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Antiestrogenic and anticancer activities of peptides derived from the active site of alpha-fetoprotein

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***Cyclo*[EKTOVNOGN] (AFPep), a cyclic 9-amino acid peptide derived from the active site of alpha-fetoprotein, has been shown to prevent carcinogen-induced mammary cancer in rats and inhibit the growth of ER$^+$ human breast cancer xenografts in mice. Recently, studies using replica exchange molecular dynamics predicted that the TOVN region of AFPep might form a dynamically stable putative Type I beta-turn, and thus be biologically active without additional amino acids. The studies presented in this paper were performed to determine whether TOVN and other small analogs of AFPep would inhibit estrogen-stimulated cancer growth and exhibit a broad effective-dose range. These peptides contained nine or fewer amino acids, and were designed to bracket or include the putative pharmacophoric region (TOVN) of AFPep. Biological activities of these peptides were evaluated using an immature mouse uterine growth inhibition assay, a T47D breast cancer cell proliferation assay, and an MCF-7 breast cancer xenograft assay. TOVN had very weak antiestrogenic activity in comparison to AFPep's activity, whereas TOVNO had antiestrogenic and anticancer activities similar to AFPep. OVNO, which does not form a putative Type I beta-turn, had virtually no antiestrogenic and anticancer activities. A putative proteolytic cleavage product of AFPep, TOVNOGNEK, significantly inhibited E_2-stimulated growth *in vivo* and *in vitro* over a wider dose range than AFPep or TOVNO. We conclude that TOVNO has anticancer potential, that TOVNOGNEK is as effective as AFPep in suppressing growth of human breast cancer cells, and that it does so over a broader effective-dose range. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: anticancer; linear peptides; dose range; alpha-fetoprotein; breast cancer

Introduction

A cyclic 9-amino acid peptide *cyclo* [EKTOVNOGN] (AFPep), derived from the active site of AFP, has been shown to prevent the growth of carcinogen-induced mammary tumors in rats [1–3] and inhibit the growth of ER + human breast cancer xenografts growing in mice [2,4]. Because this cyclic peptide was derived from AFP, it has been referred to as AFPep. AFPep has been shown to be orally active as an antiestrogenic and anticancer agent [2]. A combination of suboptimal doses of AFPep and tamoxifen was more effective than either compound alone against the growth of breast cancer cells in culture, in tumor xenografted mice and in carcinogen exposed rats [1]. AFPep is nontoxic, and inhibits uterine hyperplasia induced by estrogen or by tamoxifen [1,5]. These observations provide substantial evidence to support AFPep as a potential anticancer agent, but its cyclic nature makes its production expensive and its dose-response curve is not as broad as desired.

In earlier work describing the development of AFPep, Mesfin *et al*. [6] suggested that EMTPVNPG was the smallest fragment of AFP which maintained biological activity. These studies indicated that truncated forms of the linear peptide (MTPVNPG and EMTPV) had no biological activity [6]. They also showed that hydroxyproline (O) could be substituted for proline (EMTPVNPG → EMTOVNOG) which improved the analog's shelf life, presumably by decreasing aggregation [7]. Molecular mechanics minimization approaches of this linear form of AFPep implied that it had a horseshoe

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Abbreviations used: *AFP, Alpha-fetoprotein; AFPep, Cyclo [EKTOVNOGN], Alpha-fetoprotein-derived peptide; Boc, N-alpha-tert-butyloxycarbonyl; DCFBS, Dextran/charcoal-treated fetal bovine serum; DIPEA, N,N-diisopropylethylamine; DMEM, Dulbecco's modified Eagle's medium; DMF, Dimethylformamide; E_2, 17β-Estradiol (estrogen); EDT, 1,2-Ethanedithiol; EDTA, Ethylenediaminetetraacetic acid; ER+, Estrogen receptor-positive; Fmoc, 9-Fluorenylmethoxycarbonyl; HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-Hydroxybenzotriazole; MTT, 3-(4,5-Dimthylthiazole-2-yl)-2,5-diphenyltetra zolium bromide; PAL, 5-(4-Aminomethyl-3,5-dimethoxyphenoxy) valeric acid handle; $Pd(PPh_3)_4$, Tetrakis(triphenylphosphine) palladium; PEG-PS, Polyethylene glycol-polystyrene graft support; REMD, Replica exchange molecular dynamics; SCID, Severe combined immuno-deficient; TFA, Trifluoroacetic acid.*

shape conformation, which suggested a head-to-tail cyclization [7] as a possible stability-conforming structure. Adding a ninth amino acid, asparagine (N), to the peptide sequence so as to facilitate cyclizing this analog (to yield *cyclo* [EMTOVNOGN]), aided in altering the dose-response curve from biphasic toward a more sigmoidal shape [7]. The cyclic analog was further optimized to increase its yield and purity by replacing methionine (M) with lysine (K) [4]. DeFreest *et al*. [4] subsequently showed, through amino acid substitution studies, that the peptide's pharmacophoric region included OVNO. Recently, Kirschner *et al*. [8] using REMD, suggested that the pharmacophoric region was TOVN, and proposed that the active conformation is a putative Type I beta-turn. These studies suggested that smaller, more cost effective peptide analogs, derived from the pharmacophore of AFPep, might retain biological activity [8,9]. In the studies herein, some of these analogs were synthesized, and those containing putative Type I beta-turns were found to be antiestrogenic, i.e. to inhibit estrogen(E_2)-stimulated growth of immature mouse uterus [8]. On the basis of these findings, it was important to determine whether any of these smaller linear analogs retained anticancer activity and whether any of them yielded a sigmoidal dose-response curve.

We also posited that if orally active AFPep were to be cleaved by a trypsin-like protease, the resultant linear peptide would be TOVNOGNEK. If such cleavage occurred to a large extent when the molecule was administered orally, the linear product might be the proximal antiestrogenic agent. Thus, it became important to determine the antiestrogenic, anticancer, and dose-response profile of this potential AFPep metabolite. The antiestrogenic and anticancer properties of these analogs of AFPep are described in this report.

Materials and Methods

Materials

Fmoc-protected amino acids were obtained from Calbiochem-Novabiochem (San Diego, CA, USA). Reagents for peptide synthesis, including HATU, HOBt, Fmoc-PAL-PEG-PS resin, 20% piperidine in DMF, and DIPEA were obtained from Applied Biosystems, Inc. (Framingham, MA, USA). DMEM, crystalline bovine insulin, nonessential amino acids, penicillin/streptomycin, L-glutamine, and trypsin were obtained from GIBCO/BRL (Gaithersburg, MD, USA) and cosmic calf serum was purchased from Hyclone (Logan, UT, USA).

Peptide Synthesis

Peptides were prepared using Fmoc solid-phase synthesis as previously described by Mesfin *et al*. [7]. A Pioneer Peptide Synthesis system (Applied Biosystems, Inc.) or a Thuramed T100 Peptide Synthesis system (Advanced ChemTech) was used to assemble the growing peptide chain on Fmoc-PAL-PEG-PS resin beginning with the *C*-terminus using N^{α}-protected amino acids. Activation of the *C*-terminus of incoming amino acids was accomplished by treatment with HATU and DIPEA. Following synthesis, linear peptides were cleaved from the resin by incubation for approximately 2 h in 9.5 ml of TFA : anisole : EDT (90 : 2.5 : 2.5) per 0.1 mmol of peptide (0.5 g resin) and side-chain protective groups were removed concurrently. Peptides were precipitated by the addition of cold (−20 °C) diethyl ether and washed by repeated extraction with diethyl ether and then with ethylacetate/diethyl ether (1.5 : 1). The peptide was dissolved in a volume of deionized water to achieve a concentration of 5–10 mg/ml and lyophilized. Cyclization of AFPep was accomplished using methods previously described [4,7,10,11].

One analog studied herein, MTPVNPG, was difficult to synthesize using Fmoc solid-phase peptide chemistry (either in this laboratory or when synthesized by a commercial firm). Therefore, we used an BOC-solid-phase peptide synthesis strategy [12], which was successful. Subsequently, the related analogs (MTOVNOG, KTPVNPG, and KTOVNOG) (easily synthesized by both methods) were synthesized with both Fmoc solid-phase chemistry and BOC-solid-phase chemistry, and all four of these 7-mer peptides were compared for antiestrogenic activity.

Purification of Peptides

Peptides were purified using a reverse-phase C18 Sep-Pak cartridge (Waters, Milford, MA, USA) as previously described by DeFreest *et al*. [4]. A sample containing a peptide of unknown purity was loaded onto a prewashed cartridge and the sample was sequentially eluted with water, 10, 30, and 60% acetonitrile in water. The fraction containing peptide was then lyophilized. Peptides used for structure–activity relationship analysis were purified prior to use in biological assays, and those used in biological assays were at 95% or greater purity. Peptides were evaluated by mass spectrometry.

Mouse Uterine Growth Inhibition Assay

The antiestrotrophic activity of the peptides was determined using the immature mouse uterine growth inhibition assay as described by Bennett *et al*. [2]. Intraperitoneal (i.p.) administration of 0.5 μg of E_2 to immature mice has been demonstrated to double the uterine weight in 24 h with a corresponding increase in mitotic figures. Swiss/Webster female mice (13–15 day old, 6–8-g body weight; Taconic Farms, Germantown, NY, USA) were weighed and distributed into treatment groups typically consisting of five mice each, such that groups contained mice of comparable weight ranges. Each group received two sequential i.p. injections (0.2 ml per injection) spaced 1 h apart. The first injection contained test substance (0.001–1000 μg/mouse) or vehicle control. The second injection contained 0.5 μg/mouse E_2 or vehicle. Twenty-two hours after the second injection, the mice were sacrificed and weighed. Uteri were dissected and immediately weighed. Uterine weights were normalized to body weight (milligram of uterine weight per gram of body weight) to compensate for differences between body weights of littermates. Experiments used a minimum of five animals per group. The inhibition of estrogen-stimulated uterine growth was calculated from the average normalized uterine weights in each group using the following equation:

$$\%\ \text{Growth inhibition} = 100 \times (\text{full } E_2\text{-stimulated weight} - E_2\text{-stimulated weight in test group})/(\text{full } E_2\text{-stimulated weight} - \text{no } E_2\text{-stimulation weight})$$

T47D Cell Proliferation Assay

Confluent cultures of T47D human breast cancer cells American Type Culture Collection (ATCC) growing in culture were released from monolayer by trypsinization (0.25% trypsin and 0.25%

EDTA). T47D cells were suspended in phenol red-free DMEM supplemented with 10% DCFBS. Cells were then seeded into 24-well Collagen-IV coated tissue culture plates at a density of 5×10^4 cells/well in 1 ml of medium. The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Starting one day later, E_2, vehicle, or E_2 with test agents were added every day for 6 days with medium change every other day prior to treatment. On the seventh day after seeding, cell number and viability were quantified by MTT assay [13]. Each assay was done with 4–6 replicate wells per group. Experiments were performed at least three times.

Human Breast Cancer Xenograft Assay

An *in vivo* assay for anti-breast cancer activity was performed using the methodology of Bennett *et al*. [2]. Confluent MCF-7 human breast cancer cells growing in monolayer in DMEM without phenol red, supplemented with 5% bovine calf serum, 2-mM L-glutamine, 100-IU/ml penicillin, 100-µg/ml streptomycin, 0.1-mM nonessential amino acids, and 10-ng/ml bovine insulin were released from the flask using trypsin/EDTA (0.25%/0.25%). Cells (20 million) were pelleted by centrifugation at 200 *g* and then solidified into a fibrin clot by treatment with 10-µl fibrinogen (50 mg/ml) and 10-µl thrombin (50 units/ml). The solid tumor mass was then cut into pieces of approximately 1.5 mm diameter. Each tumor segment was implanted under the kidney capsule of a SCID male mouse (Taconic Farms) which weighed about 25 g. Estrogen supplementation was accomplished by subcutaneous (s.c.) placement of a silastic tubing implant (1.55 mm inner diameter, 3.125 mm outer diameter) containing solid E_2 (3 mm in length) inserted on the day of tumor implantation [14]. Peptides (0.2 ml) were injected i.p. or administered by oral gavage once daily at doses ranging from 10–100 µg/mouse. Tumor size was measured microscopically at the time of tumor implantation and 21 days after implantation by measurement of the short (*d*) and long (*D*) axes of the tumor using a dissecting microscope equipped with an ocular micrometer. Tumor volumes were calculated assuming the tumor shape to be an ellipsoid of revolution about the long axis (*D*) using the formula $(\pi/6)d^2D$. Mean tumor volume ±SE was calculated and plotted as growth curves. All animal care procedures were approved by the Albany Medical College Animal Care and Use Committee.

Statistical Analyses

All statistical tests were done using GraphPad Prism 4.03 (GraphPad Software). Significant differences were determined using one-way ANOVA with Tukey multiple comparisons posttest or Wilcoxon Rank-Sum test.

Results

The ability to inhibit E_2-stimulated growth of normal mouse uterus [8] was used as a screening assay for biological activity of AFPep analogs. In general, we consider inhibitory activity ≥20% to be biologically significant. Each analog's inhibitory effect on E_2-stimulated growth was compared to AFPep (a positive control) and to PGVGQ (a negative control). PGVGQ (amino acids 478–482 of AFP) is a portion of the primary structure of AFP near to the anticancer active site of AFP and is comprised of amino acids (P, V, and G) that are important components of AFPep.

Table 1. Effect of AFPep analogs on E_2-induced growth of mouse uterus or T47D human breast cancer cell proliferation

Peptide sequence	Inhibition ± S.E (%)		
	Immature mouse uterine growth		T47D cell proliferation
	Intraperitoneal	Oral gavage	
1. *cyclo*[EK**TOVN**OGN]	33 ± 7*	35 ± 4*	51 ± 4*
2. EK**TOVN**OGN	30 ± 2*	32 ± 3*	41 ± 9*
3. **TOVN**OGNEK	32 ± 2*	36 ± 6*	40 ± 7*
4. K**TOVN**OG	35 ± 6*	–	41 ± 4*
5. K**TOVN**O	32 ± 5*	–	43 ± 4*
6. K**TOVN**	30 ± 4*	–	33 ± 9*
7. **TOVN**	21 ± 4*	–	18 ± 2
8. **TOVN**O	38 ± 2*	30 ± 5*	48 ± 5*
9. OVNO	7 ± 3	12 ± 2	8 ± 3
10. OVNOG	25 ± 4*	–	24 ± 1*
11. VNOG	5 ± 3	–	16 ± 6
12. M**TPVN**PG	18 ± 1	–	–
13. K**TPVN**PG	34 ± 2*	–	–
14. M**TOVN**OG	28 ± 2*	–	–
15. EMTOV	1 ± 1	–	–
16. EKTOV	9 ± 1	–	8 ± 2
17. EKTPV	0	–	–
18. EMTPV	5 ± 4	–	–
19. **TPVN**	29 ± 1*	–	–
20. **TPVN**P	27 ± 1*	–	–
21. PVNP	6 ± 3	–	–
22. PGVGQ	0	–	3 ± 1

Bold letters represent the residues that form the putative Type I beta-turn [8,9]. All peptides were administered at a dose of 1 µg/mouse intraperitoneally or 10 µg/mouse by oral gavage. In culture, T47D cells were treated with each peptide at a concentration of 1 µM.
* $p < 0.05$ as compared to the group stimulated with E_2 alone. Wilcoxon Rank-Sum test.

We chose not to use a scrambled analog of AFPep because some scrambled peptides retain low biological activity (data not shown). In Table 1, bold letters denote the sequences containing a putative Type I beta-turn, which was postulated to be necessary for antiestrogenic activity [8,9].

Peptides 1–3 are AFPep and two linear 9-mer analogs. Analogs 4–11 are hydroxyproline-containing peptides of varying lengths used to assess the minimum size required to maintain inhibitory activity. Analogs 12–14 are 7-mers that lack the *N*-terminal amino acid (E) of AFPep. Analogs 15–18 are 5-mers from the *N*-terminus of EKTOVNOGN (analog 2), but outside the putative pharmacophoric region. Analogs 17–21 are the proline-containing peptides. Much of the REMD work [8,9] had modeled proline instead of hydroxyproline; hydroxyproline had been introduced [7] to increase hydrophilicity.

Mesfin *et al*. [6] showed that the minimum size required to maintain antiestrogenic activity was eight amino acids and that a 7-mer peptide, MTPVNPG (analog 12), had substantially reduced antiestrogenic properties. Kirschner *et al*. [8] suggested that TOVN (analog 7) would retain antiestrogenic activity. In Table 1, analogs 4–11 were used to determine the minimum size required to maintain antiestrogenic activity. In contrast to results reported by Mesfin *et al*. [6], linear analogs of AFPep that were less than eight amino acids maintained antiestrogenic activity.

Even TOVN had weak antiestrogenic activity. However, OVNO lacked antiestrogenic activity. The most effective analog was 5-mer TOVNO (analog 8), which significantly inhibited E_2-stimulated uterine growth.

REMD suggested that AFPep analogs forming putative Type I beta-turns within the TPVN/TOVN sequence should retain antiestrogenic activity [8]. Table 1 shows that all analogs containing a putative Type I beta-turn (signified by bold-face font) significantly inhibited estrogen-stimulated growth of immature mouse uterus except for MTPVNPG (analog 12). Analogs not exhibiting a putative Type I beta-turn had little or no antiestrogenic effect except for OVNOG (analog 10), which weakly inhibited E_2-stimulated uterine growth.

Proline-containing peptides (analogs 17–21) replicated the activity of hydroxyproline-containing peptides (analogs 4–11), which showed that there is no significant difference in biological activity when proline is replaced by hydroxyproline.

Noting that if AFPep (*cyclo*[EKTOVNOGN]) were to be cleaved by a trypsin-like protease *in vivo*, the product would be TOVNOGNEK (analog 3), we assessed the effectiveness of this analog as an antiestrogen. TOVNOGNEK significantly inhibited E_2-stimulated uterine growth and there was no significant difference between its inhibitory activity and that of AFPep. Analog 2 (EKTOVNOGN) was also active at this dose, which confirmed work done in previous studies.

REMD had suggested that MTPVNPG (analog 12 of Table 1) contains a putative Type I beta-turn and should significantly inhibit E_2-stimulated uterine growth. Earlier studies found that MTPVNPG was difficult to synthesize by Fmoc solid-phase peptide chemistry and had little antiestrogenic activity [6]. In part this may have led to Mesfin's [6] conclusion that EMTPVNPG was the minimum size AFP-derived peptide able to retain antiestrogenic activity. We also found this analog difficult to synthesize by Fmoc chemistry. Therefore, we used Boc-solid-phase peptide chemistry to synthesize MTPVNPG and three related linear 7-mer peptides (KTOVNOG, KTPVNPG, and MTOVNOG; analogs 4, 13, and 14 of Table 1). We found that the purity and activity of each peptide were the same after both methods of peptide synthesis – KTOVNOG, KTPVNPG, and MTOVNOG significantly inhibited E_2-stimulated uterine growth, whereas MTPVNPG showed significantly less activity.

We also assessed the antiestrogenic properties of peptides outside the pharmacophoric region (EMTOV, EKTOV, EKTPV, and EMTPV) (analogs 15–18) and found that they have little or no antiestrogenic activity (Table 1), which confirmed Mesfin's [6] and Kirschner's [8] results.

The activities of three active peptides (EKTOVNOGN, TOVNOGNEK, and TOVNO), and one inactive peptide (OVNO) were compared to AFPep for their ability to inhibit E_2-stimulated growth of mouse uterus after oral administration. As shown in Table 1, analogs active by the parenteral route were also active by the oral route of administration, and their activities were comparable to those of AFPep.

A number of AFPep analogs were assessed for *in vitro* anticancer activity. Analogs containing the putative pharmacophore directly interfered with the growth stimulatory effects of estrogen on T47D human breast cancer cells in culture (Table 1), but they did not affect the basal growth of these cells (data not shown). TOVN had very weak inhibitory activity against E_2-stimulated T47D cell growth. OVNO lacked inhibitory activity. TOVNO significantly inhibited E_2-stimulated T47D human breast cancer cell growth and was comparable to AFPep in this activity (Table 1). TOVNOGNEK was also comparable to AFPep in its ability to inhibit the E_2-stimulated growth of T47D human breast cancer cells.

As shown in Figure 1(a), the linear 9-mer precursor (analog 2) of AFPep exhibited a biphasic dose-response curve in the E_2-stimulated uterine growth assay. This is in agreement with earlier work done by Mesfin *et al*. [7]. Cyclization (to yield AFPep) attenuated but did not completely eliminate this loss of activity with increasing doses (Figure 1(b)), again reproducing earlier work [7]. We compared AFPep dose-response profile to the profiles seen from two of the more active linear peptides, TOVNO and TOVNOGNEK. Figure 1(c) shows a biphasic dose-response curve for linear TOVNO. TOVNO inhibited E_2-stimulated uterine growth in a dose-dependent manner up to 1 μg/mouse, but thereafter it lost inhibitory effect with increasing dose. Figure 1(d) shows that TOVNOGNEK inhibited E_2-stimulated uterine growth and maintained its inhibitory effect at high doses.

Figure 2 shows the dose-response profiles of these four active peptides against E_2-stimulated growth of human breast cancer cells *in vitro*. EKTOVNOGN inhibited E_2-stimulated T47D cell growth at low concentrations but lost activity at high concentrations, leading to a biphasic dose-response curve (Figure 2(a)). AFPep inhibited the E_2-stimulated growth of T47D cells in a concentration-dependent manner up to 10^{-6} M but lost activity at higher concentrations (Figure 2(b)). The shorter 5-mer peptide, TOVNO, was an effective inhibitor of E_2-stimulated growth of T47D human breast cancer cells in a concentration-dependent manner up to 10^{-7} M, but lost activity with increasing concentration thereafter (Figure 2(c)). However, TOVNOGNEK inhibited E_2-stimulated T47D cell growth in a concentration-dependent manner and maintained its activity with increasing concentrations (Figure 2(d)) at concentrations as high as 10^{-4} M.

The T47 D human breast cancer cell line is responsive in culture, but it was a less reliable tool when grown as a xenograft tumor in immune deficient mice, having a take-rate of <60% in these mice. However, we have found that the MCF-7 human breast cancer cell line has a tumor take-rate of 100% in immune deficient mice and is completely dependent on E_2 for growth in these mice [2]. Consequently, MCF-7 xenografts were used as a model for ER + human breast cancer to evaluate, *in vivo*, the effectiveness of TOVNO and TOVNOGNEK as anticancer agents. As shown in Figure 3, MCF-7 human breast cancer tumors were dependent on supplemental estrogen for growth as a xenograft in male SCID mice. AFPep, TOVNOGNEK, or TOVNO, each given once a day at a dose of 100 μg by oral gavage (Figure 3(a)) or 10 μg/mouse i.p., (Figure 3(b)) significantly inhibited the growth of ER + human MCF-7 breast cancer tumors.

Discussion

AFPep had previously been shown to be a stable, orally active anticancer peptide, but its pharmacophore and its metabolites had not been thoroughly investigated. DeFreest *et al*. [4] suggested that the pharmacophore was OVNO. However, Kirschner *et al*. [8] suggested that TOVN was the pharmacophore. We have shown in this report that OVNO lacks biological activity and that TOVN had weak activity. This may indicate that the pharmacophore needs one or more of the other amino acids (either T or the second O) to significantly inhibit E_2-stimulated growth. This report also shows that analogs as small as five amino acids significantly inhibited E_2-stimulated growth. Importantly, one of the 5-mer

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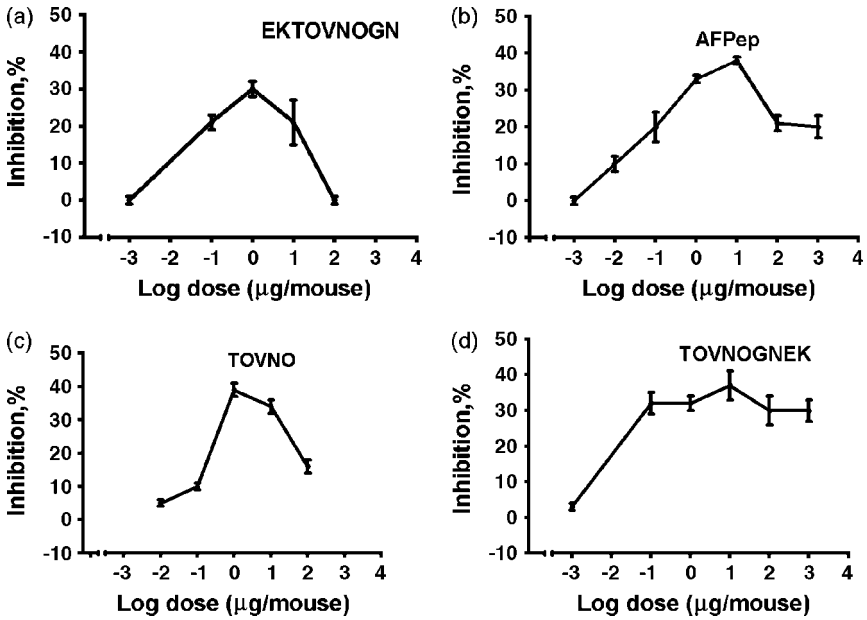

Figure 1. Dose-response profile of AFPep analogs in their inhibition of E_2-stimulated uterine growth. To assess inhibition of estrogen (E_2)-stimulated uterine growth, mice were injected i.p. with peptides (a) EKTOVNOGN, (b) AFPep, (c) TOVNO, and (d) TOVNOGNEK at various doses. One hour later mice received 0.5 μg of 17β-estradiol (E_2) i.p. Twenty-two hours later uteri were dissected and weighed. Values are reported as mean ± SEM of ≥3 independent experiments. There were five replicate mice in each group, in each experiment. Negative control mice received saline alone. Positive control mice received E_2 alone.

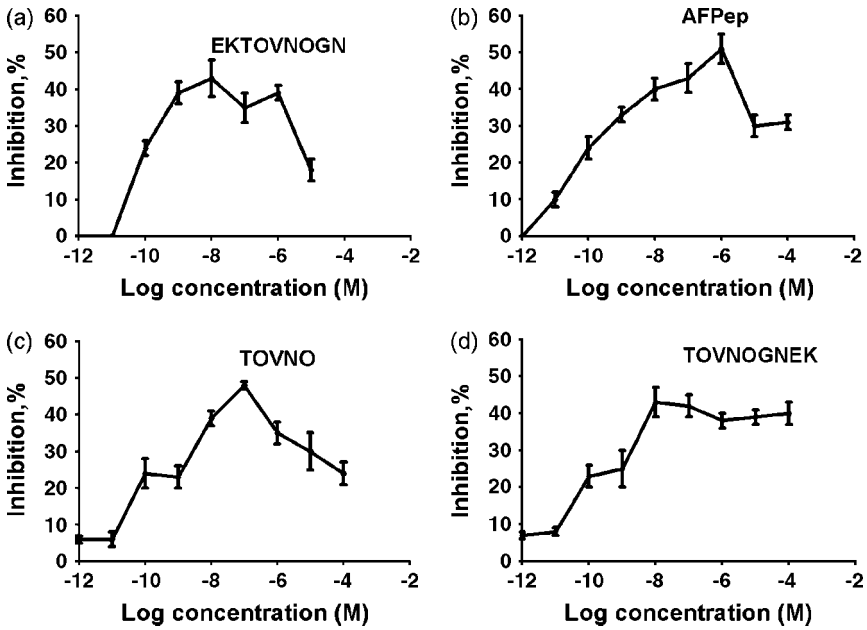

Figure 2. Dose-response profile of AFPep analogs in their inhibition of T47D human breast cancer cell proliferation. To assess inhibition of E_2-stimulated T47D cell proliferation, T47D cells were cultured on collagen-IV coated 24-well plates and treated with peptides (a) EKTOVNOGN, (b) AFPep, (c) TOVNO, and (d) TOVNOGNEK and/or 17β-estradiol (E_2). Final concentrations of each peptide were 10^{-12} to 10^{-4} M and final E_2 concentration was 1 nM. Values are reported as mean ± SEM of ≥3 independent experiments. There were 4–6 replicate wells in each experiment.

linear peptides, TOVNO, was comparable to AFPep in inhibiting E_2-stimulated growth of human breast cancer *in vitro* as well as E_2-stimulated growth of immature mouse uterus *in vivo*. Earlier studies showed that the smallest peptide analog of AFP required to sustain growth-inhibitory activity was eight amino acids [6]. Now it has been shown that five amino acids is the minimum size required to effectively inhibit E_2-stimulated growth *in vivo* and *in vitro*.

Previous results by Mesfin *et al*. [6] showed that a 5-mer peptide (EMTPV) derived from the active site of AFP was ineffective against E_2-stimulated uterine growth. This was in keeping with the concepts postulated by Kirschner *et al*. [8], who showed that this peptide lacks a putative Type I beta-turn. However, there were two peptides (MTPVNPG and OVNOG) that contradict the theory of Kirschner *et al*. OVNOG does not contain a Type I beta-turn but weakly inhibited E_2-stimutated uterine growth. OVNOG adopts a

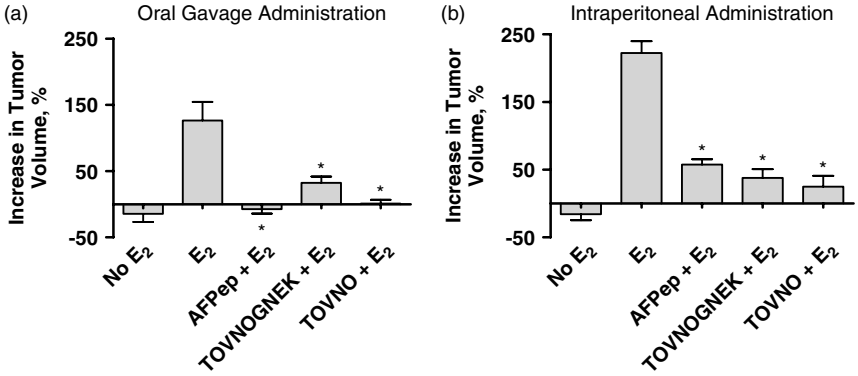

Figure 3. Linear peptide analogs of AFPep block the growth of human tumor xenografts in SCID mice. There were five replicate mice per group. Tumor-bearing mice treated with TOVNOGNEK, TOVNO, or AFPep received (a) 100 μg orally (b) 10 μg i.p. of these peptides as described in Materials and Methods. The percentage changes in tumor volume for the TOVNOGNEK + E_2 groups, TOVNO + E_2 or AFPep + E_2 group were significantly different from the E_2 alone group. Tukey multiple comparison test, $^*p < 0.01$ *versus* E_2.

Type I beta-turn for only 17% of the time, but it can also adopt an extended beta-turn. The criteria for a putative Type I beta-turn has been a 0.7-nm Cα – Cα distance between the first and fourth amino acids that are involved in the putative beta-turn as seen in the REMD simulations [8,9]. In OVNOG, the average distance is 0.83 nm. It may be that OVNOG has some inhibition of E_2-stimulated uterine growth because it can adopt an extended beta-turn-like structure which may fit into the receptor pocket.

In this report and previous studies [6], MTPVNPG was shown to have insignificant inhibition of E_2-stimulated uterine growth. However, REMD studies indicate it contains a putative Type I beta-turn and should be biologically active according to Kirschner *et al*. [8]. We had difficulty in synthesizing MTPVNPG using Fmoc solid-phase peptide chemistry. Therefore, Boc-solid-phase peptide chemistry was used to synthesize MTPVNPG and three other related 7-mer peptides (KTPVNPG, MTOVNOG, and KTOVNOG). MTPVNPG showed weak inhibition whereas the other three related 7-mer peptides significantly inhibited E_2-stimulated uterine growth. In previous studies on AFPep, O had been substituted for P (*cyclo* [EMTPVNPGN] → *cyclo* [EMTOVNOGN]) to reduce aggregation [7] and K was substituted for M (*cyclo* [EMTOVNOGN] → *cyclo* [EKTOVNOGN]) to increase the peptide's purity and yield [4]. MTPVNPG is more hydrophobic than the other three analogs, which may suggest that it forms aggregates that would decrease its availability or weaken its interaction with the receptor(s).

Reasoning that if orally active AFPep were to be cleaved by a trypsin-like protease, the product would be TOVNOGNEK, it became important to know whether that product would be an effective antiestrogenic or anticancer agent. TOVNOGNEK is as effective as AFPep as an antiestrogenic or anticancer agent. It is as orally active as AFPep against human MCF-7 breast cancer xenografts in SCID mice. Importantly, its dose-response curve is sigmoidal *in vivo* as well as *in vitro*.

This project was focused on assessment of biologically active analogs of AFPep (i.e. drug development), but not specifically on analyses of amino acid neighbor preferences or on the contribution of side chains to function. Either of these efforts would require a more exhaustive synthetic effort in order to obtain definitive outcomes. Nevertheless, it is interesting to note the extent to which deviation from the original protein sequence is tolerated, and when such deviation is not tolerated. The history of development of AFPep [*cyclo* (EKTOVNOGN)] is one of the identifying active site of AFP (EMTPVNPG, [6]), and then 'developing' the peptide into a more drug-like molecule. For AFPep, such development involved substitution of hydroxyproline for proline to increase hydrophilicity in an attempt to decrease peptide aggregation [7], substitution of lysine for methionine to improve synthetic yield, increase hydrophilicity and maintain activity [4], and cyclization to maintain stability and broaden the dose-response curve [7]. It has also meant investigation of which amino acids could not be altered [4,8]. Earlier work suggested the importance of TOVNO, and this work confirms the earlier observations. Nevertheless, related analogs (MTOVNOG, TOVNOGNEK and EKTOVNOGN) are also appreciably active. We chose to utilize PGVGQ as a control peptide rather than a scrambled analog of AFPep because some scrambled peptides retain low but appreciable activity (data not shown), sufficient to call to question the general utility of scrambled peptides. Interestingly, peptides 15 through 19 in Table 1 (EMTOV, EKTOV, EMTPV, and EKTPV) comprise a set of conservative substitutions but result in activity ranging from 0 to 9%. Thus, from the limited data here, it seems a bit hazardous to generalize about suspected 'essential' amino acids, effects of neighboring residues, and scrambled peptides. Rather, this study documents the need for careful design followed by comprehensive testing of postulated molecules.

Binding studies have shown that AFP, the parent molecule of AFPep, binds with high- and low-affinities to at least two membrane binding sites on MCF-7 cells [15], on T-lymphoma cells [16,17], and on human monocyte-derived macrophages [18,19]. However, these AFP binding sites or receptors have not been thoroughly characterized. Nevertheless, it is reasonable to suspect that AFPep might be binding to these high- and low-affinity AFP receptors. Binding to two receptors could lead to the biphasic dose-response curve for AFPep (Figures 1 and 2). This would imply that the high-affinity binding site leads to a growth-inhibitory response as it becomes saturated with AFPep. The low-affinity binding site may negate that response as it begins to load at higher ligand concentration. Thus, at low doses, AFPep would bind to the high-affinity, growth-inhibitory site and cause breast cancer growth inhibition. At high AFPep dosage, loading of the low-affinity site would operate to limit inhibition of breast cancer growth. Therefore, a drug that binds only to the high-affinity, growth-inhibitory site would be desirable for development as a pharmacological agent. TOVNOGNEK exhibits a sigmoidal dose-response curve,

Table 2. Comparison of the biological activities of peptide analogs to the shape of their dose-response curves

Peptide	Anticancer activity	Dose-response curve
EKTOVNOGN	Active	Biphasic
AFPep	Active	*Biphasic*[a]
OVNO	Inactive	–
TOVNO	Active	Biphasic
TOVNOGNEK	Active	Sigmoidal

[a] The dose-response curve of AFPep is not entirely sigmoidal, but it does not exhibit the complete fall-off activity exhibited by other analogs.

suggesting that it interacts only with the high affinity/growth-inhibitory site (Figures 1 and 2). TOVNOGNEK was able to maintain its inhibitory effect on E_2-stimulated growth over a wide dose range, suggesting that it may have greater clinical utility than AFPep.

Table 2 shows a summary of the biological activity of AFPep and four linear analogs of AFPep. We have confirmed that EKTOVNOGN (linear prototype of AFPep) is active, but that it has a biphasic dose-response curve. When cyclized to AFPep, biological activity was retained even at higher doses, but the dose-response curve is not purely sigmoidal. TOVNO inhibited breast cancer growth *in vitro* and *in vivo*, showing that there are analogs less than eight amino acids that can retain anticancer activity. However, TOVNO yielded a strongly biphasic dose-response curve which would make it less useful as a potential anticancer agent. It is interesting that TOVNOGNEK was effective as an anticancer agent and maintained its inhibitory effectiveness over a wider dose range than did AFPep. On the basis of these results TOVNOGNEK is a worthy candidate for further development as a new drug in the treatment and prevention of ER^+ breast cancer. It may be that further design considerations may improve upon the dose-response profile and efficacy of TOVNOGNEK.

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