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To test the hypothesis that a novel synthetic peptide can prevent breast cancer, we utilized a standard model to induce breast cancer in rats and initiated a dose-finding study in which four log doses of peptide were administered daily beginning 10 days after treatment with carcinogen, and lasting for 23 days, a time period that mimics pregnancy. Treatment with peptide was then discontinued, and animals were palpated for tumors daily for 100 days. The number of animals with tumors, number of tumors per animal, time to generation of palpable tumors, and mass of tumors (at autopsy) were noted as endpoints, and weight, weight gain, cage activity and fur texture were used as gross assessments of toxicity. The study was not complete at the time of report preparation, but to date no evidence of toxicity due to the peptide has been noted. Early generation of tumors associated with pregnancy was not observed, suggesting that the anti-oncotic peptide does not possess an undesirable immunosuppressive activity. Pre-100 day data indicate fewer cancers in the Peptide- compared to No Peptide groups. We conclude that the model can appropriately assess prevention capability and should generate data concerning dosages for use in assessing the gravidomimetic prevention potential of this novel drug.

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Introduction

This is the Final Report for DAMD17-01-1-0472, a CONCEPT award entitled "Gravidomimetic Prevention of Breast Cancer." The CONCEPT application was written in April of 2000 and funded for the period 14 MAY 2001 to 13 MAY 2002. The format of the CONCEPT application did not require specification of a Statement of Work, but the results described in this report are written as if a Statement of Work had been included in the original application. The amount of the award was \$50,000 of direct costs, which allowed for one prevention study (the cost of maintaining animals for hundreds of days is very high). The term "gravidomimetic" refers to an activity that is similar to that of pregnancy.

Body

Hypothesis. Many epidemiologic studies have shown that an early full-term pregnancy significantly lowers a woman's risk of developing breast cancer (1, reviewed in 2). From the earliest collection of breast cancer incidence data (3), it had been seen that parous women are at lower risk for breast cancer than nulliparous women (*c.f.*, 4). From experimental studies involving the prevention of carcinogen-induced mammary tumors, Grubbs (5) and others (6,7) have shown that pregnancy results in a decreased tumor burden in rats treated with *N*-methyl *N*-nitrosourea (NMU). While there may be many factors during the complex process of pregnancy that contribute to the decrease in breast cancer incidence later in life, it is clear that one of these factors, and probably the major factor, is alpha-fetoprotein (AFP). AFP concentrations in maternal serum are approximately 10-fold that found in the serum of a non-pregnant, normal adult. Jacobson hypothesized (8), and supported with epidemiological studies, and Richardson confirmed by direct measurement (9,10), that women who experience higher levels of maternal serum AFP during pregnancy are at lower risk of breast cancer later in life. Jacobson further hypothesized (11) that AFP acts in an endocrine manner to extinguish pre-malignant foci that, much later in life, would have gone on to develop into cancer. We have shown experimentally that AFP, in a dose-dependent manner, prevents the growth of human tumor xenografts growing in immunodeficient mice (12), that it prevents the growth of estrogen-dependent human breast cancers (MCF-7 and T47D) in cell culture (13-14), and that it is anti-estrogenic (15-17). To the extent that we would be able to show that exposure of breast tissue to exogenously added AFP would result in a decreased incidence of breast cancer, the molecule could be considered gravidomimetic.

AFP itself is unsuitable as a potential drug, either for treatment or prevention of breast cancer, due to its large size, difficulty of production, difficulty of administration, presence of multiple functions (including immunosuppression), and problems associated with its long-term storage. However, we have identified the active site of AFP that imparts its anti-cancer activity and have developed this site into a synthetic octapeptide which

retains the anti-oncotic activity of the parent molecule (13). This anti-estrogenic 8-mer peptide is effective in preventing the growth of human breast cancer xenografts growing in mice, preventing the growth of estrogen-dependent breast cancer cell lines such as MCF-7 or T47D growing in culture, and is anti-uterotrophic, inhibiting the growth of murine uteri that have been stimulated by estrogen or tamoxifen (18). This peptide, and other more effective analogs which we have recently developed (51), are easy to produce, stable during storage, and are likely candidates for novel breast cancer treatment drugs. Due to their small size, it was thought likely that peptidic and peptidomimetic analogs could be developed that would be orally active pharmaceutical agents. However, nothing was known about the ability of these peptides to *prevent* cancer. To the extent that small synthetic peptides act in the same manner as does the parent protein molecule, the peptides should prevent breast cancer gravidomimetically. Using our most potent, most stable peptide analog, it became possible to compare directly, in the Grubbs model, the active site of AFP to pregnancy itself with the endpoint being prevention of breast cancer.

Therefore, the hypothesis was that administration of peptide to *N*-methyl *N*-nitrosourea-treated rats would decrease the tumor burden induced by the carcinogen.

Background

AFP as an Active Agent in Pregnancy. Epidemiological data suggest that AFP is one of the factors in pregnancy that confer on parous women their significant reduction in risk of breast cancer. As shown in Table 1 (next page), AFP is elevated in maternal serum during pregnancy. Furthermore, there are factors in pregnancy, such as maternal race, weight, consumption of alcohol, hypertension, number of fetuses *in utero*, and neural tube defect in the fetus, where maternal serum AFP (MSAFP) is substantially altered from normal pregnancy levels. In studying the literature, Jacobson noted (11) the consistent and striking correlation that in those pregnancy conditions associated with an elevated level of MSAFP (namely race and weight), there was a significant reduction in the lifetime risk to the mother of acquiring breast cancer. Conversely, in pregnancy conditions characterized by low MSAFP (alcohol), subsequent breast cancer risk was elevated. This led Jacobson and Janerich to undertake epidemiological studies of three other pregnancy conditions (hypertension, twinning, and neural tube defects) that are associated with elevated maternal serum AFP. For each of these, a correlation between high maternal serum AFP and reduced breast cancer risk was observed. Further, Ekblom *et al.* (19) have published an epidemiological study which suggests that, at least in the case of hypertension during pregnancy, the reduction of breast cancer risk is also passed on to the fetus. All known reports of Mothers-of-Twins and breast cancer that have failed to confirm the Jacobson hypothesis have employed small populations that lacked sufficient power to confirm or negate the observation (20-22), or included older populations of women in whom malignant transformations are likely to have occurred after their final pregnancy (21-22). The report by Albrektsen *et al.* (23) using a large population of Norwegian breast cancer patients, stated explicitly that the "reduced risk of breast cancer observed among women with multiple births compared to women with singletons only is consistent with results reported by Jacobson *et al.* [(24)]. Our results

give some support to the observation of a more pronounced protective effect of a multiple last birth (Jacobson *et al.* [(24)]; Nasca *et al.* [(25)]).”

Recently, Richardson *et al.* (9) employed data from the University of California at Berkeley Child Health and Development Study to measure directly the association between maternal serum AFP concentrations and subsequent breast cancer incidence. They found that the concentration of AFP in maternal sera that had been cryogenically preserved was inversely correlated to the risk of breast cancer in these same mothers 20 to 30 years after their pregnancies. Richardson (9,26) concluded that “the results of this study agree with the protective effect reported by Jacobson *et al.* [(8)] and Thompson *et al.* [(27)] using surrogate indicators (multiple births and hypertension) for a high level of AFP during the index pregnancy.” Recently, a report by Melbye *et al.* (28) concluded that “a high level of AFP in maternal serum during any pregnancy is associated with a low overall incidence of breast cancer, and, in particular, with a low incidence of *advanced* breast cancer. This association appears particularly strong for a pregnancy occurring at a young age.”

Table 1. Association of High Maternal Serum AFP Level with Decreased Breast Cancer Risk

Maternal Condition 1	Maternal Condition 2	MSAFP	Maternal Lifetime Breast Cancer Risk
Pregnant	Non-pregnant	1>2 (29)	1<2 (30)
Pregnant, Black	Pregnant, white	1>2 (31)	1<2 (32)
Pregnant, lean	Pregnant, obese	1>2 (31)	1<2 (33)
Pregnant, consuming no alcohol	Pregnant, consuming alcohol	1>2 (34)	1<2 (35)
Pregnant, hypertensive	Pregnant, normotensive	1>2 (36)	1<2 (27)
Pregnant with multiple fetuses	Pregnant with a single fetus	1>2 (27)	1<2 (24)
Pregnant, fetus with neural tube defect	Pregnant, fetus without neural tube defect	1>2 (38)	1<2 (39)

Numbers in parenthesis are the references for the data. See also refs (11-12).

Anti-oncotic activity of AFP. In addition to the epidemiological studies which suggested to us that AFP may be an active agent in risk reduction, various clinical observations also suggested a similar interpretation. For example, it is known that hepatomas secrete AFP (40). In fact, serum AFP levels are used as a marker of tumor burdens in this disease. What is less well-known is that amenorrhea is one of the first symptoms of hepatoma in

premenopausal women, and this symptom resolves following surgical removal of the tumor (41). Moreover, hyperestrogenemia and normal-to-elevated levels of gonadotropins are present in hepatoma patients (42). Taken together, these data suggest that neither the uterus nor the hypothalamic-pituitary axis is responding to estrogen in hepatoma patients. It is our belief that elevated AFP levels could bring about this result.

Laboratory investigations also support the interpretation that AFP may protect against breast cancer. We have shown (12) that AFP inhibited the growth of estrogen-dependent human breast cancer xenografts in mice, and that estrogen-independent tumors were not affected by AFP. We have also shown that AFP inhibits the estrogen-stimulated growth of normal mouse uterus (14-16), which serves as a rapid and convenient screening assay (compared to the xenograft assay). Soto *et al.* (43) have shown that AFP-containing serum from a hepatoma-bearing rat inhibited the estrogen-stimulated induction of progesterin receptor, and that an AFP-secreting tumor induced the regression of an estrogen-dependent rat mammary tumor (44). We have shown that AFP inhibits the growth in culture of MCF-7 breast cancer cells and T47D breast cancer cells (13-14). Indeed, there can be little doubt that AFP inhibits estrogen-dependent breast cancer growth.

Active site of AFP. We (and others) noted that certain chemical environments alter the structure and activity of AFP. For example, when AFP is placed in a large molar excess of estrogen, it undergoes a change in conformation demonstrated by difference spectroscopy (17,46), and its optimally effective anti-estrotrophic dose is *decreased* by two orders of magnitude (12-16). We speculated that this was due to the exposure of an active site of AFP as a result of a conformational change. We showed that estrogen sequestration by AFP was not responsible for the observed biological effects, and we began studies to identify the portion of the protein responsible for the anti-estrogenic, anti-oncotic activities. Molecular biology approaches were used to express Domain III and various other fragments; Domain III was shown to possess full anti-estrotrophic activity, while Domain I had no activity. Further, a smaller fragment, termed Subdomain AB of Domain III had full activity (17), and this encouraged us to switch to the synthetic peptide approach to further narrow the active site.

We identified (13) the *minimal* active site of AFP, in terms of anti-oncotic and anti-estrogenic assays, as an octapeptide of the sequence EMTPVNPG. However, there was no reason to believe that the native 8-mer was the *optimal* ligand, so we prepared dozens of analogs of this peptide. Our approach to analog generation was guided by three principles. *a.)* Because the original peptide lost activity upon storage in the lyophilized state, and based on our extensive work on a precursor 34-mer peptide in which we documented aggregation of 34-mer (47), we were led to believe that 8-mer was aggregating and that the aggregates are less bioactive. Therefore, we synthesized many analogs that were more hydrophilic than the parent 8-mer in an attempt to obviate aggregation. *b.)* We used simple modeling programs to produce and assess energy-minimized structures of the 8-mer and various analogs, and this led us to produce cyclic analogs. *c.)* Finally, we utilized a directed combinatorial replacement approach for generation of additional analogs, in search of a super-agonist of the active site peptide.

Cyclic analogs were devised to provide conformational constraint and because they are also thought to be of longer biological half-life (especially those that incorporate some D-amino acids). These concepts are currently being examined by analysis of recovered peptide from serum. (Our cyclic analogs are obviously the prototypes for the peptidomimetics that will be developed in the future.) We utilized N-alpha Fmoc-L-glutamic acid-alpha-allyl ester at the C-terminus of the synthetic peptide, which we coupled to the resin *via* the gamma carboxylic acid. Removal of the 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 allyl group. This 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 to yield the gamma-carboxamido derivative (*i.e.*, Q). The cyclic peptide was then purified and characterized. To date, published data demonstrate that a small, easily-synthesized, cyclic peptide, derived from the naturally occurring protein AFP, retains the full anti-oncotic activity of the parent protein. The peptide is *cycloEMTOVNOGQ*.

Prevention models. Two chemical carcinogens relatively specific for mammary cancer induction, namely dimethylbenzanthracene (DMBA) and *N*-methyl *N*-nitrosourea (NMU) have been used frequently for the rapid induction of experimental mammary cancer in rodents (48). The procedures call for administration of the carcinogen to rats followed by treatment with the experimental drug designed to prevent cancer, and palpating the animals for several weeks to assess tumor burden. To date, no methodological surrogates for this tedious, costly, and seemingly unsophisticated approach have been devised.

Grubbs and colleagues often use the *N*-methyl-*N*-nitrosourea (NMU) model of carcinogenesis, which employs Sprague Dawley rats, 40-50 days of age. NMU causes mainly estrogen-receptor positive tumors, which is more appropriate for our study which will evaluate the chemopreventive properties of a novel anti-estrotrophic peptide. NMU is administered in a single dose in saline, pH 5.0 administered *iv* into the left jugular vein. Rats are monitored for 20 weeks or more, but can be maintained in regular cages and need no special treatment to protect the animal care workers during disposal of cage bedding. Also important is that carcinogenesis occurs at essentially a single point in time, rather than across a duration of several days as may be the case in the DMBA model. NMU has a half-life measured in minutes at the pH of serum (49) and therefore accomplishes its carcinogenic function within the few minutes available to it before it decomposes. It should be noted that both tamoxifen and Raloxifene were subjected to study using these prevention assessment models (reviewed in 50), and the data obtained provided confidence that was very helpful during clinical trials of these agents.

Results.

Task 1. Synthesis and characterization of anti-oncotic peptide.

After the submission of the CONCEPT application but prior to commencement of funding, cyclic peptide analogs were devised in order to provide conformational constraint and because they were thought to be of longer biological half-life. These analogs were shown to be active (51, included in Appendix) and became the lead compounds with which we performed all studies in this report. Correct synthesis and proper purification were confirmed by high pressure liquid chromatography for purity, amino acid analysis for identity, purity, and quantitation of amount of peptide. Mass spectroscopy and Edman degradation sequencing confirmed the correct synthesis. Biological activity of the cyclic analogs suggested that the molecules were more active than the linear precursors, and that an extended dose-response curve was obtained (51). Preliminary NMR studies to determine conformation of these cyclic molecules were initiated during the project period, but no results are available yet. To assess the serum stability of these molecules, studies were conducted in which the molecules were incubated in serum at room temperature for various times. These studies did indicate that the cyclic peptide is subject to proteolysis, preventable by the addition of a 'cocktail' of protease inhibitors. This observation led us to initiate studies designed to generate an active peptide which is not subject to proteolysis in serum, but the results from this study are not yet available.

Task 2. Prevention Trial: Dose-finding and Toxicity Assessment Experiment

To test the hypothesis that this novel synthetic peptide can prevent breast cancer, we utilized a standard model to induce breast cancer in rats and initiated a dose finding study. The cancer induction model was that of Grubbs (49, 52-54), a widely accepted model but one that was new to this laboratory. To train in the implementation of this model, the PI spent some time in the laboratory of Clinton J. Grubbs, Ph.D. at the University of Alabama and became familiar with the surgical techniques necessary to administer the carcinogen, the methods to palpate the animals, and the means of data collection and processing. This short-term training opportunity was funded by the Albany Medical College Faculty Development (Wiggers) Award. Using Sprague-Dawley rats, NMU was administered at 50 days of age, and 10 days later peptide was administered once daily for 23 days, a time period that mimics pregnancy. Treatment was then discontinued, and animals were palpated daily for 100 days.

Weight, weight gain, cage activity and fur texture were used as ongoing gross assessments of toxicity prior to autopsy, and uterine weight was assessed *post mortem*. No evidence of toxicity was noted for any animal treated with peptide, even at the highest dose of 100 ug per animal per day. There was no difference in weight of animals between any groups (carcinogen treated or not, peptide treated or not), suggesting no evident toxicity due to peptide. Cage activity was indistinguishable between groups, and there was no effect on fur texture except in control animals whose tumors grew very large and had to be sacrificed. Uterine weights were indistinguishable between groups, and

varied depending on the time of the cycle between animals, again indicating no evident toxicity in these preliminary assessments.

Number of animals with tumors, number of tumors per animal, and time to generation of palpable tumors were used as endpoints for a dose-finding study in which 0.0, 0.1, 1.0, 10.0, and 100.0 ug of peptide per animal were used. Mass of tumors at autopsy will also become available. Using pre-100 day data (necessary for report generation), there were no significant differences between the 10, 1, and 0.1 ug peptide per animal groups, but the 100 ug peptide per animal group showed significantly longer time to generation of palpable tumors: this parameter averaged 47 days in the low-dose and no-peptide groups and was 66 days in the high-dose group. Obviously the number of animals with tumors and number of tumors per animal were also significantly lower for the high-dose group. These data obtained until 70 days, at which time tumors began to appear in the high-dose group. These data are similar to what was reported for tamoxifen in the early prevention studies in this model (55,56), although those studies indicated some decrease in weight gain that is not evident in peptide-treated animals. It should be noted that the experimental design, that of limiting the peptide-treatment time to 23 days to correspond to pregnancy, is very stringent. Prolonged treatment with peptide, such as was developed for tamoxifen, may provide substantial additional benefit and will be the subject of future prevention trials.

In Grubbs early work, tumors appeared earlier in the pregnancy group than in the nulliparous group: in the pregnant group of animals, tumors first appeared at day 20 (after administration of NMU, or 10 days after insemination). This early onset of tumors is likely due to the immune suppression that accompanies pregnancy and which is necessary for maintenance of pregnancy. This immune suppression may be due to alpha-fetoprotein, and it is very important to monitor for the possibility of this effect in any AFP-derived drug. Since the peptide used in this study is designed to be the anti-oncotic active site, and not the immune suppression active site, of AFP, it was thought that the early onset of tumors would be obviated by the use of this drug. That this was the case is shown conclusively in that no animal developed tumors before 45 days post-NMU in any peptide treated group, and this was not different for the No Peptide (NMU only) group. Thus, the drug has achieved a major aspect of its design intent, namely the ability to prevent cancer in a manner that is superior to pregnancy, or to AFP itself.

Key Research Accomplishments

- Learned to implement the Grubbs model of induction of ER+ breast cancer
- Synthesized and characterized a novel, cyclic peptide with activity against human breast cancer
- Identified means to extend shelf-life indefinitely
- Identified the serum half-life of the cyclic molecules and developed plans to make molecules that will last even longer than the currently available analogs
- Conducted one prevention trial, a dose-finding study: Concluded that 0.1 mg/animal is optimal dose
- No early generation of tumors as occurs with pregnancy, indicating peptide is superior to pregnancy in terms of preventing cancer
- No evidence of toxicity of any sort has been detected at doses up to 100 ug per animal per day
- Peptide extended tumor-free-days by almost three weeks following a 3-week period during which the drug was administered. This encouraging result is similar to the early prevention studies with tamoxifen.

Reportable outcomes

Manuscripts. No manuscripts have been prepared as of the time of this report.

Abstracts. Two abstracts have been submitted:

- Era of Hope meeting in Orlando, FL for September 2002: Gravidomimetic Prevention of Breast Cancer. (Copy in Appendix)
- AACR Frontiers in Cancer Prevention Research meeting in Boston, MA for October 2002: Gravidomimetic Prevention of Breast Cancer (Copy in Appendix)

Presentations

- Rensselaer Polytechnic Institute April 2002: A Novel Peptide for the Treatment and Prevention of Breast Cancer
- Siena College March 2001: A Novel Peptide for the Treatment and Prevention of Breast Cancer
- Cancer Center of Albany Medical Center Hospital. September 2001: Novel Peptide that Stops the Growth of Estrogen-Dependent Breast Cancer

Patents

- Andersen, T.T., Bennett, J.A., Jacobson, H.I., Mesfin, F.B AFP Peptides and Uses Thereof. Filed 1 June 2001
- Andersen, T.T., Bennett, J.A., Jacobson, H.I., and Mesfin, F.B. Alpha-Fetoprotein Peptides and Uses Thereof. Provisional application filed November 22, 2001.

These are two different patents that have the same name.

Funding applied for

- NIH FIRCA. 1 R03 TW006124-01 Application submitted 15 MAR 2002. Title: Elucidating a Novel Breast Cancer Inhibition Mechanism.
- Charitable Leadership Foundation. Application submitted 15 SEP 2001. Title: Analogs of a Synthetic Peptide with Anti-Cancer Activity
- NIH NIBIB Application submitted 24 APR 2002. Title: REACT: A Novel MR Platform for Molecular Imaging
- Army IDEA award. Application due 13 JUN 2002. Title: Gravidomimetic Prevention of Breast Cancer
- NIH Prevention grant. Application due 1 OCT 2002. Title: Prevention of Breast Cancer with a Novel Cyclic Peptide

Conclusions

Importance of the completed work. The work to date suggests that the anti-oncotic peptide, which has been shown to be useful for the *treatment* of existing breast cancers (in animals), may also be effective for the *prevention* of breast cancer. The peptide appears to prevent cancer to an extent similar to that reported for tamoxifen under similar experimental design. A second study, ongoing at the time of report writing and funded by the NIH, will provide one complete study of longer duration and with an experimental designed improved upon as a result of these initial studies.

Recommended changes: For future work, it is recommended that the experimental design not concentrate on testing the *gravidomimetic* portion of the hypothesis, but rather should concentrate on drug development. Although it is important to the PI to demonstrate that the AFP peptide acts in a fashion similar to AFP and that AFP is *the* active agent of pregnancy that decreases subsequent risk of breast cancer, the most important aspect of the work is to get a new preventive agent to clinical trials as quickly as possible. Thus, if time and funding permit, the gravidomimetic portion (comparing peptide to pregnancy) may be completed, but the focus will be on drug development. In addition, drug development work must focus on generating an orally active agent, as prevention agents must be convenient and of very low risk.

So What? : Part of the significance of this novel AFP-related peptide has not been reported in this summary because it was supported by other funds. We have shown in ref 18 (see Appendix) that the peptide works by a mechanism that is different from that of tamoxifen, and that the peptide is effective against tamoxifen-resistant forms of breast cancer. The potential for such a drug in *treatment* of breast cancer is impressive: a.) the peptide could be used after cancers have become tamoxifen-resistant (and when the patient has very few options), or b.) the peptide could be used in conjunction with tamoxifen, especially if we could show that lower doses of tamoxifen (or of both drugs) would be efficacious, thereby delaying onset of resistance to tamoxifen, or c.) the peptide

could be used alone, in replacement of tamoxifen, especially since the peptide, unlike tamoxifen, is not uterotrophic (18). Similar potential presumably exists for the *prevention* aspects of the drug *vis a vis* tamoxifen. The potential significance of this peptide, and of superior molecules based on the structure of the existing peptide, is impressive.

In other studies, we plan to test the peptide and some of its linear analogs for the ability to assist in the early detection of breast cancer. The grant application to the NIBIB (listed above) outlines a proposal by which the peptide could be a targeting ligand useful for the early detection of breast cancer by magnetic resonance imaging. Thus, the peptide has potential for treatment, prevention, and early diagnosis of breast cancer, and it will be important to develop all three arms.

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APPENDICES

Bibliography of Abstracts

One abstract has been submitted:

- Era of Hope meeting in Orlando, FL for September 2002:
Gravidomimetic Prevention of Breast Cancer. (Copy in Appendix)

Two papers are included in the Appendix:

- Mesfin, FB, Andersen, TT, Jacobson, HI, Zhu, SJ, and Bennett, JA. Development of a cyclized peptide derived from alpha-fetoprotein that prevents the growth of human breast cancer. *J. Peptide Res.* 58: 246-256, 2001.
- Bennett, JA, Mesfin, FB, Andersen, TT, Gierthy, JF, and 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., USA* 99: 2211-2215, 2002.

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GRAVIDOMIMETIC PREVENTION OF BREAST CANCER

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From population studies, it is clear that pregnancy reduces the lifetime risk of breast cancer (BrCa). The major pregnancy-associated factor that contributes to this decrease in risk of BrCa is alpha-fetoprotein (AFP). We hypothesized that AFP acts in an endocrine manner to extinguish pre-malignant foci that would, later in life, develop into cancer. We have shown that AFP prevents the growth of human tumor xenografts growing in mice, that it is anti-estrogenic, and that it prevents the growth of estrogen-dependent cancers in cell culture. AFP itself is unsuitable as a potential drug for either the treatment or prevention of BrCa, not only because it is a large protein but also because it has been shown to have an immunosuppressive function. It has been suggested that this immunosuppressive function leads to the early appearance of a small number of aggressive breast cancers shortly after pregnancy, but because of AFP's anti-estrotrophic activity, there is an overall decrease in tumor burden (in animal models) and tumor incidence (in women). We have identified and synthesized the active site of AFP (an octapeptide) and shown that it retains the full anti-oncotic activity of AFP. It prevents human BrCa growth in culture and as xenografts in immune deficient mice. It prevents the growth of tamoxifen-resistant MCF-7 human breast cancer xenografts and impedes the trophic effects of estrogen or tamoxifen on the uterus. Due to its small size, ease of synthesis, stability during storage, single (anti-oncotic) function, potency, and lack of apparent toxicity, this peptide may serve as a lead compound from which orally active agents for the treatment and/or prevention of BrCa could be generated. The peptide, in so far as it has ability to serve as a surrogate for intact AFP, should be gravidomimetic, and should prevent BrCa.

To test the hypothesis that the peptide can prevent BrCa, we utilized the N-Nitroso-N-methyl urea (NMU)-induced breast cancer model in rats. This model generates a high percentage of estrogen-receptor-positive breast cancers. We have initiated a dose-finding study in which four log doses of peptide were administered daily beginning 10 days after NMU treatment, and lasting for 23 days, a time period that mimics pregnancy. Treatment with peptide was then discontinued, and animals were palpated daily for 100 days. The number of animals with tumors, number of tumors per animal, time to generation of palpable tumors, and mass of tumors (at autopsy) were noted as endpoints, and weight, weight gain, cage activity and fur texture were used as gross assessments of toxicity. The study was not complete at the time of Abstract submission, but no evidence of toxicity due to peptide has yet been noted. Early generation of tumors associated with pregnancy was not observed, suggesting that the anti-oncotic site of AFP does not possess immunosuppressive activity. Pre-100 day data indicate fewer cancers in the Peptide-compared to No Peptide groups. We conclude that the model can appropriately assess prevention capability and should generate data concerning dosages for use in assessing the gravidomimetic potential of this AFP-derived peptide.

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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, EMTPVNPQ, 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 EMTPVNPQ 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 EMTPVNPQ (maximal activity at 1 μ g/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; E₂, estradiol; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, O-(7-azabenzotriazol-1-yl)-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.

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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 $1 \mu\text{g}/\text{mouse}$ 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 N²-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 (OtBu), Hyp (tBu), Thr (tBu) 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 N²-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(PPh₃) in CHCl₃/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 C₁₈ (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 (E₂) or vehicle control for E₂ 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 (%)

$$= \frac{(\text{Full E}_2 \text{ stimulation} - \text{E}_2 \text{ stimulation in test group})}{(\text{Full E}_2 \text{ stimulation} - \text{No E}_2 \text{ stimulation})} \times 100\%$$

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 E₂ 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 \pm 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-[³H]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 [³H]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

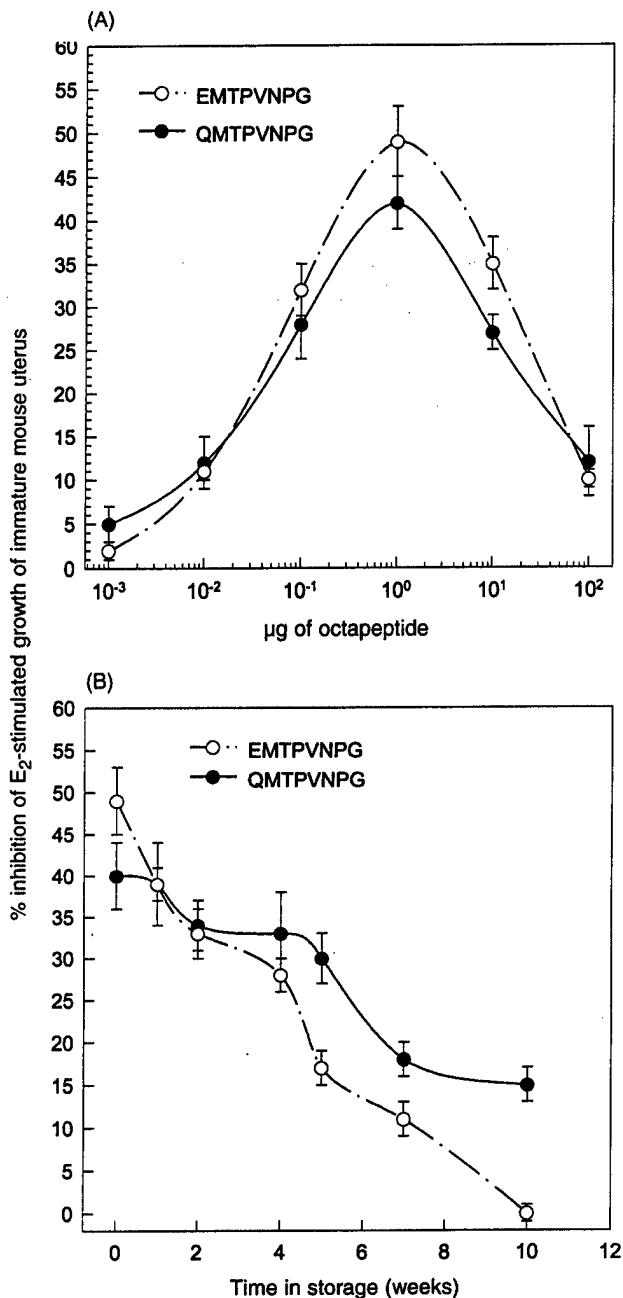


Figure 1. Anti-uterotrophic activity of octapeptide, QMTPVNPG, measured in the immature mouse uterine growth assay. Peptide or vehicle control was injected i.p. into immature female Swiss mice. One hour later 0.5 µg of E₂ or vehicle control was injected i.p. into these mice. Twenty-two hours later, uteri were dissected and weighed. Percent inhibition of E₂-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.

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

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.

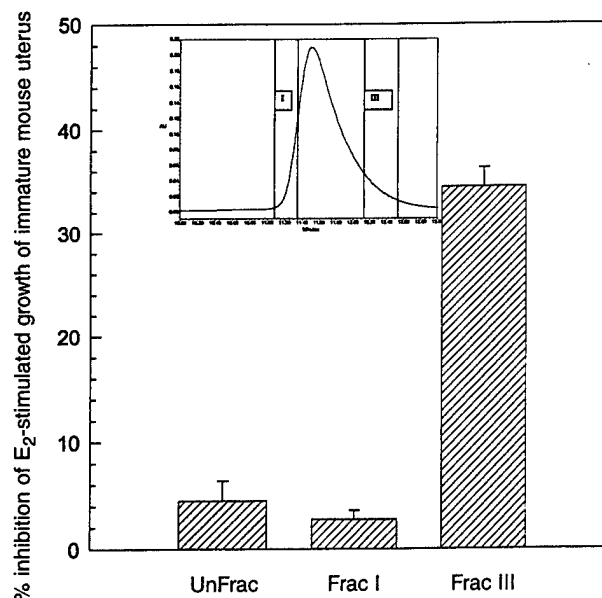


Figure 2. Anti-uterotrophic activity of fractions from gel-filtration chromatography of stored octapeptide, QMTPVNPG. Peptide, QMTPVNPG, was fractionated using a Waters SW 200 gel-filtration column using phosphate-buffered saline pH 7.4 as mobile phase. Fractions which had significant UV absorbance at 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 E₂-stimulated growth was measured.

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 $\mu\text{g}/\text{mouse}$ (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 $\mu\text{g}/\text{mouse}$ (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

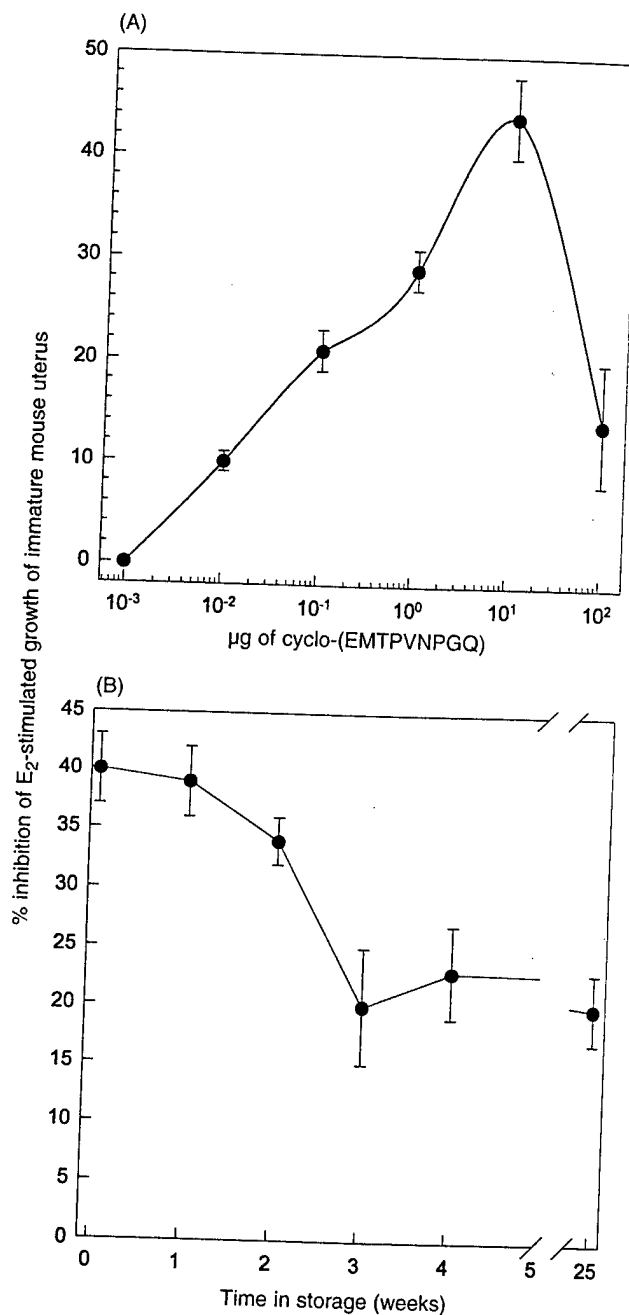


Figure 3. Anti-uterotrophic activity of *cyclo*-(EMTPVNPGQ). (A) Dose-response. (B) Effect of time in storage.

hydrophilic species exhibited dose-dependent inhibition of estrogen-stimulated growth of immature mouse uterus with maximal effect at a dose of 1 $\mu\text{g}/\text{mouse}$ (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).

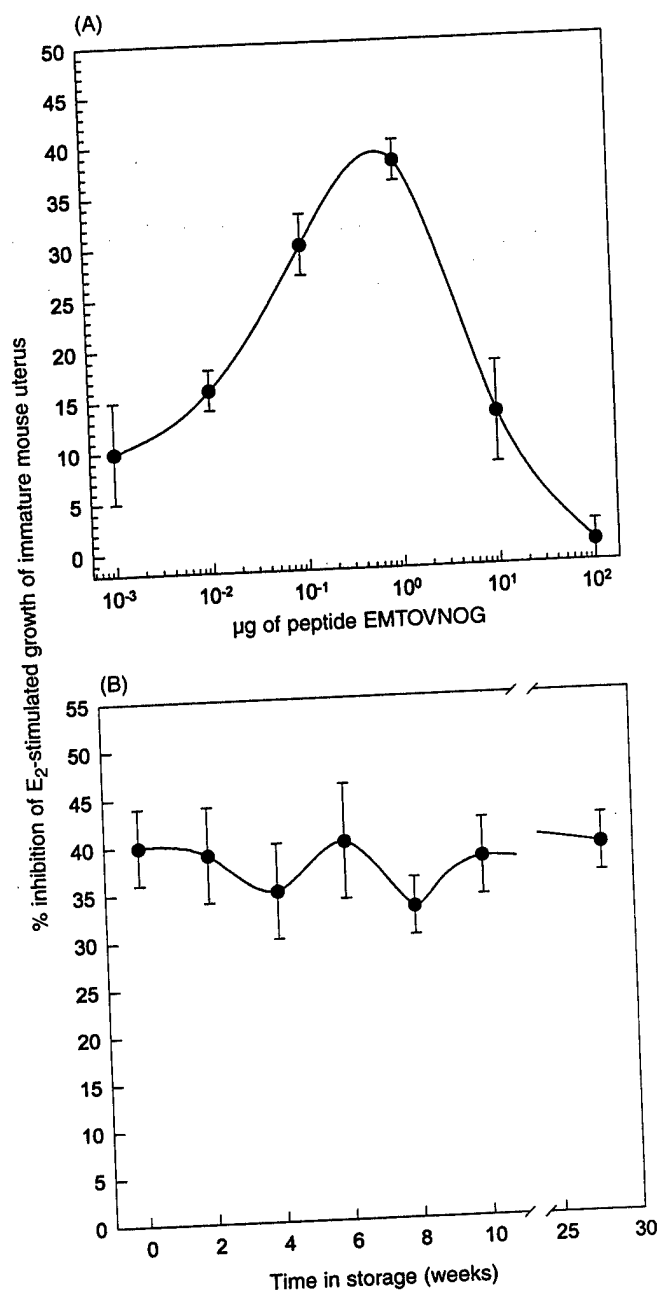


Figure 4. Anti-uterotropic activity of peptide with hydroxyproline substituted for proline. (A) Dose-response. (B) Effect of time in storage.

Cyclo-(EMTVNOGQ) 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*-(EMTVNOGQ) 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

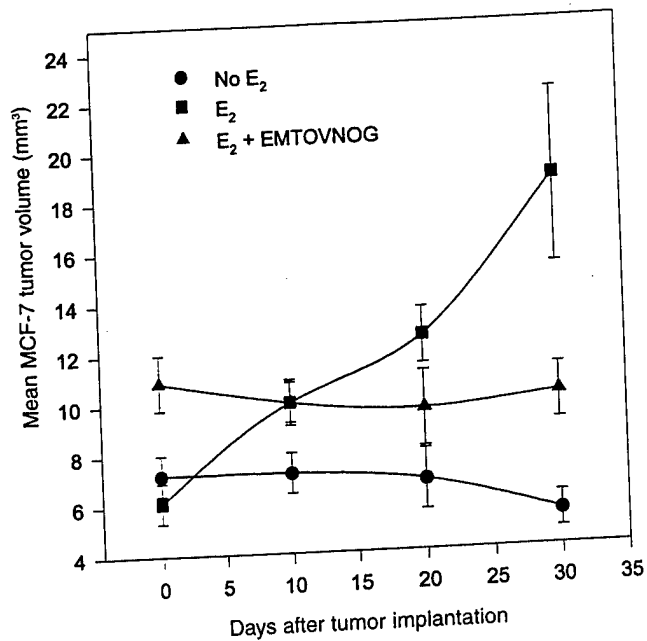


Figure 5. Anti-estrotrophic activity of hydroxyproline-substituted linear peptide against MCF-7 human breast cancer xenografts. There were 5-8 replicate mice per treatment group. Estrogen was provided via a slow release pellet implanted subcutaneously. Peptide was given twice a day i.p. at a dose of 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 E₂+ peptide group were significantly different from tumor volumes in the E₂ alone group, $P \leq 0.05$; Wilcoxon ranks sum test.

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*-(EMTVNOGQ) 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).

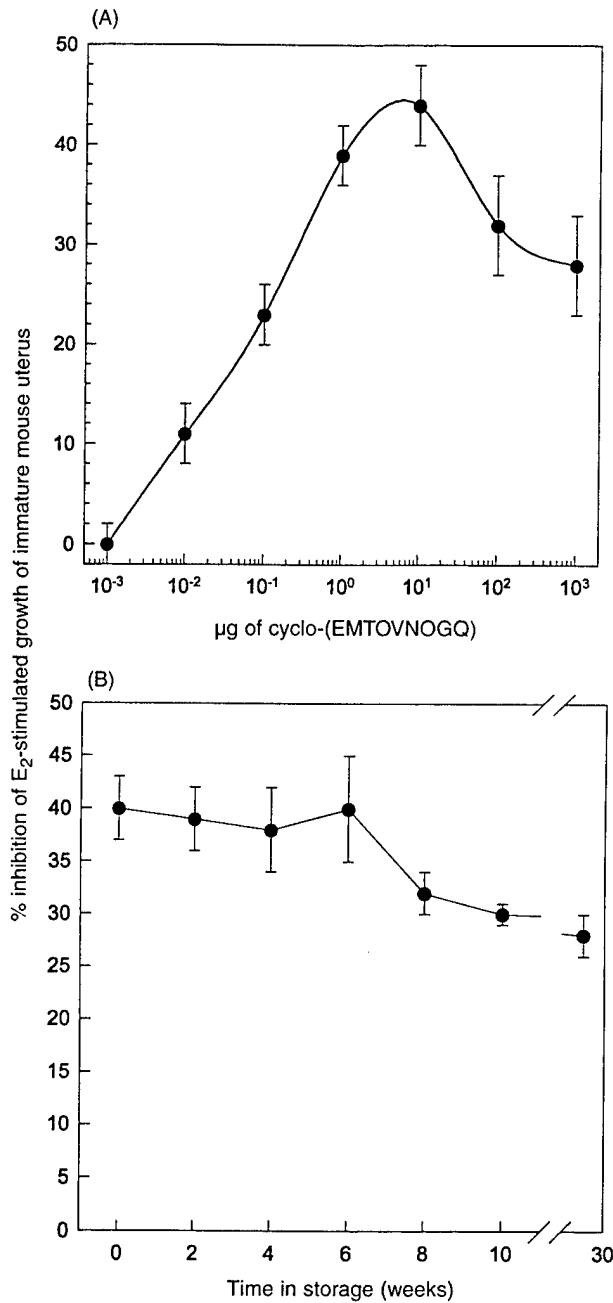


Figure 6. Anti-uterotrophic activity of cyclized peptide with hydroxyproline substituted for proline. (A) Dose-response. (B) Effect of time in storage.

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

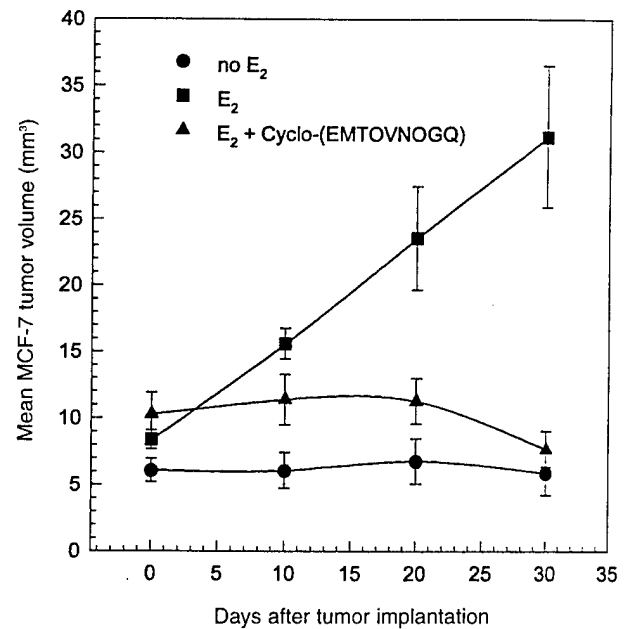


Figure 7. Anti-estrotrophic activity of cyclized peptide with hydroxyproline substituted for proline against MCF-7 human breast cancer xenografts. Experimental protocol is described in legend to Fig. 5 and Experimental Procedures. At 20 and 30 days after tumor implantation tumor volumes in the E₂+ peptide group were significantly different from tumor volumes in the E₂ alone group, $P < 0.05$; Wilcoxon ranks sum test.

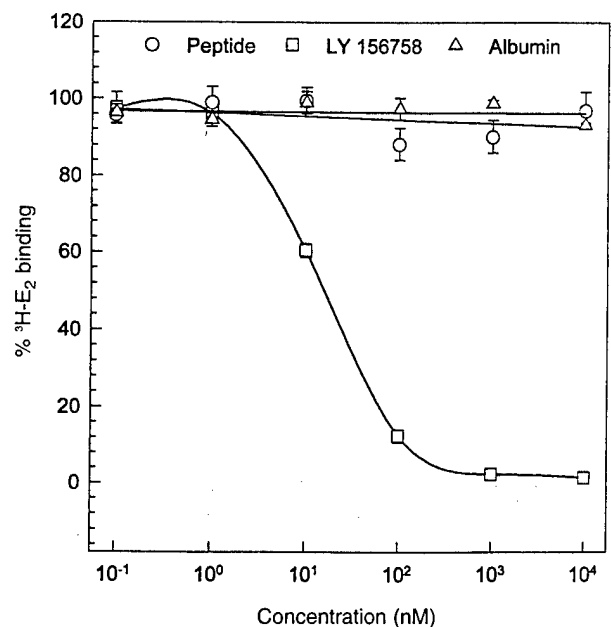


Figure 8. Effect of linear hydroxyproline-substituted peptide on binding of E₂ to its receptor. Rabbit uterine cytosol was used as a source of estrogen receptor. All incubations were performed in triplicate, each containing 100 µL of cytosol, 20 µL of 10 nM 6,7-[³H]estradiol (50 Ci/mmol), and 80 µL of test agent at the final concentrations indicated on the abscissa. Details of the assay are described in Experimental Procedures. Concentration of [³H]E₂-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.

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 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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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 [³H]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 μ g/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 μ g/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 17 β -estradiol (E₂) 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 μ M tamoxifen citrate during routine culture conditions (1:10 sub-

Abbreviations: AFP, α -fetoprotein; ER, estrogen receptor; E₂, 17 β -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 $200 \times g$. The pellet of each cell line then was solidified into a fibrin clot by exposing it to $10 \mu\text{l}$ of fibrinogen (50 mg/ml) and $10 \mu\text{l}$ 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 $1 \mu\text{g}$ 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 $(\pi/6)(d)^2D$, 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 \pm 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 antiestrogenic activity was performed by using an immature mouse uterine growth assay based on previous studies, which demonstrated that i.p. administration of $0.5 \mu\text{g}$ 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 \pm 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 ($50,000 \times g$) 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 $100 \mu\text{l}$ of cytosol, $20 \mu\text{l}$ of 10 mM 6,7- ^3H estradiol [50 Ci/mmol (1 Ci = 37 GBq); NEN], and $80 \mu\text{l}$ of putative antagonist in assay buffer. Total count tubes received $20 \mu\text{l}$ of ^3H estradiol and $180 \mu\text{l}$ of assay buffer. After incubation overnight at 4°C , all but the total count tubes received $300 \mu\text{l}$ of dextran-coated charcoal suspension; tubes were agitated for 15 min and then centrifuged ($1,000 \times g$) 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 antiestrogenic dose of AFPep was 0.1 – $1.0 \mu\text{g}$ 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 $1.0 \mu\text{g}$ 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. 1A, 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 $1 \mu\text{g}$ of AFPep, tumor growth was prevented over the 30-day observation period. Similarly, when E_2 -supplemented tumors were given once-daily injections of $50 \mu\text{g}$ 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. 1B). 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. 1B). 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

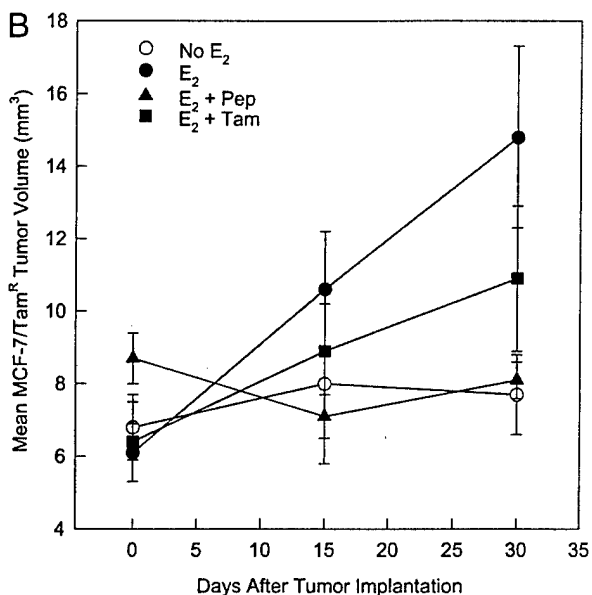
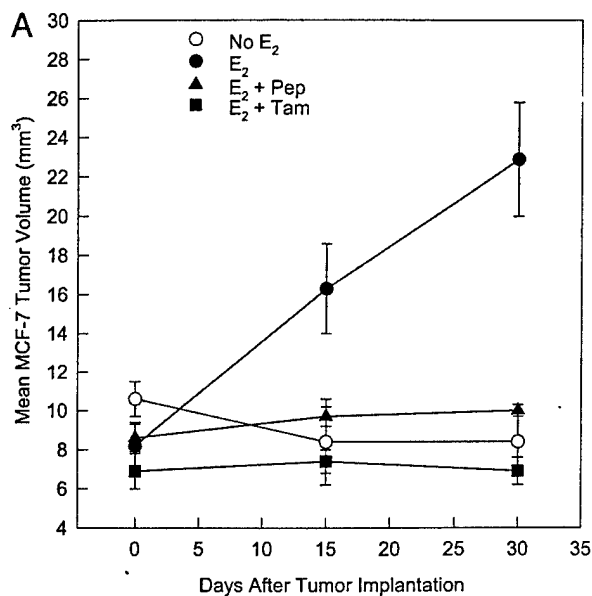


Fig. 1. Effect of AFP-derived peptide on growth of ER-positive MCF-7 and MCF-7/Tam human breast cancer xenografts. Tumors were implanted as described in *Materials and Methods*. Estrogen (solid symbols) was provided by means of a slow-release pellet of E₂ implanted s.c. ▲, Peptide (Pep) was given twice 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 E₂ + Pep group and in the E₂ + Tam group were significantly different from tumor volumes in the E₂ 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 E₂ + Pep group but not in the E₂ + Tam group were significantly different from tumor volumes in the E₂ 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

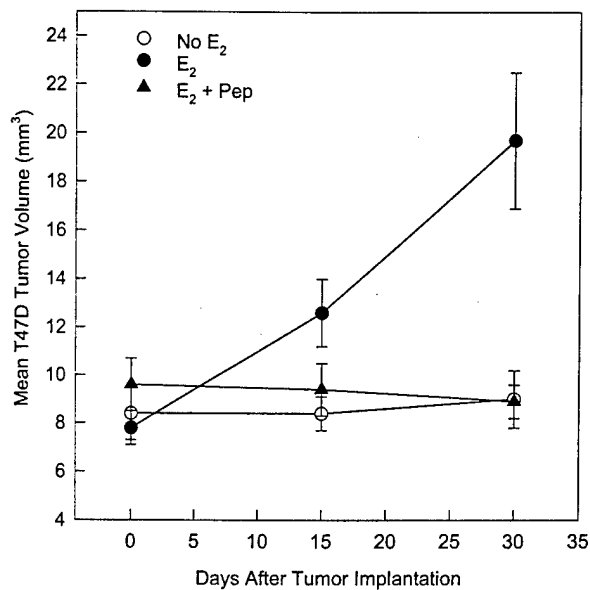


Fig. 2. Effect of AFP-derived peptide on growth of estrogen-receptor-positive T47D human breast cancer xenografts. See legend to Fig. 1 for experimental protocol. At day 30 after tumor implantation, tumor volumes in the E₂ + Pep group were significantly different from tumor volumes in the E₂ alone group, *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 E₂ for binding to ER. As shown in Fig. 4, both 4-hydroxytamoxifen and raloxifene exhibit their well documented interference with E₂ binding to ER. In contrast, AFPep produced no interference with E₂ 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 E₂ 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. 5A, 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 E₂, but nevertheless, Fig. 5A 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. 5A), 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. 5B).

Discussion

The results of this study demonstrate that a synthetic 8-mer peptide derived from AFP prevented the E₂-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+

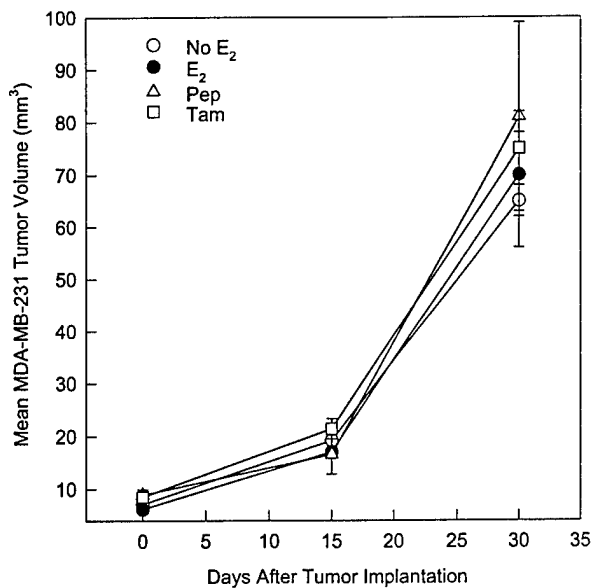


Fig. 3. Effect of AFP-derived peptide on growth of estrogen-receptor-negative MDA-MB-231 human breast cancer xenografts. See legend to Fig. 1 for experimental protocol. There were no differences in tumor volumes between any of the groups.

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

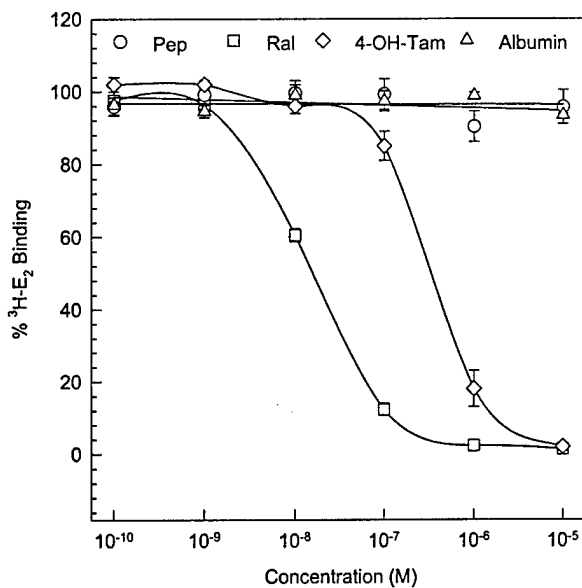


Fig. 4. Effect of AFP-derived peptide on binding of E₂ to its receptor. Rabbit uterine cytosol was used as a source of estrogen receptor. All incubations were performed in triplicate, each containing 100 μ l of cytosol, 20 μ l of 10 nM 6,7-³H]estradiol (50 Ci/mmol), and 80 μ l of test agent at the final concentrations indicated on the abscissa. Concentration of ³H]E₂-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.

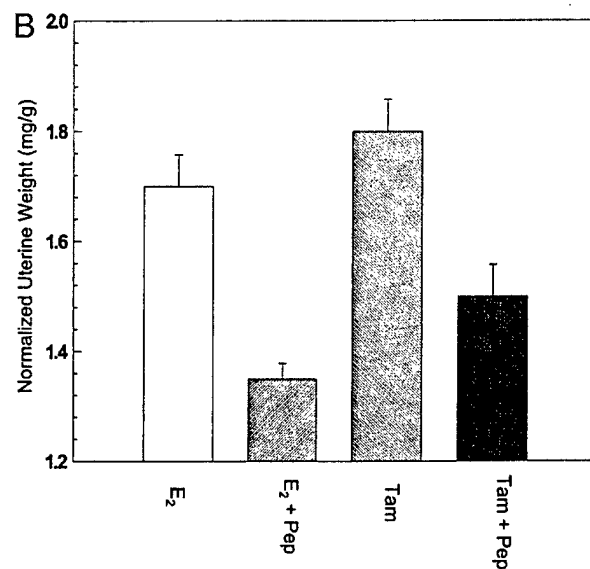
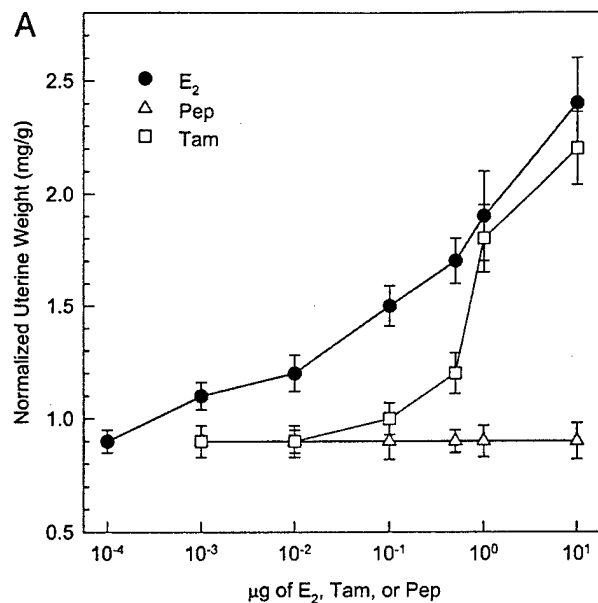


Fig. 5. (A) Effect of E₂, AFP-derived peptide (Pep) and tamoxifen (Tam) on the growth of the immature mouse uterus. The assay procedure is described in *Materials and Methods*. Various doses indicated on the abscissa of each test agent were injected i.p. Twenty-two hours later, uteri were harvested and weighed. Mean normalized uterine weights (mg uterine weight/g mouse body weight) for each group are shown on the ordinate. (B) Pep (1 μ g) or vehicle (saline, 0.2 ml) were injected i.p. One hour later, E₂ (0.5 μ g) or Tam (1.0 μ g) was injected i.p. Twenty-two hours later, uteri were harvested and weighed. Normalized uterine weights in the E₂ + Pep and Tam + Pep groups were significantly different, respectively, from normalized uterine weights in the E₂ 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 E₂ for binding to ER. Furthermore, in earlier studies we have shown that AFP administered to rodents did not reduce serum E₂ levels (1), and, in preliminary studies, AFPep also did not reduce serum E₂ 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 $\approx 30\%$ of the women who receive this drug (19). Moreover, it has been reported that uterine cancer

develops in $\approx 0.2\%$ 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 antiestrogenic 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. 5A 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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