See legend to Fig. 1 for experimental protocol. At day 30 after tumor implantation, tumor volumes in the E_2 + Pep group were significantly different from tumor volumes in the E_2 alone group, $P < 0.05$.

30-day observation period (Fig. 3). Similarly, tamoxifen did not affect the growth of this ER− tumor.

Although the spectrum of tumors that have been tested for sensitivity to AFPep is somewhat limited thus far, it appears that this peptide interferes with E-dependent, but not E-independent, breast cancer growth. As a first step in evaluating the mechanism of action of this peptide, it was compared with 4-hydroxytamoxifen and raloxifene as a competitor of E_2 for binding to ER. As shown in Fig. 4, both 4-hydroxytamoxifen and raloxifene exhibit their well documented interference with E_2 binding to ER. In contrast, AFPep produced no interference with E_2 binding to ER over a peptide concentration range of 10^{-10} M to 10^{-5} M. Thus, the mechanism by which AFPep interferes with response to estrogen is clearly different from that of tamoxifen and other agents that directly compete with E_2 for binding to ER.

A troublesome side effect of tamoxifen in women has been its hypertrophic effect on the uterus (19). It is likewise an estrogen agonist in the murine uterus. As shown in Fig. 5*A*, tamoxifen stimulated the growth of immature mouse uterus by 50% at a dose of 1 μg/mouse. Tamoxifen's potency was approximately one-tenth that of E_2, but nevertheless, Fig. 5*A* reaffirms that tamoxifen acts as an estrogen agonist on the murine uterus, even though it antagonizes the effect of estrogen on cancer of the breast. AFPep, on the other hand, had no uterotrophic effect whatsoever (Fig. 5*A*), even at a dose of 10 μg/mouse, which is 10-fold greater than the dose used to prevent breast cancer growth (Figs. 1 and 2). Moreover, peptide inhibited the uterotrophic effect of tamoxifen as well as that of estradiol (Fig. 5*B*).

Discussion

The results of this study demonstrate that a synthetic 8-mer peptide derived from AFP prevented the E_2-stimulated growth of human breast cancer xenografts, including an ER+ breast cancer line that had become resistant to tamoxifen. Although tamoxifen has been the mainstay of medical treatment for ER+ breast cancers and has provided significant clinical benefit (10, 11, 20), additional drugs are needed for the treatment of ER+

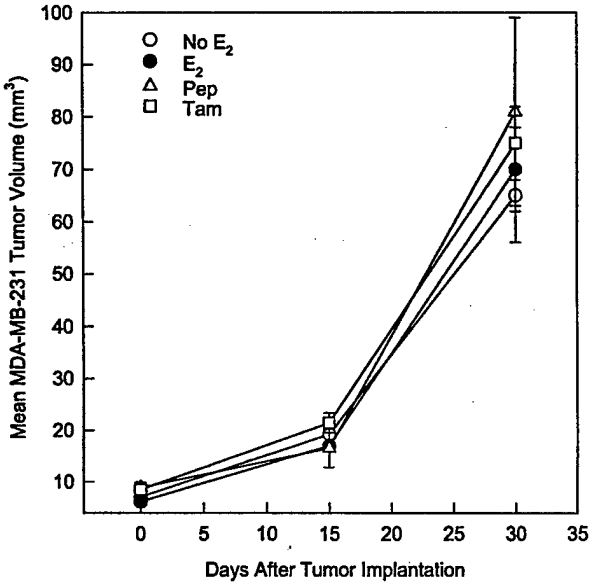

Fig. 3. Effect of AFP-derived peptide on growth of estrogen-receptor-negative MDA-MB-231 human breast cancer xenografts. See legend to Fig. 1 for experimental protocol. There were no differences in tumor volumes between any of the groups.

breast cancer, especially when these cancers are found to be refractory to tamoxifen. AFPep has the potential to fill this niche. There are other drugs further along in development that also could fill this niche. Letrozole, which blocks estrogen synthesis by inhibiting aromatase, and goserelin, which stifles ovarian release of estrogen by inhibiting gonadotropin release, are both being tested for this purpose (21, 22). AFPep, on the other hand, seems to represent a new class of agents able to inhibit the growth of ER+ breast cancer. How AFPep effects this

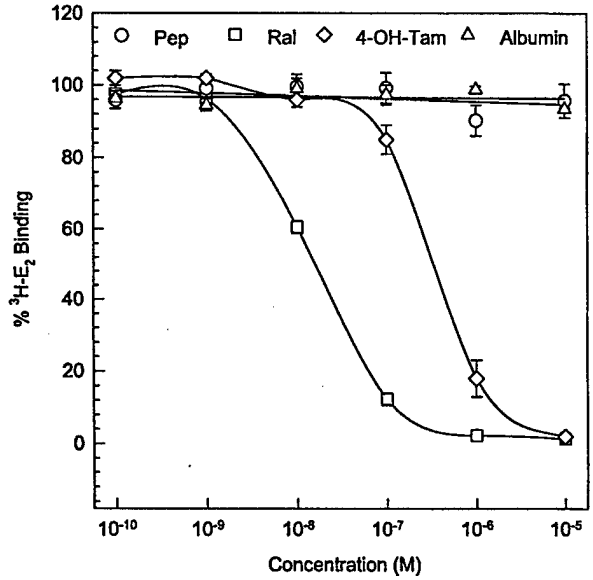

Fig. 4. Effect of AFP-derived peptide on binding of E_2 to its receptor. Rabbit uterine cytosol was used as a source of estrogen receptor. All incubations were performed in triplicate, each containing 100 μl of cytosol, 20 μl of 10 nM 6,7-[^{3}H]estradiol (50 Ci/mmol), and 80 μl of test agent at the final concentrations indicated on the abscissa. Concentration of [^{3}H]E_2-complex with receptor in the presence of different concentrations of test agent is expressed as a percentage of the amount of complex formed in the absence of test agent.

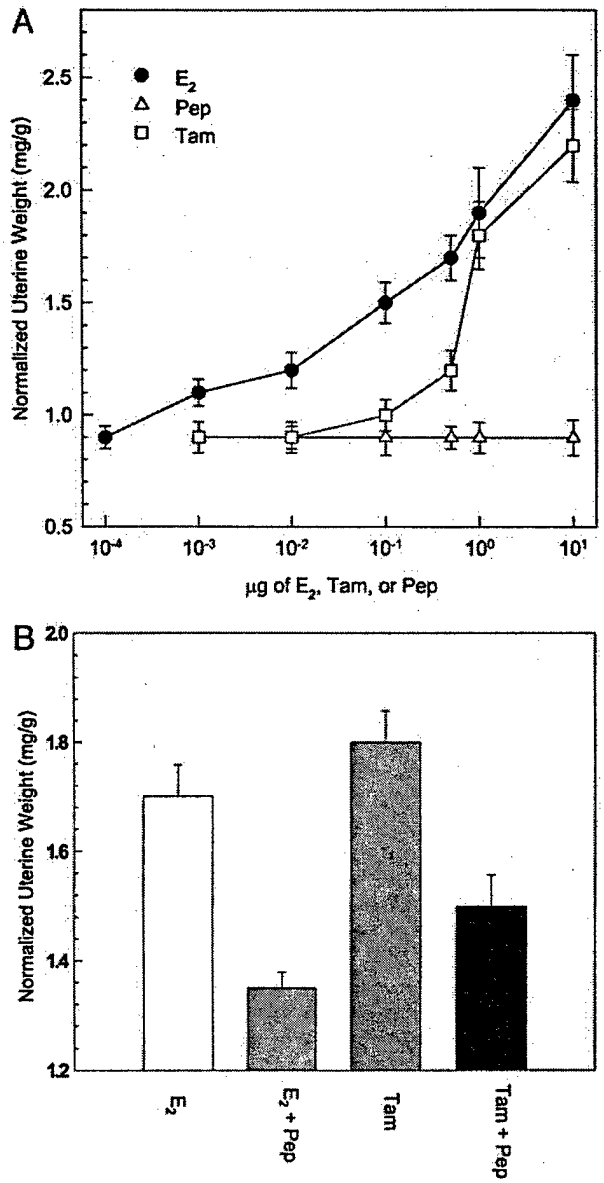

Fig. 5. (*A*) Effect of E_2, AFP-derived peptide (Pep) and tamoxifen (Tam) on the growth of the immature mouse uterus. The assay procedure is described in *Materials and Methods*. Various doses indicated on the abscissa of each test agent were injected i.p. Twenty-two hours later, uteri were harvested and weighed. Mean normalized uterine weights (mg uterine weight/g mouse body weight) for each group are shown on the ordinate. (*B*) Pep (1 μg) or vehicle (saline, 0.2 ml) were injected i.p. One hour later, E_2 (0.5 μg) or Tam (1.0 μg) was injected i.p. Twenty-two hours later, uteri were harvested and weighed. Normalized uterine weights in the E_2 + Pep and Tam + Pep groups were significantly different, respectively, from normalized uterine weights in the E_2 group and Tam group, $P < 0.05$.

inhibition is not clear at this time. It is clear from the data shown in Fig. 4, however, that its mechanism is different from that of tamoxifen in that it does not compete with E_2 for binding to ER. Furthermore, in earlier studies we have shown that AFP administered to rodents did not reduce serum E_2 levels (1), and, in preliminary studies, AFPep also did not reduce serum E_2 levels, making its mechanism different from that of both letrozole and goserelin. In preliminary studies, we have found that this peptide reduces the level of MAPK kinase. This would restrict the phosphorylation of ER, which is MAPK kinase-dependent (23), and phosphorylation of ER is needed to fully operationalize this

receptor. We are continuing to investigate this signal transduction pathway for additional information that would elucidate further the mechanism by which this peptide acts. There is no agent currently in use for the treatment of breast cancer that utilizes this signal transduction pathway for interfering with estrogen response, so this peptide truly represents a novel breast cancer therapeutic both in structure and in the biological mechanism through which it operates.

There is still a great deal of developmental work that remains with regard to this peptide. Optimization of its structure, as well as its dose, route, and schedule, and understanding its mechanism all remain to be worked out; its toxicology also needs to be explored. With regard to toxicology, we have observed no adverse effects of this peptide in the mice used for either the xenograft assays or the uterine growth assays. The cancer xenograft assays are important from a toxicological perspective because AFPep was given twice a day for 30 days in these assays, during which we observed no change in mouse body weight, cage activity, fur texture, or body temperature. Furthermore, on necropsy, there was no change in size or appearance of major organs relative to mice in the control group. Similarly, the uterine growth studies were important from a toxicological perspective because treatment with AFPep, unlike tamoxifen, did not stimulate murine uterine growth and interfered with the uterine growth stimulated by tamoxifen. This is significant because tamoxifen treatment of breast cancer patients induces uterine hypertrophy in ≈30% of the women who receive this drug (19). Moreover, it has been reported that uterine cancer develops in ≈0.2% of the women who are treated with tamoxifen (24). Although there are newer drugs in preclinical development that compete at the ER and have minimal agonist activity on the uterus (25), that AFPep acts at a different site than these drugs to mediate its antiestrotrophic activity and actually interferes with the uterotrophic effect of tamoxifen suggests that it may have use not only alone, but also in combination with ER competitors, even if these agents have partial agonist activity on the uterus. For example, the tamoxifen-stimulated uterine growth curve shown in Fig. 5*A* indicates that a slight lowering of tamoxifen dose substantially blunts uterine stimulation. Although we do not know the tamoxifen dose-response curve for uterine hypertrophy in women, this murine data suggest that AFPep in combination with lower doses of tamoxifen might well maintain full anti-breast cancer activity while reducing tamoxifen-induced uterine hypertrophy.

In summary, AFP is known to inhibit estrogen-dependent responses. We have isolated the active site in AFP responsible for this activity and have synthesized it as a biologically active peptide. This 8-mer peptide prevented E_2-dependent human breast cancer xenograft growth, including breast cancers that had become resistant to tamoxifen. Its mechanism is different from that of tamoxifen and appears to be well tolerated in mice. Thus, this 8-mer peptide or peptidomimetics derived therefrom warrant further development as novel agents for the treatment of human breast cancer.

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Development of a synthetic cyclized peptide derived from α-fetoprotein that prevents the growth of human breast cancer

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Key words: alpha-fetoprotein; antibreast cancer peptides; anti-estrogens; cyclic peptide

Abstract: The peptide, EMTPVNPG, derived from alpha-fetoprotein, inhibits estrogen-stimulated growth of immature mouse uterus and estrogen-dependent proliferation of human breast cancer cells. However, the biological activities of the peptide diminish over time in storage, even when in the lyophilized state, probably because of peptide aggregation through hydrophobic interaction among monomers. Two analogs of EMTPVNPG were designed with the intent of minimizing aggregation and retaining biological activity during prolonged storage. EMTOVNOG, where O is 4-hydroxyproline, is a linear peptide generated by substituting 4-hydroxyproline for the two prolines, thereby increasing peptide hydrophilicity. This analog exhibited a dose-dependent inhibition of estrogen-stimulated growth of immature mouse uterus similar to that of EMTPVNPG (maximal activity at 1 μg/mouse). A second analog, *cyclo*-(EMTOVNOGQ), a hydrophilic, cyclic analog with increased conformational constraint, was as potent as the other peptides in its inhibition of estrogen-dependent growth of immature mouse uterus, and had an expanded effective dose range. Both linear and cyclized hydroxyproline-substituted analogs exhibited indefinite shelf-life. Furthermore, both analogs inhibited the estrogen-dependent growth of MCF-7 human breast cancer growing as a xenograft in SCID mice. These analogs may become significant, novel agents for the treatment of breast cancer.

Abbreviations: AFP, alpha-fetoprotein; E_2, estradiol; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, *O*-(7-azabenzotriazol-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high-pressure liquid chromatography; i.p., intraperitoneal; O, 4-hydroxy proline; OtBu, *tert*-butyl ester; SCID, severe combined immune deficient; tBu, *tert*-butyl; TFA, trifluoroacetic acid.

Recently, Mesfin *et al.* (1) reported that within the 590 amino acid alpha-fetoprotein (AFP) molecule, there is an 8 amino acid sequence (EMTPVNPG, amino acids 472–479 in AFP) which retained all of the anti-estrotrophic activity found with full-length AFP. This peptide inhibited the estrogen-dependent growth of immature mouse uterus as well as the estrogen-dependent proliferation of human breast cancer cells (1). Its mechanism of action appears to be different from those attributed to agents currently in use for the treatment of breast cancer, which enhances its attractiveness for clinical development. However, there were two properties of this peptide which diminished its attractiveness for clinical development. The first was its rather limited shelf-life of ≈ 4 weeks. Loss of activity occurred even if the peptide was stored in the lyophilized state. The second disadvantageous property was the biphasic dose–response curve of the peptide. Anti-estrotrophic activity was dose dependent up to 1 μg/mouse but higher doses resulted in decreasing biological effect.

These properties are by no means unique to this peptide. Loss of biological activity during storage has been seen with insulin (2), calcitonin (3), interferon-β_1 (4), interferon-γ (5) and fibroblast growth factor (6). Biphasic dose–response curves have been seen with angiotensin II (7), glucagon-like peptide (8), and D-globin-derived synthetic peptide (9), to name just a few. Typically, shelf-life problems have been addressed by the use of excipients (10) or by direct amino acid substitution in the peptide (11). The biphasic dose–response curve is more problematic because it is less well understood but has been addressed previously using rational design approaches (12).

Strategies of rational design of peptides were used in this investigation in an attempt to increase shelf-life and expand the effective dose range of the AFP-derived octapeptide. More specifically, hydrophilic substitutions were evaluated as an attempt to increase shelf-life as previous studies had shown that AFP, as well as fragments of AFP, aggregated, presumably through hydrophobic interactions in the β-sheet portion of these molecules (13–16). Also, cyclization of peptide was evaluated as an approach to expand the effective dose range by limiting the number of conformational possibilities available to the peptide. We report results which indicate that both of these strategies were successful in yielding an analog with substantially greater clinical translatability as a novel agent for the treatment of breast cancer.

Experimental Procedures

Peptide synthesis

Peptides were synthesized using Fmoc solid-phase peptide synthesis on a Pioneer Peptide Synthesis System (PerSeptive Biosystem Inc., Framingham, MA, USA). Briefly, peptides were assembled on Fmoc-PAL-PEG-PS-resin (Applied Biosystems, Inc.) from the C-terminus, reacting the deblocked N-terminus of support-bound amino acid with the activated C-terminus of the incoming amino acid to form an amide bond. Amino acids used in the synthesis had their N^{α}-amino group protected by the 9-fluorenylmethyloxycarbonyl (Fmoc) group, which was removed by piperidine at the end of each cycle in the synthesis. Side-chain protecting groups of amino acids were Asn (Trt), Gln (Trt), Glu (O*t*Bu), Hyp (*t*Bu), Thr (*t*Bu) which were deprotected by trifluoroacetic acid (TFA) after peptide synthesis. The carboxyl-group of the amino acid was activated with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) obtained from PerSeptive Biosystems Inc. The specific amino acid derivatives, supports, and reagents used in the synthesis were purchased from PerSeptive Biosystems Inc. and NovaBiochem (San Diego, CA, USA).

After synthesis was completed, the resin was washed three times with 100% propanol and the cleavage reaction was achieved by incubating the resin in 10 mL TFA/thioanisole/anisole/1,2-ethanedithiol (90:5:2:3) per 0.5 g resin for 5 h. The cleavage reaction mixture was filtered using a sintered glass funnel to separate the solid resin from the peptide solution. Filtrate volume was reduced to 1 mL by evaporation facilitated with a gentle stream of air and the peptides were precipitated by addition of 15 mL dry-ice-chilled ethyl ether. The peptides were allowed to settle for 5 min at –80°C, and the supernatant was aspirated. The peptides were then washed twice in similar manner with 15 mL of ethyl ether. After three further washings with 15 mL of ethyl acetate/diethylether (1.5:1, room temperature), the peptides were dissolved in deionized water, purified by reverse-phase HPLC (see details below), lyophilized, and stored at –20°C.

Cyclization of the peptides

Cyclization of peptides was accomplished using methods described by Kates *et al.* (17,18). Briefly, N-alpha Fmoc-L-glutamic acid-alpha-allyl ester at the C-terminus of the synthetic peptide was coupled to the resin via the gamma carboxylic acid. Removal of the N^{α}-Fmoc allowed the

remaining amino acids to be incorporated sequentially into the growing peptide. A free alpha-carboxyl group was then generated upon removal of the allyl group from the C-terminal Glu (18). Peptidyl-resin was dried in an incubator and the tube was flushed with nitrogen delivered through a septum. A catalyst solution was prepared separately by mixing 3 eq. of $Pd(PPh_3)$ in $CHCl_3$/acetic acid/*N*-methylmorpholine (37:2:1) (15 mL/g of resin) and dissolved by bubbling nitrogen through the solution. The catalyst was transferred to the tube containing peptidyl-resin using a gas-tight syringe, and mixed for 2 h. Peptidyl-resin was washed consecutively with 0.5% diisopropylethyl amine in DMF and 0.5% w/w sodium diethyldithiocarbamate in DMF to remove the catalyst. Fmoc was removed from the N-terminus and the free alpha-carboxyl group was then coupled to the free N-terminal residue of the peptide (while on the resin) in order to generate the cyclic peptide, which was then removed from the resin in such a way as to yield the gamma-carboxamido derivative (i.e. Q). The cyclic peptide was then purified and characterized as described below.

Purification of peptides

Purification of peptides was accomplished using a Waters Delta-Pak C_{18} (19 mm×30 cm) reverse-phase column with a pore diameter of 300 Å on a Waters 650E liquid chromatography system equipped with a 486 adjustable absorbance detector and a 600E controller. The column was operated with gradient using a 0.1% TFA in water as solvent A and 0.1% TFA in acetonitrile as solvent B. The gradient was set as follows: 100% solvent A for the first 4 min, followed by increasing acetonitrile from 0 to 40% solvent B over the next 35 min then isocratically at 40% B for 11 min, and followed by a linear gradient of 40–100% B over 10 min all with a flow rate of 7 mL/min. Peptide was monitored at 230 nm and fractions containing pure peptide (>95% purity) were pooled together and lyophilized.

Peptide characterization

Amino acid analyses of all peptides were performed using the Waters AccQ-Tag amino acid analysis system (19,20). Peptides were analyzed by mass spectrometry using standard alpha-cyano-4-hydroxysinnipinic acid and sinnipinic acid matrices. The integrity of the cyclized peptides was further validated using the Kaiser test (21) to ensure absence of free terminal amino group.

Immature mouse uterine growth assay

A bioassay for anti-estrotrophic activity was performed using an immature mouse uterine growth assay (22). Swiss/Webster female mice, 6–8 g in body weight (13–15 days old), were obtained from Taconic Farms (Germantown, NY, USA). Mice were weighed and distributed into treatment groups (typically 5 mice/group) such that each group contained the same range of body weight. In a typical experiment, each group received two sequential intraperitoneal injections 1 h apart. Test material or vehicle control for that material was contained in the first injectant. Estradiol (E_2) or vehicle control for E_2 was contained in the second injectant. Twenty-two hours after the second injection, uteri were dissected, trimmed free of mesenteries, and immediately weighed. The uterine weights were normalized to mouse body weights (mg uterine weight/g of body weight) to compensate for differences in body weight among litters of the same age. Experiments employed a minimum of five mice per group, and the mean normalized uterine weight ±SE for each group was calculated. Percent growth inhibition in a test group was calculated from the normalized uterine wet weights as described below.

Growth inhibition (%)
= (Full E_2 stimulation – E_2 stimulation in test group)/
(Full E_2 stimulation – No E_2 stimulation) ×100%

Differences between groups were evaluated, employing the nonparametric Wilcoxon ranks sum test (one-sided). In all cases, growth inhibitions that were >25% were significant at $P \leq 0.05$.

Human breast cancer xenograft assay

A bioassay for antibreast cancer activity was performed according to Bennett and co-workers (23,24). Confluent MCF-7 human breast cancer cells were trypsinized into suspension and pelleted by centrifugation at 200 g. The pellet was then solidified into a fibrin clot by exposing it to 10 µL of fibrinogen (50 mg/mL) and 10 µL of thrombin (50 units/mL). The solid mass of MCF-7 cells was then cut into pieces 1.5 mm in diameter. A tumor segment of ≈1.5 mm in diameter was implanted under the kidney capsule of an immunodeficient ICR-SCID male mouse (Taconic Farms) that weighed ≈25 g. Small variations in the initial size of the implant occur but are almost irrelevant, as it is tumor growth, or lack thereof, that is

the measured variable. Estrogen supplementation was accomplished by s.c. implantation of a silastic tubing capsule containing solid E_2 inserted on the day of tumor implantation. Peptide was injected i.p. every 12 h at a dose of 1 μg/mouse. Tumor growth was monitored during survival laparotomy at 10-day intervals by measurement of the diameters of the short (d) and long axes (D) of each tumor, using a dissecting microscope equipped with an ocular micrometer. Tumor volumes were calculated using the formula $(\Pi/6)(d)^2D$, assuming the tumor shape to be an ellipsoid of revolution around its long axis (D). There were 5–7 replicate mice included in each treatment group. Mean tumor volume ±SE in each group was calculated for display of growth curves. Significance of differences between groups was tested using the one-sided Wilcoxon ranks sum test.

Assessment of estrogen receptor antagonism

Commercially obtained rabbit uteri (Pel-Freez Biological, Rogers, AR) were used as a source of estrogen receptor. Uteri were pulverized in a stainless steel impact mortar under liquid nitrogen and homogenized (20% w/v) in assay buffer [10 mM Tris (pH 7.4), 1.5 mM EDTA, 10% glycerol, 10 mM monothioglycerol, and 10 mM sodium molybdate] on ice. Centrifugation (50 000 *g*) for 1 h yielded a supernatant containing cytosol, which was adjusted with assay buffer to 2.5 mg protein/mL. All incubations were carried out in triplicate, each containing 100 μL of cytosol, 20 μL of 10 mM 6,7-[^{3}H]estradiol (50 Ci/mmol; DuPont Pharmaceuticals Company, Wilmington, DE, USA), and 80 μL of putative antagonist in assay buffer. Total count tubes received 20 μL of [^{3}H]estradiol and 180 μL of assay buffer. After incubation overnight at 4°C, all but the total count tubes received 300 μL of dextran-coated charcoal suspension; tubes were agitated for 15 min and then centrifuged (1000 *g*) for 15 min. Supernatants were decanted into counting vials, scintillant was added, and protein-bound tritium was determined by liquid scintillation counting.

Results

Previously, we showed that an energy-minimized structure of octapeptide EMTPVNPG indicated that the peptide had potential to form a horseshoe-shaped structure (1). Energy-minimization studies of an analog of this peptide, which would be generated by substitution of the N-terminal glutamic acid with glutamine (QMTPVNPG), indicated that this product would have the potential to bow even further inward and form a pseudo-cyclic structure (data not shown). This pseudo-cyclic structure may have greater structural stability because of hydrogen bonding between the N-terminal glutamine gamma-carboxamide group and the C-terminal glycine alpha-carboxamide. This linear analog (QMTPVNPG) was therefore synthesized, and its biological activity was compared with EMTPVNPG in the estrogen-dependent immature mouse uterine growth assay. QMTPVNPG inhibited the estrogen-stimulated growth of mouse uterus with an optimal dose of 1 μg/mouse (Fig. 1A), similar to the native octapeptide EMTPVNPG. These results suggested that the substitution of glutamic acid to glutamine did not detract from the biological activity and also did not change the biphasic nature of the dose–response curve. Shelf-life studies indicated that QMTPVNPG stored somewhat better than the native octapeptide (EMTPVNPG), but its anti-estrotrophic activity also diminished to insignificant levels after 5 weeks of storage (Fig. 1B), indicating that the putative stabilization was not sufficient to prevent loss of biological activity during storage.

As shown in Table 1, aged octapeptide QMTPVNPG, stored in the lyophilized state at –20°C for over 1 year, was completely biologically inactive. However, brief treatment with 4 M urea restored its biological activity, suggesting that this peptide might have aggregated during storage, resulting in loss of biological activity. A scrambled form of the Q octapeptide had no biological activity either with or without urea treatment. The biological activity of stored inactive EMTPVNPG was likewise regenerated by 4 M urea (data not shown).

Gel-filtration column chromatography of aged peptide (QMTPVNPG) yielded a single peak (Fig. 2, inset) which became broader as a function of time in storage. This suggested that small aggregates (dimers, trimers) were forming during storage. Although gel-filtration chromatography has low resolution for monomers, dimers and trimers in this size range (841–2523 Da), the width of the peak suggested that aggregates might be separating from monomer. Fractions from different portions of the broad peak from aged, chromatographed peptide were therefore tested for biological activity. The higher molecular mass fraction (Fig. 2, inset, left side of peak) was biologically inactive, whereas the lower molecular mass fraction (Fig. 2, inset, right side of peak) was active in the estrogen-dependent immature mouse uterine growth assay. This suggested that the octapeptide QMTPVNPG, like its parent protein and precursor 34-mer peptide (13,14), aggregated during prolonged storage in the lyophilized state and only partially dissociated during chromatography, and that the monomeric form of the

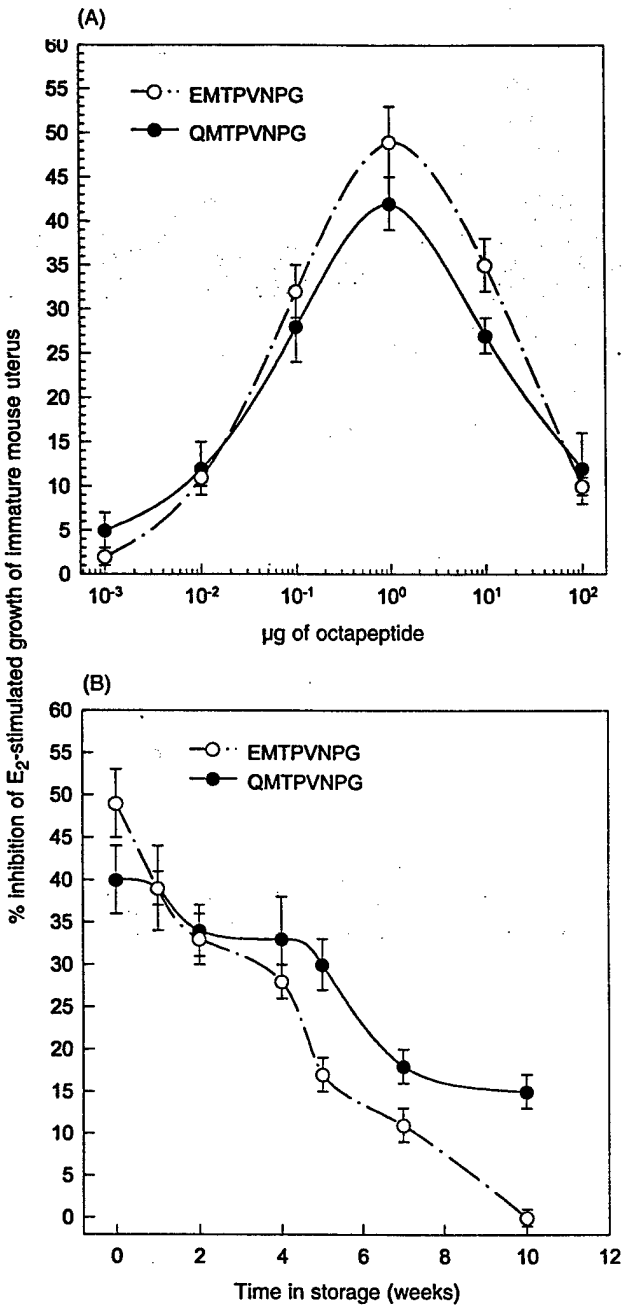

Figure 1. Anti-uterotrophic activity of octapeptide, QMTPVNPG, measured in the immature mouse uterine growth assay. Peptide or vehicle control was injected i.p. into immature female Swiss mice. One hour later 0.5 µg of E_2 or vehicle control was injected i.p. into these mice. Twenty-two hours later, uteri were dissected and weighed. Percent inhibition of E_2-stimulated growth of uterus by peptide was calculated as described in Experimental Procedures. There were 5–8 replicate mice per treatment group. (A) Peptide dose–response. (B) Anti-estrotrophic activity as a function of peptide storage time at –20°C in the lyophilized state, 1 µg peptide/mouse.

***Table 1.* Effect of urea on the biological activity of stored peptide**

	Test agent	Storage time	% Inhibition of E_2-stimulated growth of immature mouse uterus ±SE[a]
I	Octapeptide QMTPVNPG	Fresh	38±3
II	Octapeptide QMTPVNPG	Stored >1 year	0±2
III	II after urea treatment[b]	None	34±4
IV	Scrambled octapeptide	Fresh	2±5
V	IV after urea treatment	None	0±4

a. Assessed as described in legend to Fig. 1.
b. Peptides were dissolved in phosphate buffered saline pH 7.4 at a concentration of 200 µg/mL. They were then diluted to 20 µg/mL in 4 M urea and incubated at room temperature for 1 h. After incubation they were diluted to 2 µg/mL in buffer and 0.5 mL of this preparation (1 µg) was injected into mice as described in legend to Fig. 1. This diluted dose of urea had no adverse effects on mice.

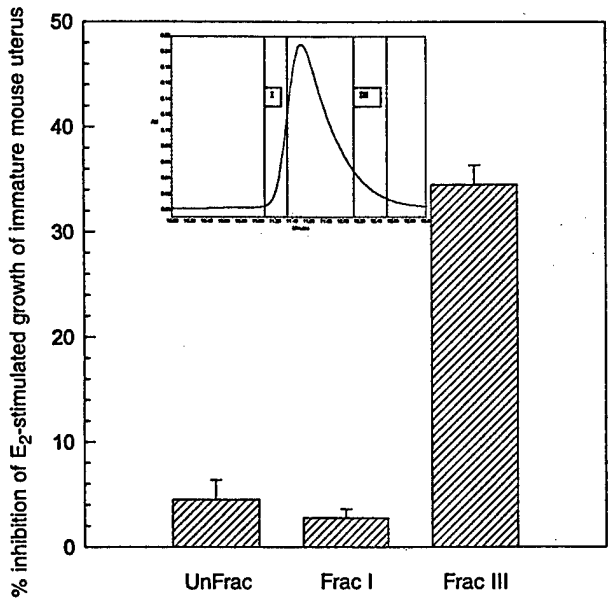

Figure 2. Anti-uterotrophic activity of fractions from gel-filtration chromatography of stored octapeptide, QMTPVNPG. Peptide, QMTPVNPG, was fractionated using a Waters SW 200 gel-filtration column using phosphate-buffered saline pH 7.4 as mobile phase. Fractions which had significant UV absorbance at 230 nm were collected at 20-s intervals. The first fraction (Frac I), the last fraction (Frac III), and the starting material (UnFrac) were all tested in the immature mouse uterine growth assay as described in Fig. 1. One microgram of peptide was injected i.p. into mice in all cases, and percent inhibition of E_2-stimulated growth was measured.

peptide was the active species. We showed previously (Ref. 14, Table 1) that aggregates of the 34-mer peptide were able to inhibit the activity of the monomeric 34-mer peptide, and would anticipate similar results with the smaller peptides. While not especially hydrophobic, the 8-mer peptide carries a net charge of only +1 at neutral pH, and taken together with the chromatography and urea evidence, it is reasonable to conclude that hydrophobicity played a role in its aggregation.

In addition to aggregation, small peptides such as octapeptide EMTPVNPG or QMTPVNPG have structural flexibility that allows them to attain a variety of different structural conformations. As it was thought unlikely that all structural conformers of octapeptide EMTPVNPG or QMTPVNPG would be biologically active, it seemed appropriate to employ the strategy of conformational constraint in an effort to produce stable analogs. Therefore, cyclic analogs were generated to limit the number of conformers. Also hydrophilic analogs were generated to reduce the aggregation potential of the peptide by minimizing possible hydrophobic interactions discussed above.

A linear peptide precursor for a potential cyclic peptide analog was synthesized by adding a glutamine residue to the C-terminus of the native octapeptide. This strategy provides a number of advantages: (i) the glycine residue, previously shown to be essential for biological activity (1), would not be involved in a cyclizing peptide bond, thereby retaining a conformation more like that in the native protein; (ii) addition of glutamine to the C-terminus and retention of glutamic acid at the N-terminus maintains a charge (–1) on the peptide after cyclization, which may be advantageous in diminishing hydrophobically induced aggregation; and (iii) the allyl-protection of the C-terminal amino acid facilitates the chemistry necessary to effect cyclization. This linear nonapeptide (EMTPVNPGQ) was found to inhibit the estrogen-stimulated growth of mouse uterus with maximal inhibition at dose of 1 μg/mouse (data not shown). Thus adding a glutamine residue to the C-terminal of octapeptide EMTPVNPG did not diminish its biological activity. *Cyclo*-(EMTPVNPGQ) was then synthesized by a head-to-tail cyclization reaction of the precursor nonapeptide as described in Experimental Procedures. *Cyclo*-(EMTPVNPGQ) exhibited dose-dependent inhibition of estrogen-stimulated growth of immature mouse uterus with maximal inhibitory activity at a dose of 10 μg/mouse (Fig. 3A), and was also very active at the optimal dose of the linear peptides (1 μg). Nevertheless, storage experiments indicated that this cyclized peptide had somewhat extended, but still rather limited, shelf-life (Fig. 3B). After prolonged storage of 6 months, *cyclo*-(EMTPVNPGQ) exhibited significant, albeit not optimal, biological activity (Fig. 3B). Treatment of aged *cyclo*-(EMTPVNPGQ) with 4 M urea restored the optimal biological activity (data not shown) which suggested that the cyclic peptide might also have aggregated during storage in the lyophilized state.

In order to generate a more hydrophilic analog, the linear octapeptide EMTOVNOG, where O is 4-hydroxyproline, was produced. Like the native octapeptide, this more

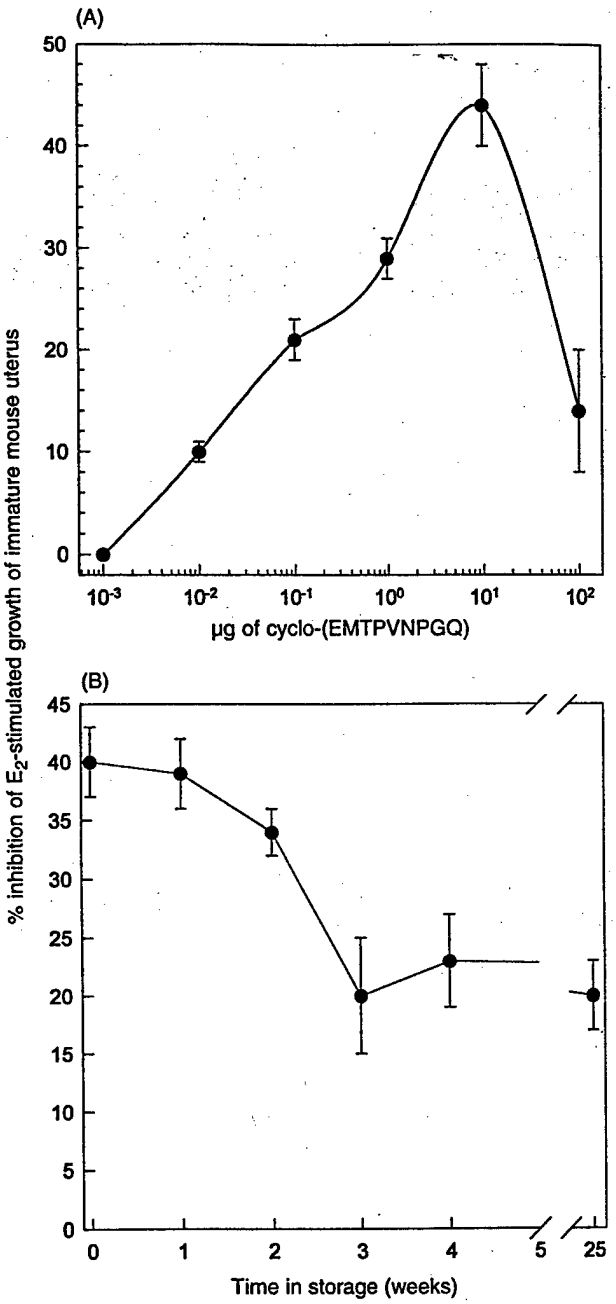

Figure 3. Anti-uterotrophic activity of *cyclo*-(EMTPVNPGQ). (A) Dose–response. (B) Effect of time in storage.

hydrophilic species exhibited dose-dependent inhibition of estrogen-stimulated growth of immature mouse uterus with maximal effect at a dose of 1 μg/mouse (Fig. 4A). This result showed that the substitution (proline to 4-hydroxyproline) did not affect biological activity. Of critical importance, this hydrophilic octapeptide exhibited indefinite shelf-life when tested in the immature mouse uterine growth assay (Fig. 4B). Furthermore, octapeptide EMTOVNOG inhibited the estrogen-dependent growth of MCF-7 human breast cancer xenografts indicating that peptide activity extended to human breast cancer tissue (Fig. 5).

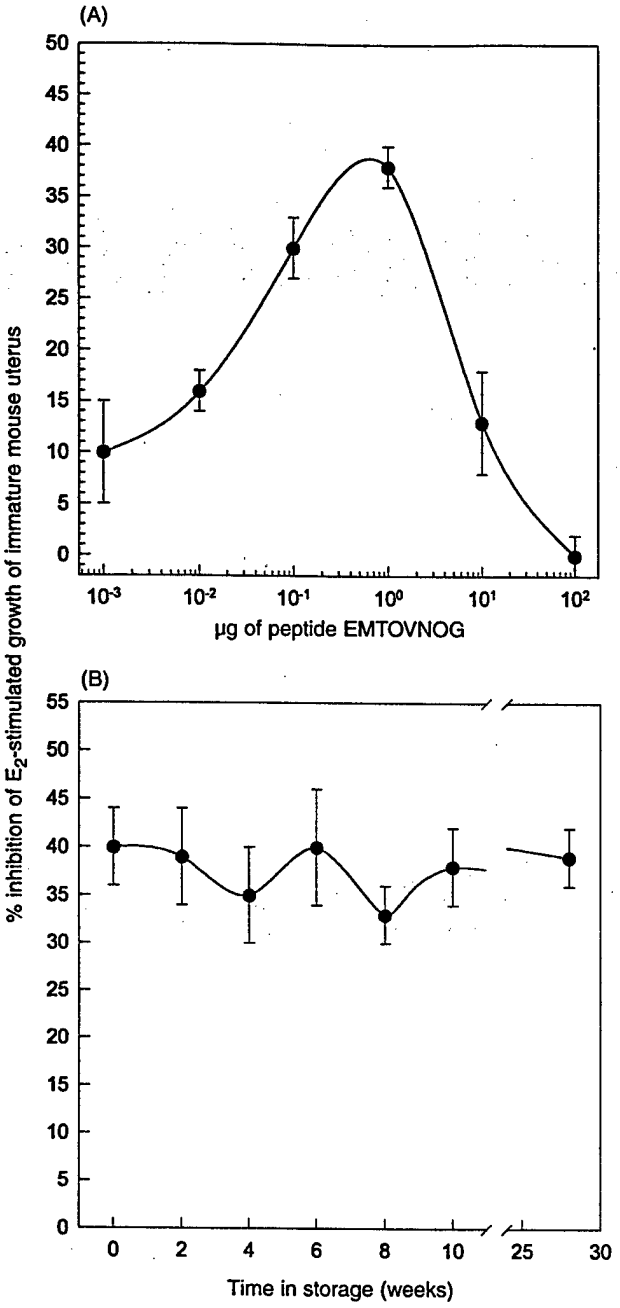

Figure 4. Anti-uterotrophic activity of peptide with hydroxyproline substituted for proline. (A) Dose–response. (B) Effect of time in storage.

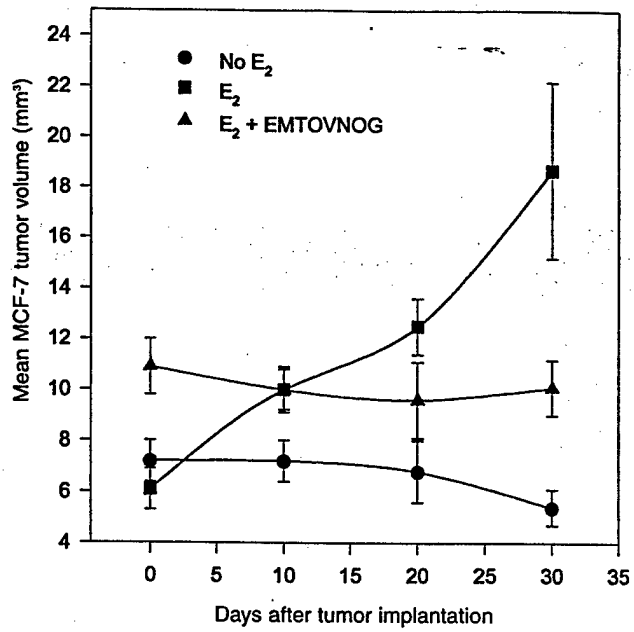

Figure 5. Anti-estrotrophic activity of hydroxyproline-substituted linear peptide against MCF-7 human breast cancer xenografts. There were 5–8 replicate mice per treatment group. Estrogen was provided via a slow release pellet implanted subcutaneously. Peptide was given twice a day i.p. at a dose of 1 µg/mouse. Tumor volumes in each mouse were measured at the time of tumor implantation and at 10-day intervals thereafter during survival laparotomies. At 30 days after tumor implantation, tumor volumes in the E_2+ peptide group were significantly different from tumor volumes in the E_2 alone group, $P \leq 0.05$; Wilcoxon ranks sum test.

Cyclo-(EMTOVNOGQ) was synthesized by head-to-tail cyclization of the precursor nonapeptide as described in Experimental Procedures. This analog incorporates conformational constraint as well as hydrophilic substitution of amino acids. *Cyclo*-(EMTOVNOGQ) exhibited dose-dependent inhibition of estrogen-stimulated growth of immature mouse uterus with the maximum inhibitory activity at a dose of 10 µg/mouse (Fig. 6A). Interestingly, this peptide retained significant anti-estrotrophic activity at doses >10 µg/mouse, leading to a rather broad active dose range. There was no evidence of toxicity to the mice, even at an effective dose of 1 mg/mouse. Furthermore, storage experiments indicated that *cyclo*-(EMTOVNOGQ) had indefinite shelf-life (Fig. 6B). This peptide was therefore tested for antibreast cancer activity, and like the linear form it significantly inhibited the estrogen-dependent growth of MCF-7 human breast cancer xenografts (Fig. 7).

The mechanism by which these peptides mediate anti-estrogenic activity is now under investigation. As a first step in the study of mechanism, it seemed reasonable to explore whether this peptide behaved like classical estrogen receptor antagonists currently in clinical use. As shown in Fig. 8, the linear hydroxyproline-containing octapeptide did not interfere with estradiol binding to its receptor over a broad peptide concentration range, whereas raloxifene at a concentration of 100 nM completely inhibited this association. Thus, the mechanism of action of AFP-derived octapeptide is different from classical estrogen receptor antagonists and requires further investigation to disclose its biochemical target(s).

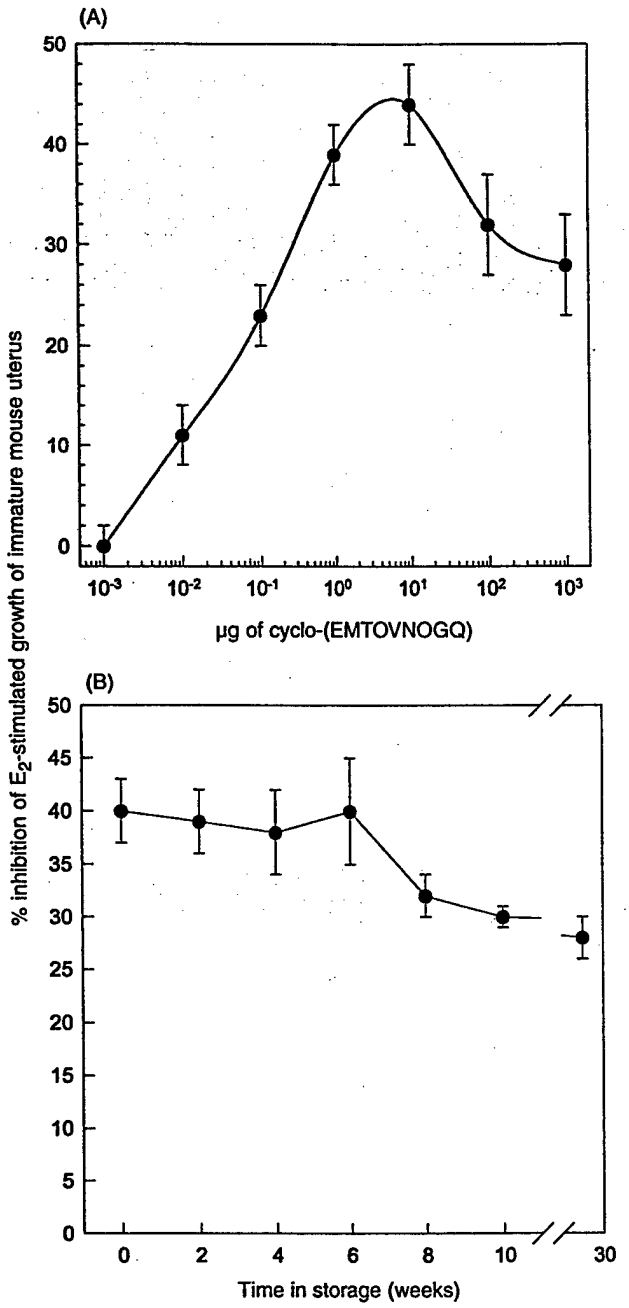

Figure 6. Anti-uterotrophic activity of cyclized peptide with hydroxyproline substituted for proline. (A) Dose–response. (B) Effect of time in storage.

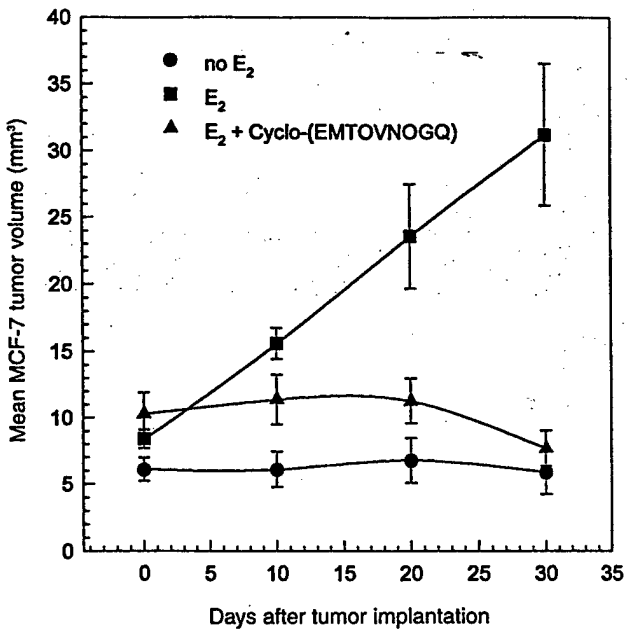

Figure 7. Anti-estrotrophic activity of cyclized peptide with hydroxyproline substituted for proline against MCF-7 human breast cancer xenografts. Experimental protocol is described in legend to Fig. 5 and Experimental Procedures. At 20 and 30 days after tumor implantation tumor volumes in the E_2+ peptide group were significantly different from tumor volumes in the E_2 alone group, $P < 0.05$; Wilcoxon ranks sum test.

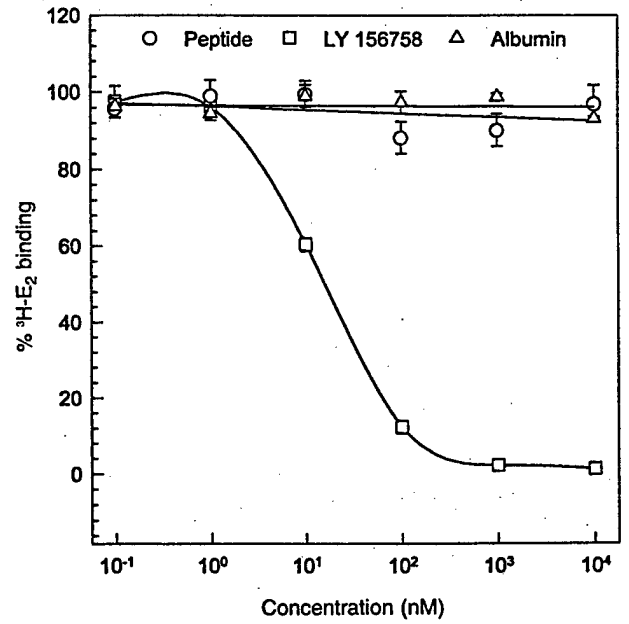

Figure 8. Effect of linear hydroxyproline-substituted peptide on binding of E_2 to its receptor. Rabbit uterine cytosol was used as a source of estrogen receptor. All incubations were performed in triplicate, each containing 100 μL of cytosol, 20 μL of 10 nM 6,7-[^{3}H]estradiol (50 Ci/mmol), and 80 μL of test agent at the final concentrations indicated on the abscissa. Details of the assay are described in Experimental Procedures. Concentration of [^{3}H]E_2-complex with receptor in the presence of different concentrations of test agent is expressed as a percentage of the amount of complex formed in the absence of test agent.

Discussion

The results of this study demonstrated that rational design approaches led to stepwise improvements in the therapeutic usefulness of an anti-estrotrophic peptide derived from AFP. The native peptide, EMTPVNPG, and an analog with a minor modification, QMTPVNPG, were problematic in that they lost their anti-estrotrophic activity after a relatively short time of 4–5 weeks in storage. The urea, as well as the

chromatography, data suggested that monomeric units of these peptides were aggregating during storage, leading to biologically inactive oligomers. Using mass spectrometry analysis, there was no evidence of other possible changes such as asparagine deamidation, methionine oxidation, or pyroglutamate formation to account for loss in biological activity. In these octapeptides, it is likely that the hydrocarbon side-chains of proline and valine created a hydrophobic pocket that associated with like pockets on adjacent monomers resulting in aggregation during storage. However, by increasing the peptide's hydrophilicity by replacing the two prolines with 4-hydroxyprolines, this hydrophobic interaction was apparently impeded as the biological activity of the 4-hydroxyproline-containing peptide did not diminish over time in storage. The net result is that this minor, but novel, design modification yielded a peptide with indefinite shelf-life that is eminently more translatable to the clinic in comparison with a peptide with only 4 weeks of shelf-life.

Aggregation of proteins and peptides is not unusual and in fact has been seen with full-length AFP as well as with subunits of AFP. Wu & Knight (13) showed that AFP tends to form aggregates, which may contribute to its loss of anti-estrotrophic activity during storage. Eisele *et al.* (14) reported that oligomers of various sizes formed during storage of a 34-mer peptide (amino acids 447–480) derived from AFP. Similar aggregation behavior has been seen with a number of other protein and peptide pharmaceuticals including human interferon gamma (5), human calcitonin (3), insulin (2), and synthetic beta-amyloid peptide (16,25,26). Recent studies have shown that increasing peptide hydrophilicity can impede peptide aggregation. Hughes *et al.* (10) and Hilbich *et al.* (27) reported inhibition of amyloid peptide aggregation by substitution of hydrophobic phenylalanine with hydrophilic threonine or by adding poly lysine at the C-terminus of the amyloid peptide. It seems clear that increasing the hydrophilicity of our peptides impeded their aggregation which sustained their biological activity.

Aggregation has been and continues to be a problem in the development of protein- or peptide-based pharmaceuticals. One way of dealing with this problem has been with the use of excipients (28). Although the data are not shown here, we evaluated a variety of excipients as cryoprotectants and lyoprotectants for the native AFP-derived octapeptide EMTPVNPG. Mannitol, as well as dodecylmaltoside, significantly prolonged shelf-life of the peptide, whereas sucrose did not. However, there are complicating factors associated with the use of excipients. Their presence can confound studies of mechanism of action of the primary agent, which is more troublesome during peptide development than during clinical use. Moreover, there is also a risk of toxicity from the excipient. We noted toxic effects of the excipient dodecylmaltoside as its dose was increased to 4 mg/mouse. Therefore, rational peptide design was the preferred approach and was utilized to achieve improved bioactivity, and to extend substantially the shelf-life of these peptides.

It was considered that cyclization of this peptide would limit its flexibility and thereby reduce the number of possible conformations it could assume and that this may in turn broaden the effective dose range assuming that different conformations are not all biologically active and may in fact interfere with each other. However, we did not want to lose the advantages accrued from the hydroxyproline substitutions, and therefore the two approaches were combined. This was remarkably successful. The cyclic, hydrophilic analog, *cyclo*-(EMTOVNOGQ), has full biological activity and indefinite shelf-life. What was even more beneficial about this analog was that its dose–response curve was broadened substantially, greatly increasing the range of doses over which the agent was effective. With linear peptide the dose–response curve was biphasic. A dose of 1.0 μg/mouse produced maximal inhibition, whereas higher doses showed reduced anti-estrotrophic activity. 'Biphasic' dose–response profiles are not unusual with growth regulatory agents. Certainly estrogen itself is biphasic with lower doses stimulating growth and higher doses actually retarding growth (29). Similarly, angiotensin II (7), glucagon-like peptide (8), D-globin-derived synthetic peptide (9), and other protein pharmaceuticals are biphasic in that the optimal biological response modifying dose is less than and yields more biological activity than the maximally tolerated dose. By contrast, with *cyclo*-(EMTOVNOGQ) the shape of the dose–response curve was sigmoidal, with 1.0 μg to 1.0 mg per mouse providing similar anti-estrotrophic activity. This greatly expands the active dose range and increases the probability of maintaining an effective dose as this peptide is translated to humans. Understanding the biochemical basis for the different dose–response curves generated with these peptides requires further investigation.

The finding that both linear and cyclized peptides completely stopped the growth of human MCF-7 breast cancer xenografts is highly significant and certainly demonstrates the relevance of these peptides to breast cancer therapeutics. The magnitude of their inhibitory effect was similar to that of tamoxifen, which was also shown to stop MCF-7 breast cancer xenograft growth in an earlier study

(23). However, their mechanism of action of seems to be different from that of tamoxifen in that they did not interfere with estrogen binding to its receptor. This opens the possibility of combining these agents with tamoxifen or using them in place of tamoxifen when, as so often happens, an estrogen receptor-positive breast cancer becomes resistant to tamoxifen (30).

It is intriguing that development of these peptides constitutes another example of mining a biologically active agent from a much larger parent protein. Multiple cases of this have been described. Recent anticancer examples are angiostatin (31) and endostatin (32), which were mined from plasminogen and collagen XVIII, respectively. This study additionally demonstrates that active agents successfully mined can be further improved by rational design approaches. Simple substitution of hydroxyproline for proline in these AFP-derived peptides significantly prolonged peptide shelf-life. Cyclization substantially broadened the effective dose range. Combination of these approaches yielded an analog with markedly enhanced usability without compromising biological activity. It is hypothesized that continued development of these peptides will yield a new class of valuable agents for the effective treatment of breast cancer.

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