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AFPep prevents estrogen receptor-positive breast cancer in ACI rats

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ABSTRACT

Breast cancer is a leading cause of death in the US, affecting more than 200,000 women each year. About 80% of these cancers are estrogen receptor positive (ER+). Several therapeutic options exist for ER+ patients, including selective estrogen-receptor modulators (SERMs) such as tamoxifen; however, these drugs are known to have serious side effects. Tolerability has been a major barrier for development of preventative agents. AFPep is a 9-mer cyclized peptide derived from alpha-fetoprotein (AFP), a protein naturally expressed during fetal development. AFPep has been shown to have therapeutic efficacy against ER+ breast cancer. The aim of this project is to assess the safety and efficacy of AFPep as a preventive agent for breast cancer. Preventative efficacy of AFPep was assessed in August Copenhagen Irish (ACI) rats, a strain that develops breast cancer when exposed to high but physiological levels of estrogen. Female rats were exposed to estrogen through subcutaneous estradiol implants for 24 weeks. Rats were treated once daily with saline or 25 µg AFPep for 4 weeks to mimic pregnancy. Tumors were monitored twice weekly for 24 weeks. Tolerability was assessed using animal weight, behavioral parameters, and organ weights at necropsy. AFPep significantly decreased formation of mammary tumors under estrogen exposure and showed no signs of adverse effects after lengthy duration of administration. A comparison of cancer prevention models in rats delineates the advantages and disadvantages of two commonly used approaches.

KEYWORDS: breast cancer, prevention, estrogen receptor positive, prevention models, tolerability, peptide, drug development.

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INTRODUCTION

Beginning with the observation that women who experience pregnancy are at lower risk for breast cancer than are nulliparous women [1-8], and ascribing this observation to the effects of α-fetoprotein (AFP) [1, 9-11], it became possible and imperative to capture this important biological activity of AFP, a pregnancy-associated molecule, in an effort to produce the cancer protective effect on demand. The anti-estrogenic, anti-cancer active site of AFP was identified [12-19] and developed into a small cyclic peptide [12, 17, 18] called AFPep. AFPep exhibits anti-estrogenic activity (inhibition of estrogen-stimulated growth of uterus in immature mice) [15, 20-22], anti-cancer activity (stopping the growth of human tumor xenografts growing in mice [12, 21, 23-25] or *in vitro*) [12, 15, 17, 20, 21, 26-29], and cancer prevention activity (decreasing incidence and increasing latency in carcinogen-induced mammary cancer in rats) [20, 23, 30]. Being a single epitope fragment of a natural protein, AFPep has exhibited no toxicity or side effects after administration of 0.125 mg/kg to 400 mg/kg and treatment durations 1 to 200 days in four species [20, 23, 30] (manuscript in review). Being a cyclic

peptide, AFPep maintains its biological activity even after oral administration [23, 25]. Additional information about the cancer preventive potential of AFPep is needed to facilitate entry into clinical trials and utility. Especially important would be to determine if the molecule could prevent cancers that were induced by substances other than strong chemical carcinogens, such as methyl nitrosourea (MNU).

The MNU-induced cancer model has been used for many studies [31]. MNU is a direct-acting alkylating agent that has induced mammary cancer in several species and is believed to be a human carcinogen as well, although there are no human case reports. Cell types that are susceptible to MNU experience alterations in DNA structure and accumulate mutations that either increase the chance of developing cancer or lead to apoptosis. The MNU-induced mammary cancer model provided sufficient justification to allow tamoxifen to proceed to clinical trials, for therapeutic uses as well as for preventive uses of that drug [32]. We have shown that AFPep can prevent MNU-induced mammary cancer [20, 23, 30].

When investing in the expensive process of drug development, it is important before entering clinical trials to be able to expect efficacy against the usual forms of human breast cancer. We felt it important to assess the preventive efficacy of AFPep in the estrogen-induced breast cancer model in ACI rats, over and above its demonstrated ability to prevent MNU-induced cancer. The ACI rat is genetically predisposed to develop mammary cancer under the influence of physiological steady state levels of estrogen [33-35]. The neoplasia and ultimate cancers that develop phenotypically and histologically resemble human breast cancer [33]. This long-duration model enables staging of cancer development under the promotional effects of estrogen [36-39] as opposed to models that use a single bolus of oxidizing or alkylating agents that may be less relevant to humans. The model also offers opportunity to assess tolerability of test agents after lengthy durations of drug administration. Assessment of AFPep efficacy in the ACI model would also afford the opportunity to compare the carcinogen-induced and estrogen-induced cancer prevention models.

We show here that AFPep prevents breast cancer in two rat models and that it is comparable in efficacy to tamoxifen but without the tolerability issues associated with tamoxifen. We discuss the trends in approaches to cancer treatment, the trends in the use of both the estrogen-induced and MNU-induced prevention models, and the trends in drug development that lead to molecules that can provide efficacy without toxicity.

MATERIALS AND METHODS

AFPep, sequence *cyclo* (EKTOVNOGN) where O is hydroxyproline, was synthesized by AmbioPharm, Inc. (Augusta, S.C.) and assessed by mass spectrometry, as described elsewhere (manuscript in review). Bioactivity of AFPep was confirmed as a function of time in storage using an assay designed to measure the inhibition of estrogen-stimulated growth of the uterus in an immature mouse [15, 17, 20, 21, 40]. AFPep maintained biological activity and structural integrity throughout the duration of the 28-week repeat dosing tolerability study described below (data not shown; manuscripts in review).

Silastic tubing (0.078 in ID, 0.125 in OD, Dow Corning) was cut to 16 mm and mounted on a thin dowel rod (Puritan applicator) leaving one end open. The open end was dipped into powdered 17β-estradiol (estrogen, Sigma) until the tubing was packed. The open end was stoppered with another dowel rod, the wooden rods cut flush with the tubing, and the ends sealed with Silicone Type A medical adhesive (Dow Corning). Implants made in this manner contain approximately 9 mg of estrogen [41]. For control animals, empty Silastic tubing implants were made in a similar fashion.

Animals

All animals were housed in facilities certified by the American Association for the Accreditation of Laboratory Animal Care. The animal studies were carried out in adherence to the guidelines established in the Guide for the Care and the Use of Laboratory Animals with approval of the Albany Medical College (AMC) Animal Care and Use Committee.

ACI rats

The August Copenhagen Irish (ACI) rat is a cross between the August and Copenhagen-Irish strains of rat [41-43]. A number of investigators have used this strain of rat to assess breast cancer preventive

potential of various substances [36, 37, 41]. To ensure a 94% probability of detecting a difference of 50% between treatment groups, 30 animals were included. Intact female outbred ACI rats, weighing 95-100 g, were supplied by Envigo, Inc. (formerly Harlan Sprague Dawley) (Indianapolis, IN) and were handled daily during their 1- to 2-week acclimation period. Envigo can supply a limited number of ACI rats each week, but not a number sufficient to accommodate a large study. Therefore, this study was necessarily non-synchronous. Upon arrival each week at AMC for ten weeks, animals were randomized such that 10% of each shipment of animals was allotted to each experimental treatment group. When rats achieved a weight > 105 g, they were anesthetized with 45 mg/kg of Brevital (15 mg/ml methohexital sodium solution). A small incision was made on the shaved area on the back and a single 16 mm estradiol implant was inserted subcutaneously over the scapula of each rat. Wounds were closed with a single 9 mm wound clip (AUTOCLIP, Becton Dickinson) and animals were allowed to recover in their home cage. For assessment of prevention, animals were injected with 0.2 ml of saline containing 25 µg AFPep daily (0.15 mg/kg, 5 days on, 2 days off) for 24 days to mimic the duration of pregnancy in rats. For assessment of tolerability, animals were injected (0.2 ml) daily (5 days on, 2 days off) for 28 weeks with either saline or various doses of AFPep administered subcutaneously. Animals were observed daily for behavioral endpoints, weighed weekly and sacrificed after 28 weeks by CO_2 inhalation. Upon necropsy, organs were collected and weighed.

Estrogen concentration in rat blood was measured in the clinical chemistry laboratory at Albany Medical Center Hospital. The reportable range for estrogen under standard conditions in that laboratory is 25 to 1000 pg/ml.

RESULTS

Figure 1 shows the incidence of tumors palpated in ACI rats as a function of time after insertion of estrogen-containing implants. Estrogen-supplemented animals treated only with s.c. saline injection for 24 days incurred breast cancer with an incidence

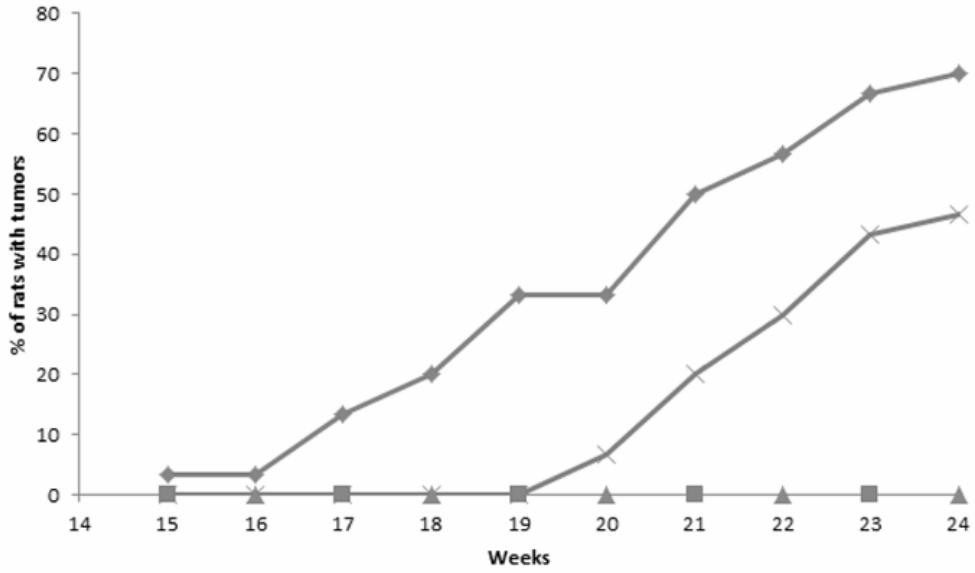

Figure 1. AFPep prevents estrogen-induced breast cancer. ACI rats received Silastic tubing implants containing 9 mg estrogen at Week 0. Control animals (n = 30, diamonds) were treated with s.c. injection of 0.2 ml saline, once daily for 4 weeks (5 days on, 2 days off) beginning on the day of estrogen implantation. AFPep-treated rats (n = 30, X) were treated with 25 µg of AFPep in 0.2 ml saline once daily for 4 weeks (5 days on, 2 days off) beginning on the day of estrogen implantation. Some animals received an empty Silastic tubing (no estrogen), and were treated with saline (n = 10, triangles) or with 1000 µg AFPep in 0.2 ml (n = 10, squares). All animals were weighed weekly and palpated twice per week for 24 weeks (diamonds). The difference in incidence at 24 weeks is significant (p = 0.024, Fisher's exact).

of 70% after 24 weeks; this number will increase to 100% if the animals live to 28-30 weeks (data not shown). Estrogen-supplemented animals treated with once-daily s.c. injection of AFPep (25 µg in 0.2 ml) for 24 days beginning on the day the estrogen implant was inserted experienced a decreased incidence (43% of animals had tumors, $p = 0.024$, Fisher's exact) and an increase in latency. Animals not supplemented with estrogen-containing Silastic implants had no tumors.

In a separate study to assess the tolerability of AFPep (manuscript in review), ACI rats were exposed to estrogen using implants as described above, or not exposed to estrogen (using empty Silastic tubing implants), and treated with various doses of AFPep for 28 weeks (5 days on, 2 days off) or not treated with AFPep. After 28 weeks of AFPep treatment, animals were sacrificed and subjected to full body necropsy. Organ weights were obtained for heart, lung, liver, and kidney. Table 1 shows organ weight and normalized organ weights (organ weight/body weight) for animals exposed to estrogen (or not) and treated with AFPep (or not). There were no significant differences attributable to AFPep for any of these parameters and no indication of toxicity due to AFPep.

After 8 weeks of exposure, animals that bore empty Silastic tubing implants had serum levels of estrogen of 57.2 ± 21 pg/ml, while animals that bore an estrogen-containing implant had 131.5 ± 39 pg/ml of estrogen. For all animals exposed to estrogen, some powdered estrogen remained in the Silastic implant at the end of the study, suggesting that these steady state levels of estrogen prevailed for the duration of the study. ACI rats exhibit some anatomical anomalies [44] including renal agenesis. Of the 310 ACI rats subjected to necropsy in these studies, 12 had only one kidney (including 1 of 70 animals not treated with AFPep, 11 of 240 animals treated with AFPep), an incidence below that cited in the literature. One rat had urinary tract stones; there were no other anomalies, and no behavioral changes in rats treated with AFPep or not, or exposed to estrogen or not.

DISCUSSION

Prevention strategies in the management of cancer have shown striking success against some cancer types, but discovery of new, well-tolerated chemopreventive strategies is greatly needed to reduce the overall incidence of this disease. For example, vaccines against the Hepatitis B virus have reduced incidence of liver cancer. Vaccines for the papilloma virus, as well as Pap smears, have reduced the incidence of cervical cancer. Smoking cessation campaigns have reduced the incidence of lung cancer. Colonoscopies with polyp removal have reduced the incidence of colon cancer, while better food preparation has reduced the incidence of stomach cancer.

With regard to breast cancer, it is clear that the disease can be prevented. Epidemiology studies indicate that multiple pregnancies lead to a reduction in breast cancer incidence later in life [1-8], but an intervention based on this observation seems impractical. Some women who carry the *BRCA1* gene have opted for bilateral mastectomy, which certainly prevents breast cancer but is associated with significant morbidity. Studies in high-risk individuals using tamoxifen or aromatase inhibitors have prevented the incidence of breast cancer by over 50% [45-47]. However, side effects of tamoxifen make this drug somewhat unattractive for preventive use. Nevertheless, interfering with estrogenic promotional activities of breast cancer development is a confirmed strategy that facilitates the prevention of breast cancer. What is required is a pharmacological agent that is better tolerated than currently available drugs. Agents that interfere with the growth promoting effects of estrogen, and which have no side effects, should be a welcome addition to the pharmacopoeia.

AFPep may be such an agent. We show here that AFPep prevents breast cancer in an ACI rat model, and have shown earlier [20, 23, 30] that it prevents breast cancer in the MNU cancer model, even when administered by an oral route (gavage) [23]. Because AFPep has its origins as an active site of α-fetoprotein (AFP), a protein of pregnancy, we studied the ability of AFPep to prevent breast cancer when administered for a duration designed to mimic pregnancy in the rat. Treatment of estrogen-supplemented ACI rats with AFPep for 24 days over the course of the month that began with the implantation of the estrogen-containing Silastic tubing significantly reduced incidence of breast cancer and provided additional tumor-free days compared to animals not treated with AFPep. These data may imply that a short-duration

Table 1. AFPep has no effect on animal weight or organ weights.

Group	n	Estrogen	AFPep (ug/rat/day)	Body Weight (g)	Lung Weight (g)	Normalized Lung/Body	Liver Weight (g)	Normalized Liver/Body	Kidney Weight (g)	Normalized Kidney/Body	Heart Weight (g)	Normalized Heart/Body
1	10	-	-	186.2 ± 5.5	1.2 ± 0.06	6.3	7.06 ± 0.30	38	1.52 ± 0.05	8.2	0.79 ± 0.02	4.2
2	29	+	-	191.3 ± 2.7	1.2 ± 0.05	6.1	8.26 ± 0.22	43	1.64 ± 0.04	8.6	0.78 ± 0.03	4.1
3	16	+	10	187.3 ± 3.2	1.1 ± 0.08	6.0	7.70 ± 0.43	41	1.55 ± 0.05	8.3	0.71 ± 0.04	3.8
4	15	+	100	190.6 ± 2.9	1.1 ± 0.05	5.7	7.81 ± 0.23	41	1.54 ± 0.05	8.1	0.68 ± 0.02	3.5
5	12	+	500	185.2 ± 5.2	1.0 ± 0.06	5.5	7.22 ± 0.38	39	1.53 ± 0.08	8.3	0.70 ± 0.03	3.8
6	29	+	1000	189.9 ± 2.8	1.1 ± 0.03	6.0	8.11 ± 0.22	43	1.56 ± 0.04	8.2	0.75 ± 0.03	4.0
7	9	-	1000	185.9 ± 4.5	1.2 ± 0.08	6.5	6.88 ± 0.33	37	1.47 ± 0.04	7.9	0.76 ± 0.05	4.1

ACI rats were exposed to estrogen (or not) and treated with AFPep (or not) at the indicated doses. AFPep was administered in 0.2 ml saline by s.c. injection, 5 days/week for 28 weeks. Animal and organ weights were obtained at necropsy. Normalized values (ratios of the means) were multiplied by 1000. There are no significant differences for any of the parameters listed.

intervention with orally administered AFPep at an early age may add significant protection against breast cancer for women, similar, and perhaps in addition to the protection afforded by pregnancy [8, 9]. Should this prove to be the case, breast cancer prevention may become both feasible and acceptable.

For assessing the potential of pharmaceutical agents to prevent breast cancer, there may be a trend toward use of the ACI rat model, in preference to the MNU model. There are few publications that directly compare the two models [33]. Therefore, it may be of interest to use AFPep in different studies to compare these cancer prevention models. Prevention of estrogen-induced cancer by AFPep (this study) could be compared to prevention by AFPep of MNU-induced breast cancer [20, 23, 30]. Despite the numerous technical differences between those studies, and despite the fact that none of the studies were designed to optimize preventive potential of AFPep, all of these studies yielded similar results in that AFPep caused a reduction in cancer incidence (from 70% of animals with one or more tumors to about 40% of animals with tumors). It is of interest to note that, when tested in the MNU model [32], tamoxifen yielded prevention efficacy of similar scope. Those observations were part of the justification for assessment of tamoxifen in human clinical trials for prevention of breast cancer. Nevertheless, data are currently insufficient to assess definitively these two prevention models, or to assert that one is preferable to the other. Advantages of the estrogen-induced breast cancer model in ACI rats include the lengthy time frame which inherently offers the opportunity to assess tolerability in repeat dosing studies, and to assess late intervention strategies [48]. A disadvantage is that it is not clear that it is possible to achieve maximal prevention in the face of the continual presence of estrogen. Removal of the estrogen implant does not allow the study to continue, as tumors shrink even in the absence of test substance [49]. Advantages of the MNU-induced breast cancer in Sprague Dawley (or other) rats include a more rapid assessment and the observation that an agent is able to interdict cancer driven by powerful alkylating agents.

Finally, it could be noted that, while there will always be a need for cytotoxic agents, there should also be a trend toward developing drugs that have efficacy without toxicity. Well-tolerated cytostatic agents sufficient to keep cancer in stasis for many years could offer patients the opportunity to enjoy a cancer-free lifestyle. Agents that could provide preventive efficacy, lasting as many years as does the protection pregnancy provides, could enjoy the success and acceptance comparable to the cancer vaccination interventions. Agents used as adjuvants to surgery that could keep cancer from progressing to malignancy and that led to no discernable side effects or toxicity would find acceptance for use in an adjuvant therapeutic intervention. In this respect, it is of interest to compare the therapeutic index of AFPep and other agents, as shown in Table 2. Based on effective dose and lethal doses in rodent studies,

Table 2. Therapeutic index of common breast cancer drugs.

Product	Therapeutic index*	Mechanism of action
AFPep	>1000	Multikinase inhibitor
Tamoxifen	16 [50, 51]	Binds to ER
Examestane	25 [52]	Aromatase inhibitor
Faslodex	72 [53, 54]	Downregulates ER
Herceptin	54	Binds to receptor for epidermal growth factor
Getfitinib	15 [55]	Binds to tyrosine kinase domain of epidermal growth factor receptor
Cyclophosphamide	3 [56]	Inhibits DNA replication
Paclitaxel	4 [57]	Inhibits DNA replication
Adriamycin	3 [57]	Inhibits DNA replication

*Taken from rodent toxicology studies.

it is clear that AFPep enjoys a very broad therapeutic index. Efficacy without toxicity may be a major challenge, but it is possible and it is the demand of cancer survivors. Peptides patterned after natural proteins offer the possibility of excellent tolerability and enjoy the advantage that metabolites (*e.g*., amino acids) are as non-toxic as is the parent drug.

CONCLUSION

From the work reported herein, together with the data reported elsewhere [12, 15, 17, 20, 21, 23-25], we conclude that AFPep is effective for treatment and prevention of breast cancer in rats and mice. AFPep is similar to tamoxifen in terms of breast cancer preventive potential and yet this work and earlier work (manuscript in review) suggests that tolerability of AFPep is especially high in all species examined (rats, mice, dogs, and primates). While every agent must be tested in human studies, the work reported to date indicates that AFPep possesses the desirable attributes of an agent that should continue in the drug development process toward clinical trials in humans.

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CONFLICT OF INTEREST STATEMENT

Each author individually declares that there are no conflicts of interest. All applicable international and institutional guidelines for the care and use of animals were followed.

ABBREVIATIONS

AFP: α-fetoprotein; AFPep: 9-amino acid analog of the anti-breast cancer active site of AFP; MNU: methyl nitrosourea; ACI: August-Copenhagen strain of rat; ID: inner diameter; OD: outer diameter.

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