Prevention of N-Methyl-N-Nitrosourea–Induced Breast Cancer by α-Fetoprotein (AFP)–Derived Peptide, a Peptide Derived from the Active Site of AFP


Abstract

Purpose: α-Fetoprotein (AFP) is a protein of pregnancy associated with a decrease in lifetime risk of breast cancer in parous women. A synthetic, cyclic nonapeptide has been developed that mimics the antioncogenic active site of AFP. To test the hypothesis that the AFP-derived peptide (AFPepp) can prevent breast cancer, the N-methyl-N-nitrosourea – induced breast cancer model was used in rats.

Experimental Design: AFPepp was given daily by injection beginning 10 days after N-methyl-N-nitrosourea treatment and continued for 23 days (a time designed to mimic pregnancy) or for other times to assess efficacy as a function of drug duration. Tumor incidence, multiplicity, and latency were noted as end points. At necropsy, pathology analysis of tumors and major organs were obtained.

Results: AFPepp prevented cancer in a dose-dependent fashion. Significantly longer mean tumor-free days (P < 0.02), lower tumor incidence (P = 0.004), and lower tumor multiplicity were observed for AFPepp-treated groups. No evidence of host toxicity as measured by body weight, cage activity, fur texture, and organ weights (liver, uterus, heart, kidney, and spleen) were found in animals treated with AFPepp. Mechanistic studies using transplantable human breast cancer xenografts showed that the peptide interfered with estrogen-dependent breast cancer growth inhibited the phosphorylation of the estrogen receptor and activated phosphorylation of p53.

Conclusions: AFPepp is a well-tolerated, mechanistically novel, chemopreventive agent in models of breast cancer and warrants further development for the prevention and treatment of this disease in humans.

 Approximately 200,000 women yearly are diagnosed with breast cancer in the United States (1). Although treatment of this disease has improved, a death rate of 40,000 breast cancer patients yearly is still anticipated. Prevention of breast cancer is an important objective because prevention can be more effective, less traumatic, and less expensive than therapy. However, to gain acceptance in clinical utility, preventive agents that are given chronically would be required to have minimal or no toxicity. Tamoxifen, currently used as a hormonal therapeutic for estrogen receptor–positive breast cancer (2), has also been used effectively as a chemopreventive agent in patients at high risk for acquiring breast cancer (3). However, tamoxifen may be accompanied by significant host toxicity (4, 5). For example, women taking tamoxifen have more than twice the chance of developing uterine cancer as do women taking placebo (4, 5). Cardiovascular side effects of tamoxifen, including thromboembolism (5), have been reported. Then, too, breast cancer cells are often intrinsically resistant or become resistant to tamoxifen during treatment (6), accentuating the need for additional drugs in the treatment and prevention of breast cancer. Other drugs are being developed as potential preventive agents, but they are not without appreciable toxicity. However, if there were a molecule that could prevent cancer with minimal or no associated host toxicity, it may find widespread clinical utility, especially if it worked through a mechanism that was distinct from that of tamoxifen or other drugs currently in use for breast cancer.

Many epidemiologic studies have shown that an early full-term pregnancy significantly lowers a woman’s risk of developing breast cancer (7). This very strong association between pregnancy and reduced breast cancer risk has been validated in experimental studies that have shown that pregnancy protects rats against carcinogen-induced mammary cancer (8–10). Although 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 (11)
that one of these factors is α-fetoprotein (AFP). AFP is a 69,000 molecular weight glycoprotein product of the fetal liver (12). AFP crosses the placenta and enters into the maternal circulation (13), although its expression is repressed at parturition (13). Jacobson et al. hypothesized (14) and supported with epidemiologic studies (11) and Richardson et al. confirmed by direct measurement (15) that pregnancy-associated protection from breast cancer is proportional to exposure level to AFP. Jacobson et al. further hypothesized that AFP acts in an endocrine manner to extinguish premalignant foci that, much later in life, would have gone on to develop into cancer (11). Sonnenschein et al. (16) showed that growth of a transplantable rat mammary cancer was inhibited by an AFP-secreting hepatoma. Bennett et al. (17) showed that AFP purified from cultured human hepatoma cells inhibited human breast cancer xenograft growth.

However, AFP itself would be unsuitable as a potential drug, either for treatment or for prevention of breast cancer, due to its large size, difficulty of production, difficulty of administration, presence of multiple functions (including immunosuppression; ref. 18), and problems associated with its long-term storage. Studies to identify the antioncogenic active site of AFP yielded an 8- amino acid peptide that retained the antiestrogenic and anticancer activity of intact AFP (19). The peptide was shown to stop the growth of ER-positive human breast cancer cell lines, such as T47D, growing in culture (19) and to be antiuterotrophic. It inhibited estrogen-induced or tamoxifen-induced uterine growth (19–21). The peptide was modified further for improved stability into a synthetic, cyclic nonapeptide (21), which has been shown, in a model of therapeutic intervention, to stop the growth of ER-positive human breast cancer xenografts growing in mice (22) even when the cancer was tamoxifen-resistant (20). AF Pep has a mechanism that is different from that of tamoxifen (20). Because AF Pep is a simple peptide mimic of a single-function active site of a naturally occurring protein, it was reasonable to test the ability of the peptide to prevent breast cancer and to suppose that toxicity would be very low.

The purpose of this study was to assess the ability of AF Pep to “prevent” breast cancer in the N-methyl-N-nitrosourea (MNU)-induced breast cancer model in rats. We report here that AF Pep leads to a lower incidence of palpable tumors, longer latency period, and lower tumor multiplicity and that there is no observable toxicity to animals.

**Materials and Methods**

**Materials**

MNU was obtained from the National Cancer Institute Carcinogen Repository (MIRI, Inc., Kansas City, MO) and was dissolved in sterile physiologic saline (1% w/v) buffered to pH 5.0 with 3% acetic acid. Female Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY) at 34 days of age and were placed immediately on a controlled diet (Agway Pro-Lab 2000; Agway Corp., Syracuse, NY), allowed free access to food and water, and maintained on a 12-hour light-dark cycle at a constant temperature (22°C) for the duration of the study. Severe combined immunodeficient mice and Swiss-Webster mice were obtained from Taconic Farms at 6 weeks of age and were maintained in individually ventilated cages. Cages, bedding, food, and water for the mice were autoclaved. Mice were handled using sterile technique in a laminar flow biosafety cabinet.

**Methods**

**Peptide synthesis.** The AF Pep cyclo[EKTVNOGN], where O is hydroxyproline, was generated using N-(9-fluorenyl)methoxycarbonyl solid-phase peptide synthesis as described (19–22), employing the head-to-tail cyclization method of Kates et al. (23, 24). 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 trifluoroacetic acid/thioanisole/anisole/ethanedithiol (90:5:2:3) per 0.5 g resin for 5 hours. 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 high-performance liquid chromatography, and then lyophilized. Peptide quality was ascertained by amino acid analysis and mass spectrometry. All peptides were screened for antiestrogenic activity in an immature mouse uterine growth inhibition assay (19). This assay was done biweekly during the time of peptide administration in prevention studies to ensure there had been no loss of the biological activity of the peptide. Biologically active AF Pep can also be purchased from PolyPeptide Laboratories (Torrance, CA) after synthesis by the t-butoxycarbonyl method or from Advanced ChemTech (Louisville, KY) after synthesis by the N-(9-fluorenyl)methoxycarbonyl method.

**Prevention assay.** This prevention study used the methodology of Gnbbs et al. (9, 10, 25–27) to test the ability of AF Pep to prevent MNU-induced breast cancers in rats. There were 30 rats in each experimental group (unless otherwise specified) to assure a 95% probability of detecting a difference between groups (ratios) of 40%, which was the difference seen for pregnancy (ref. 9; power analysis by SOLO software, BMDP Statistical Software, Inc., Los Angeles, CA). Female rats were housed three per cage in a room maintained at 72 ± 2°F and artificially lighted for 12 hours daily. At 50 days of age, rats received a single injection of MNU (50 mg/kg body weight) or vehicle in the jugular vein. MNU was given to animals from the various treatment groups according to a predetermined randomization chart to ensure uniform distribution of the carcinogen across the groups. Beginning 10 days after MNU exposure, treatment with AF Pep by s.c. injection occurred once daily for 23 days, a time chosen to mimic the gestation period of rats, or for longer or shorter times, as specified. The peptide was diluted in saline and was given in an investigator-blinded manner at doses between 0.03 and 0.27 mg/rat daily in a volume of 0.2 mL. The control group of animals received daily 0.2 mL s.c. injections of saline for the same time as AF Pep administration. Animals in the positive control group received only MNU treatment and experienced the maximal number of tumors. The negative control group of rats received no MNU and no AF Pep. These animals generated no spontaneous tumors throughout the course of the study. Additional groups of animals received MNU and AF Pep treatment as described in Results. Beginning 30 days after MNU treatment, all rats were palpated twice weekly for detection of mammary tumors, noting number, location, and size. Tumor burden was determined noninvasively with calipers by measuring the long (D) and short (d) diameters. Assuming that tumors were ellipsoid shaped, tumor volume was estimated as \((\pi / 6)(d^3)(D)\) (17). All animals were checked daily for signs of toxicity. Most studies were terminated 100 days following MNU administration, and at necropsy, tumors were dissected and weighed. Heart, liver, uterus, spleen, and kidney were collected, weighed, and assessed for toxicity. The Albany Medical College Institutional Animal Care and Use Committee approved all animal care procedures.

**Toxicity studies.** To assess the toxicity of AF Pep, three formats were used in two species. High-dose AF Pep (10 mg/mouse, equivalent to 400 mg/kg or >1,000 times the therapeutically effective dose in mice) was given as a bolus by tail vein injection to groups \((n = 10)\) of mice. High dose (up to 2 mg/mouse or 80 mg/kg) was given i.v. once daily for 5 days to groups \((n = 10)\) of mice. Therapeutic or preventive doses (100 μg/rat) were given once daily for 45 days to groups \((n = 30)\) of rats. At termination, animals were sacrificed and subjected to necropsy. Organ weights, body weights, and tissue morphology were noted as end points.

**Histologic and immunohistochemical analysis.** For histologic evaluation, tumors and organs were prepared from paraffin-embedded
blocks and stained using a regressive H&E staining method and examined by light microscopy. For immunohistochemical analysis, sections of tumors and organs were prepared from cryoembedded tissue frozen in Tissue-Tek OCT compound at −80°C following necropsy. Tissue sections (5 μm) on slides from each tissue were fixed in cold acetone for 10 minutes, air dried, and stored at −20°C until stained.

**Estrogen receptor and progesterone receptor staining.** For identification of the presence of ERα and progesterone receptor (PR), standard immunohistochemical procedures were followed. Briefly, tumor sections were rehydrated in PBS and endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide. Tissue sections were blocked with 1.5% normal goat serum solution followed by incubation overnight at 4°C in primary antibody, at 1 μg/mL, against human ER (rabbit polyclonal ERα) or human PR (rabbit polyclonal PR) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). These antibodies are known to cross-react with rat ER and PR. Sections were incubated with a biotinylated secondary antibody at 1 μg/mL (goat anti-rabbit IgG; Santa Cruz Biotechnology) and labeled with a streptavidin-peroxidase solution using the Vectastain Elite ABC System (Vector Laboratories, Burlingame, CA) following the manufacturer’s protocol. Color development was detected using 3,3′-diaminobenzidine (3,3′-Diaminobenzidine Peroxidase Substrate kit; Vector Laboratories) as substrate. Tissues were counterstained with Harris hematoxylin, dehydrated in a series of 95% and 100% ethanol, and cleared in xylene, and coverslips were mounted with Permount mounting medium (Fisher Scientific, Pittsburgh, PA). Positively stained cells were identified by darkly stained nuclei, whereas those stained with a nonspecific rabbit IgG remained blue and stained only with hematoxylin. MCF-7 breast cancer tumor sections were used as positive controls, whereas those weakly stained were considered negative.

**Cytokeratin staining.** Cytokeratin, a cytoskeletal protein that is present in the cytoskeleton of all epithelial cells, was detected with a mouse monoclonal anti-human cytokeratin antibody (clone AE1/AE3; DAKO Corp., Carpinteria, CA) known to cross-react with rat cytokeratin. Positive staining assists in identification of most carcinomas.

A DAKO ARK peroxidase kit (animal research kit) was used to stain tumors with mouse primary antibodies, which is designed to minimize reactivity of secondary anti-mouse antibodies with endogenous immunoglobulin in the tissues. Fixed, frozen tumor tissue sections were rinsed and rehydrated in TBS [50 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 7.6)] blocked with 0.03% hydrogen peroxide and rinsed. Sections were incubated with a biotinylated primary antibody solution containing normal mouse serum blocker, made according to the manufacturer’s protocol, followed by a streptavidin-peroxidase solution. Color detection was monitored with the 3,3′-diaminobenzidine-buffered substrate followed by counterstaining with Harris hematoxylin. Positive staining was detected by the presence of brown staining in the cytoplasm of epithelial cells. MCF-7 breast cancer tumors served as positive controls, whereas rat uteri tissue sections were used as negative controls.

**Xenograft assay.** MCF-7 and MDA-MB-231 human breast cancer cells from culture were solidified into a fibrin clot and implanted under the kidney capsule of severe combined immunodeficient mice as described previously (17). Tumor size was evaluated during survival laparotomy using a dissecting microscope at the time of tumor implantation and at days 15 and 30 after tumor implantation. For biochemical studies where more tumor tissue was needed than that yielded from tumors implanted under the kidney capsule, tumors were grown s.c. and used −50 days after tumor implantation. Size measurements of tumors growing s.c. were obtained using a Vernier caliper.

**PAGE assay.** Tumor samples were minced and incubated on ice for 30 minutes in 200 μl ice-cold buffer composed of 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L KCl, 10 mmol/L MgCl2, 0.5 mmol/L DTT, 0.1% Igepal CA-630, and 0.5 mmol/L phenylmethylsulfonyl fluoride. The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 13,000 rpm at 4°C for 1 hour. Nuclear extracts were prepared by resuspension of the crude nuclei in high-salt buffer (420 mmol/L NaCl, 20% glycerol) at 4°C with rocking overnight, and the supernatants were collected after subsequent centrifugation at 4°C and 13,000 rpm for 10 minutes. Nucleoproteins were separated on discontinuous SDS-PAGE (9% gels) and then transferred by electroblotting to Immobilon membranes (Millipore, Bedford, MA). After blocking with 5% milk in TBS containing 0.1% Tween, the membranes were incubated with polyclonal rabbit anti-p53 (phospho-Ser15) or monoclonal mouse anti-phospho-ER (Ser118) antibodies overnight. Secondary antibodies were either goat anti-rabbit IgG (1:1,000; DAKO) or rabbit anti-mouse IgG (1:1,000; DAKO) depending on the origin of the primary antibody. Immunoreactive proteins were detected by chemiluminescence.

**Data analysis.** Fisher’s exact test was used to compare the number of animals with tumors; the log-rank test was used to compare tumor volume of one of three replicate studies. D, MNU only; ▲, MNU + AFPep at 0.1 mg/animal daily; ○, no MNU, no AFPep.
incidence data (28). Comparison of AFPep-treated animals with untreated animals was done using the \( m^2 \) test. Student’s unpaired \( t \) test was used to assess latency and toxicity data. The repeated-measures ANOVA was employed to assess differences between treated and untreated groups with respect to tumor burden. SDs of frequency of tumors in the duration study were calculated using the SEs of estimate analysis, which combined error for the two sample means. \( P < 0.05 \) were specified as statistically significant.

**Results**

This study used a well-developed assay (9, 10, 25–27) to assess the ability of the putative preventive agent (AFPep) to prevent MNU-induced breast cancer in rats. No mammary tumors were found in rats not receiving carcinogen, but animals exposed to the carcinogen exhibited a mammary cancer incidence of 70% at termination of the study (100 days) similar to that found in other studies (25). Carcinogen-exposed rats treated with AFPep showed a decrease in tumor incidence from 70% in the control group to 40% in the AFPep-treated group (Fig. 1; \( P = 0.004 \), Fisher’s exact test). Figure 1 also shows a delayed onset of tumors (increased latency) for AFPep-treated animals when compared with untreated animals. Palpable tumors were detected as early as 40 days after MNU administration in rats exposed to carcinogen alone, and these animals had a mean tumor-free value of 77.5 ± 8.8 days. AFPep delayed development of carcinogen-induced tumors until 56 days and these animals had a mean tumor-free value of 90.2 ± 20.7 days (when calculated at day 100). This difference is significant at \( P < 0.02 \).

In addition to the number and location of tumors, the size of each tumor was determined by measuring long and short diameters of each tumor. Tumor burden, defined as the sum of tumor volume for all animals in the group, was significantly \( (P = 0.034) \) reduced in the AFPep group as depicted in Fig. 2.

Results of a dose-response study are shown in Fig. 3. AFPep decreased tumor incidence (percent of rats with tumors; Fig. 3A), increased tumor latency (mean tumor-free days; Fig. 3B), and decreased tumor multiplicity (number of tumors per animal; Fig. 3C) in a dose-dependent fashion at doses up to 0.27 mg/animal daily.

A study was done to determine the optimal duration of treatment with AFPep. As shown in Fig. 4, AFPep is effective for prevention of tumors (decreased incidence) when given for durations between 10 and 33 days. Treatment for only 5 days was not significantly different from control (no peptide).

A representative sample of tumors was examined histologically to distinguish between adenocarcinomas and fibroadenomas. Using the cytokeratin stain, >80% of tumors were seen to be adenocarcinomas, similar to others who have used this model (27). Tumor sections were stained for ER and PR: 90% of all tumors were positive for ER and PR: 90% of all tumors were positive for ER and PR and there was no...
difference in the percent of tumors expressing ER and PR between groups treated and not treated with AFPep (Fig. 5).

During all prevention trials, rats were monitored for outward signs of toxicity and were weighed beginning at 50 days of age and continuing until termination of study. MNU was given once (at age of 50 days) and peptide was given for 23 days (beginning at 60 days of age). Body weights of animals were used as one measure of toxicity. As illustrated in Fig. 6, there was no impairment of weight gain or final body weight due to MNU or peptide. Additional indicators of toxicity included cage activity and fur texture, and uterus weight, liver weight, and heart weight were obtained at necropsy. There were no significant differences between treated and untreated groups of animals in any of these indicators (Fig. 7). More acute toxicity studies in mice showed no change in body weight or organ weights following i.v. injection of 10 mg peptide with necropsy 10 days after treatment or following daily i.v. injection of 2 mg peptide daily for 5 days with necropsy 24 hours after the final injection of peptide (Fig. 8).

The design of the prevention studies described above did not lend itself to study of mechanism of action of peptide at the tumor cell level given the delayed emergence of MNU-induced tumors relative to the cessation of peptide treatment. However, to begin to address mechanistic issues, human tumor cell lines were transplanted into immunodeficient mice and those mice were treated on a daily basis with AFPep. As shown in Fig. 9, peptide prevented the growth of the ER-positive MCF-7 breast cancer xenografts but did not affect the growth of the ER-negative MDA-MB-231 breast cancer xenografts. In a separate study, s.c. implanted MCF-7 breast cancer xenografts were

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Fig. 5. ER/PR staining of AFPep-induced tumors. Tumors were harvested on necropsy, and sections (5 μm) stained with anti-ER or anti-PR antibodies (A; magnification, ×400) or anti-cytokeratin (clone AE1/AE3; CK) antibody (B; magnification, ×200) and counterstained with hematoxylin. Positive staining is detected as dark nuclei in (A), whereas tissues stained with a nonspecific IgG remained blue. Presence of cytokeratin appeared as brown staining in the cytoplasm (B). MCF-7 tumors were used as a positive control. The mouse uterus remained unstained for cytokeratin.
allowed to grow to a size of 1.5 cm in average diameter and then treated on a daily basis for 8 days with peptide. AFPep again prevented further tumor growth. Examination of these tumors 2 hours after the last treatment with AFPep showed a decrease in the phosphorylation of the ER and an increase in the phosphorylation of p53 in the tumors from mice treated with peptide (Fig. 10).

Discussion

The MNU-induced cancer model has been used extensively (9, 10, 25–33) to test the preventive potential of a variety of chemical compounds and interventions. Tamoxifen (28), aromatase inhibitors (29), 9-cis-retinoic acid (30), vitamin D3 or D5, indole-3-carbinol (27), cabbage extracts, and many other compounds (31, 32) have been shown to decrease tumor incidence, as has dietary folate deficiency (33). Few of these agents, except tamoxifen and the aromatase inhibitors, are moving into the clinic as preventive agents, and the limitation is usually that of toxicity rather than efficacy.

In an early study, Grubbs et al. (10) showed that pregnancy inhibited the development of MNU-induced rat mammary cancers. Since then, several pregnancy-associated factors have been examined in an effort to identify and exploit the natural protective agents associated with pregnancy. Grubbs et al. (26) and Rajkumar et al. (34) have shown that treatment of MNU-exposed animals with high-dose estrogen plus progesterone is effective at reducing tumor incidence. Russo et al. (35) have shown that human chorionic gonadotropin can reduce tumor incidence in the 7,12-dimethylbenz(a)anthracene and MNU models. However, the use of estrogen plus progesterone as a preventive regimen in women would be compromised by the many side effects that could be expected, and the use of the pituitary glycoprotein hormone human chorionic gonadotropin would be less than an optimal preventive approach.

Another pregnancy-associated factor is AFP, a 69,000 molecular weight glycoprotein product of the fetal liver (12).

Richardson et al. (15) 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, suggesting an AFP dose-related induction of a molecular signature that provided these women with a long-term protection against development of breast cancer. Richardson et al. (15) concluded that the results of that study were in agreement with those reported earlier by Jacobson et al. (11), in which surrogate indicators (multiple births and hypertension) for high levels of AFP during pregnancy had been used. In the earlier studies, Jacobson et al. (11, 14) had hypothesized that gravidic exposure to AFP acts in an endocrine manner to prevent premalignant foci from going on to develop into breast cancer and that this protection was proportional to the exposure level to AFP.

As with other intact proteins, AFP itself would be unsuitable as a potential drug, either for treatment or for 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, the active site of AFP was identified as an 8-amino acid peptide that retained the antiestrogenic and anticancer activity of the intact protein in therapeutic models and was subsequently developed further into a stable, cyclic synthetic peptide (19–22). Therefore, it was of interest to assess the ability of AFPep as a preventive agent.

Treatment of MNU-exposed rats with AFPep for 23 days (the gestation period in rats) resulted in a significant decrease in tumor incidence, a significant increase in tumor latency, and no indication of gross toxicity. This is actually an improvement in the protection provided by pregnancy in this model, since in the earlier studies by Grubbs et al. (9) it was shown that pregnancy resulted in an early increase followed later by an overall decrease in the incidence of MNU-induced mammary cancers.
cancers, whereas in the study reported herein there was no
evidence of an early increased incidence of mammary cancers
in rats treated with AF Pep. This may reflect an advantage of
using an active site peptide over the full-length protein because
the peptide is the minimum sequence in AFP needed for
antiestrogenic activity (19–22), whereas the overall protein
has been shown to have additional biological activities, such as
immune suppression (18).

The data in the study described herein indicate that the
protection provided by AF Pep was proportional to the exposure
level to AF Pep, which is in agreement with the conclusions of
Jacobson et al. (11, 14) and Richardson et al. (15) in their
epidemiologic studies of AFP. At least for incidence (Fig. 3A)
and latency (Fig. 3B), if perhaps not for burden (Fig. 3C), there
seems to be a rectangular hyperbolic dose-response curve.

Cytotoxic drugs, such as cisplatin, would be expected to show
a linear (or at least monotonically increasing) dose-response
curve. AF Pep is not cytotoxic (see below) and should act more
like a growth regulator than like a toxic molecule. Some
growth regulators, such as monoclonal antibodies, exhibit a
rectangular hyperbolic curve, achieving a plateau in the dose-
response curve, and others (e.g., estrogen) may even exhibit a
biphasic curve. Alternatively, the flat dose-response curve seen
in Fig. 3C could be the result of rapid metabolism of AF Pep,
and detailed pharmacokinetic studies will be needed to
elucidate that point.

Because AF Pep is derived from AFP, a naturally occurring
protein synthesized by the fetal liver and found in milligram/
milliliter levels in the fetal serum and at nanogram/milliliter
levels in the serum of pregnant women, it is reasonable to
postulate that it may be nontoxic. Active doses of the peptide
were well below the serum levels of AFP found in fetal serum
(13). Throughout this study, there was no evidence of toxicity
at active doses of peptide when given for ≥23 days. As depicted
in Fig. 7, there was no impairment in the weights of the whole
body, uterus, liver, or heart in rats receiving AF Pep. Specifically,
no evidence of uterotrophic response was seen, as happens with
tamoxifen. For all animals, both cage activity and fur texture
were monitored as normal at all times. Hundreds of rats in
several repeated prevention assays and thousands of mice in
screening bioassays have been subjected to AF Pep administra-
tion in the study of this molecule, and no evidence of gross
toxicity has ever been detected. In fact, in studies specifically
designed to find the dose-limiting toxicity of AF Pep, no adverse
effects were found following single i.v. administration of 1,000
times the active dose in mice or following multiple i.v.
administrations of 200 times the active dose in mice (Fig. 7).

Fig. 8. Effect of AF Pep on toxicity indicators in mice. Groups of mice (n = 10 per
group) were treated with saline (open columns), 10 mg AF Pep (hatched columns)
as a single i.v. bolus, or 2 mg AF Pep (checked columns) i.v. daily for 5 days.
The experiment was terminated 10 days after treatment with 10 mg AF Pep or 1 day
after the final treatment with 2 mg of AF Pep. There are no significant differences
between treatment groups.

Fig. 9. Effect of AF Pep on growth of MCF-7 and MDA-MB-231 human breast
cancer xenografts. Tumors were implanted under the kidney capsule of severe
combined immunodeficient mice. Closed symbols (○ and ▲), supplementation with
s.c. implanted estradiol pellets. AF Pep (∆ and ▲) was given twice daily i.p. at a dose
of 1 μg/injection. There was a minimum of five mice per group. There is no significant
difference between any group in the ER-negative tumors (○). In the ER-positive
tumors (▲) at day 30 after tumor implantation, tumor volumes in the estradiol +
AF Pep group were significantly different from tumor volumes in the estradiol alone
group (P < 0.05, Wilcoxon rank-sum test).
If AF Pep is metabolized in vivo, the products would be free amino acids and would have no toxicity at all unlike the metabolites of most small-molecule drugs. Thus, as expected, AF Pep is very well tolerated.

Bennett et al. (20) have shown, in a mouse model, that AF Pep does not induce uterine hypertrophy, as does tamoxifen, and that the peptide actually suppresses tamoxifen-induced (or estrogen-induced) uterine hypertrophy. Those workers suggested that AF Pep might be proven useful in combination therapy with tamoxifen. The present studies would support that suggestion and extend it by postulating the utility of the peptide as a primary or combination preventive agent.

In previous studies (20) and in this study, it was shown that AF Pep inhibited estrogen-dependent but not estrogen-independent growth of human breast cancer xenografts. Mechanistically, AF Pep does not interfere with estrogen binding to its receptor (20) as does tamoxifen (2), but it does interfere with the phosphorylation of the ER. Interestingly, in the prevention studies reported herein, although AF Pep delayed the emergence of MNU-induced mammary cancers, the cancers that appeared were ER positive. This is not inconsistent with other studies of MNU-induced mammary cancers in rats, which have shown that MNU produces mainly ER-positive mammary adenocarcinomas (36). Given that AF Pep inhibits estrogen-dependent growth, it seems that this mechanistic pressure would favor the emergence of ER-negative mammary adenocarcinomas. However, even with tamoxifen, this has not been the case in the MNU model (37). All of these studies indicate that the pressure of the carcinogen on carcinogenesis, although retarded by the preventive agent, can eventually break through the pressure of these antiestrogenic agents and induce tumors. It would be interesting to study the effects of intermittent dosing of AF Pep, which improved the effectiveness of arzoxifene and rineodixone (38) and to study the biochemical make up of the ER (39–41) in MNU-induced tumors in the absence and presence of antiestrogens. It is possible that the ER may be different in the latter case, making it less sensitive to estrogen as well as antiestrogens. Alternatively, it may be that inhibitors, such as tamoxifen and AF Pep, are retardants and not annulants and may require a multipronged attack on the tumor growth process to completely prevent emergence of tumors. The combination approach has been an effective strategy in the treatment of cancer and may be worth consideration in the prevention of cancers. However, the nature of prevention studies requires effective agents that do little or no harm given that the harmful presence of a tumor is only a potential not an actual part of the diagnostic profile. The data in this study endorse AF Pep as a relatively nontoxic agent, distinguishing it from many other molecules (25–33), given that it is an extremely well-tolerated breast cancer chemopreventive agent that has a novel mechanism of action. Further studies of AF Pep alone and in combination are indicated for its clinical translation.

References


Prevention of N-Methyl-N-Nitrosourea–Induced Breast Cancer by α-Fetoprotein (AFP)–Derived Peptide, a Peptide Derived from the Active Site of AFP


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