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Original Article

Tolerability and efficacy of AFPep in multiple models of breast cancer including spontaneous canine mammary cancer

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ABSTRACT

AFPep is a 9-amino acid, cyclic peptide derivative of the anti-estrogenic/anti-breast cancer active site of alpha-fetoprotein [AFP]. The purpose of this study was to determine the tolerability of AFPep in normal and tumor-bearing dogs and assess its effect on biomarkers of estrogen-promoted growth in several models of breast cancer. Fourteen normal beagle dogs were treated with various doses of AFPep through i.v., s.c., and p.o. routes. Blood levels of AFPep and clinical signs of tolerability were monitored. Expression of ERα, pERα, TRIM25, and KLF5 were assessed in three estrogen-sensitive tissue models: immature mouse uterus, MCF7 xenografts, and spontaneous canine mammary tumors excised from AFPep-treated animals. In normal and tumor-bearing dogs, AFPep was well tolerated at all doses and through all routes. Uteri and MCF7 xenografts from mice treated with AFPep were growth-inhibited with suppression of the aforementioned biomarkers. In tumor-bearing dogs, one of six dogs had tumors expressing ERα. In that animal, AFPep resulted in a decrease in the expression of KLF5 and TRIM25 as well as a decrease in the ratio of pERα/Total ERα in tumor tissue compared to pre-treatment levels in tumor samples from the same animal. AFPep is well tolerated in multiple animal models, inhibits estrogen-mediated growth, and decreases pERα/ERα, KLF5 and TRIM25 expression.

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KEYWORDS: AFPep, breast cancer, experimental therapeutics, biomarkers, tolerability, estrogen receptor.

1. INTRODUCTION

AFPep is a first-in-class agent being developed for the treatment and prevention of breast cancer [1]. AFPep is a 9-amino acid, cyclic peptide derivative of the anti-breast cancer active site of alpha-fetoprotein (AFP) and is devoid of the other activities associated with endogenous AFP [2]. AFPep is well tolerated in rodent models [2] and has a unique mechanism of action [2, 3]. It is a multikinase inhibitor that blocks the activation of ERα, FAK, and c-kit [4]. AFPep is additive in combination with tamoxifen and mitigates some of the toxicity [uterine hypertrophy] of tamoxifen [5]. AFPep blocks the growth of ER+ human breast cancer xenografts including those resistant to tamoxifen [5] and prevents the development of carcinogen-induced mammary cancer in rats [6-8]. AFPep is an orally active peptide [4] whose metabolites are simple amino acids, demonstrates efficacy against ER+ breast cancer, and is non-toxic in rodents. It should be considered for development for treatment and prevention of breast cancer. Due to the limitations of rodent models available for the study of new cancer therapeutics, spontaneously occurring breast cancer in outbred dogs provides a unique model [9] for preclinical study of AFPep as an anticancer agent.

Tolerability of drugs, the extent to which they are acceptable to a patient, is a critical characteristic for newly developed agents. Information about the tolerability of AFPep in higher mammals is lacking, although in rodents AFPep has a broad therapeutic index [2, 8], does not affect the estrous cycle or fertility in rats [10], and has no effect on the liver [2]. A purpose of this study was to assess the tolerability of AFPep in dogs.

For clinical trials, clear assessments of efficacy are essential especially for agents such as tamoxifen or AFPep which are cytostatic, not cytotoxic. Several studies have shown that AFPep inhibits the activation of ERα in ER positive tumor cell lines [3, 4, 11-14]. No studies have shown this in intact normal or cancerous tissue. Moreover, the consequences of ERα inhibition have not been shown on biomarkers downstream from ERα related to tumor aggressiveness. TRIM25 and KLF5 are two biomarkers downstream from ERα, and their inhibition has been correlated with inhibition of breast cancer tumor growth, invasion, and metastasis [15, 16]. It seemed logical to probe the ability of ERα, TRIM25, and KLF5 to serve as reporters for the anti-proliferative effects of AFPep in intact normal and cancerous ER+ tissue.

Often, as tumor models become more complex and tumors become more heterogenous, biomarkers of efficacy in simpler models no longer change in the more complex models [17]. A purpose of this study was to identify biomarkers that reliably report anti-estrogenic and anti-breast cancer activities of AFPep across various species and models. These models include noncancerous estrogen-dependent intact mouse uterus, cancerous estrogen-dependent human MCF7 xenografts, and intact spontaneous mammary tumor tissue from dogs. Markers that are robust enough to be identified across this spectrum of species heterogeneity might serve well to report efficacy of AFPep during clinical trials in humans. We report here that AFPep is as well tolerated in dogs as it is in rodents, and that robust biomarkers can be identified for reporting efficacy of AFPep.

2. MATERIALS AND METHODS

2.1. AFPep

AFPep, sequence *cyclo*[EKTOVNOGN] where O is hydroxyproline, was synthesized using solid phase peptide synthesis approaches as described earlier [18]. The agent was synthesized by AmbioPharm, Inc. (Augusta, SC) and assessed by mass spectroscopy [19]. AFPep was 99% pure and exhibited a molecular weight of 969.01 Da (expected 968.46 Da, monoisotopic). The 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 immature mice [5, 19].

2.2. Animals

Immature Swiss/Webster female mice, 14-days old, 6-8 grams in body weight were obtained with nursing mothers from Taconic Farms (Germantown, NY). ICR-SCID female mice 6-7 weeks old were obtained from Taconic Farms. All mice were housed in micro-isolate cages fitted with stainless steel wire lids and air filters and supported on ventilated racks supplying hepa-filtered air exchange. Sexually mature female beagle dogs were purchased from Marshall Farms, North Rose, NY. and were singly housed in large indoor pens. 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 Animal Care and Use Committees at the Albany Medical College in Albany and at The Cancer Institute of The Animal Medical Center in New York City.

2.3. Assays for estrogen-dependent growth

Efficacy of AFPep was demonstrated by a uterine growth-inhibition assay in immature Swiss Webster female mice as described earlier [5]. AFPep is defined as biologically active [anti-estrogenic] when it is found to result in significant inhibition of estrogen-stimulated growth of mouse uterus as calculated by the following formula:

% Growth Inhibition = 100 x (Full E_2 Stimulation – E_2 Stimulation in test group)/
(Full E_2 Stimulation – No E_2 Stimulation)

Efficacy of AFPep was also assessed by mouse/ human breast cancer xenograft studies. MCF-7 human breast cancer cells were adapted for growth in SCID mice with subcutaneous estrogen implants, as previously described [3, 5]. Briefly, five tumor

pieces (each 1.5 mm in diameter) were loaded into a 16-gauge trocar and deposited into the thoracic mammary fat pad. Tumors became palpable after approximately 21 days. Thereafter tumor size was measured once daily with a Vernier caliper. Mice were randomized into control(saline) or treatment (AFPep 100 µg/mouse) groups and were treated through oral gavage administration of AFPep once daily for 14 days. Tumor volumes were calculated using the formula $v = 0.52\ (d)^2D$ assuming the tumor shape to be an ellipsoid of revolution around its long axis D.

2.4. Tolerability in mice and dogs

Following administration of AFPep all mice were carefully observed for any changes in appearance and in-cage activity. Body weights were monitored throughout the studies. At necropsy organ weights and appearance were examined [2, 6, 7]. Normal beagle dogs weighing approximately 10 kg each were injected with various doses of AFPep in a single application study through either the intravenous [i.v.], subcutaneous [s.c] or oral [p.o. enteric capsules] routes. Dogs were carefully observed for any changes in behavior, appearance or activity for 24 hours after the administration of AFPep by the veterinary staff at Albany Medical College. Aliquots of blood were analyzed at IDEXX Laboratories Inc (Westbrook, ME) using Sysmex XT and Beckman AU5800 systems. For the canine studies, endpoints included Complete Blood Count variables (white blood cell count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, automated platelet counts, neutrophils, lymphocytes, monocytes, eosinophils, and basophils), and Blood Chemistry variables (globin, calcium, bilirubin, phosphorous, bicarbonate, creatine kinase, blood urea nitrogen, chloride, creatinine, potassium, albumin, cholesterol, sodium, total protein, glucose, albumin/globin ratio, BUN/creatinine ratio, hemolysis index, lipemia index, Na/K ratio, anion gap, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and gamma glutamyl transferase). Blood levels of AFPep were assessed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) in our laboratories as previously described [19]. Each assessment in normal dogs was repeated at least three times. At the end of these studies, dogs were adopted by local families.

2.5. Clinical studies in dogs with mammary cancer

This study was approved by the Animal Medical Center IACUC and informed consent was obtained from all owners. Female dogs with one or more mammary gland tumors greater than 2 cm in diameter presenting to the Cancer Institute at the Animal Medical Center in New York City were screened for inclusion in the study. Dogs were included if they had a life expectancy of more than three months, no major organ dysfunction or metastatic disease precluding general anesthesia for biopsy and mastectomy. All dogs underwent complete clinical staging consisting of a complete blood count [white blood cell count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, automated platelet count, neutrophils, lymphocytes, monocytes, eosinophils, and basophils], biochemical profile (globin, calcium, bilirubin, phosphorous, bicarbonate, creatine kinase, blood urea nitrogen, chloride, creatinine, potassium, albumin, cholesterol, sodium, total protein, glucose, albumin/globin ratio, BUN/creatinine ratio, hemolysis index, lipemia index, Na/K ratio, anion gap, ALP, ALT, AST, and GGT), urinalysis, three-view thoracic radiographs, and an abdominal ultrasound. Dogs were housed at their home and observed by their families during the 8-day study.

Tumors were biopsied prior to administration of AFPep and on Day 8 after the last injection of AFPep. Dogs were administered AFPep 10 mg s.c. once daily for 8 consecutive days. Blood levels of AFPep were measured before and 30 min following its administration on the first and eighth day of treatment with AFPep. Histopathology was obtained on all mammary gland tumors. Tolerability was assessed using the Veterinary Cooperative Oncology Group common terminology criteria for adverse events [20].

2.6. Biomarker analysis

Uterus, xenograft, and canine biopsy tissues were homogenized with standard RIPA [radioimmunoprecipitation assay] buffer supplemented with 1 mM dithiothreitol and a cocktail of protease inhibitors in Precellys lysis tubes using a Bertin MiniLys homogenizer [21]. Whole cell lysates were then sonicated and cleared by centrifugation at 14,000 g for 5 minutes at 4 °C.

Lysates were resolved on SDS-PAGE and immunoblotted as previously described [22]. Primary antibodies for ER, pER, TRIM25, KLF5 and β-actin were acquired through Cell Signaling and Santa Cruz Biotechnology. HRP-conjugated secondary antibodies were obtained from Promega and used at manufacturer-recommended dilutions. The immunoblots were developed using West Pico plus [Thermo Fisher] chemiluminescent reagents, imaged using a Bio Rad ChemiDoc MP (Hercules, CA), and quantified for relative protein expressions by normalizing to β-actin levels.

2.7. Statistical analysis

Mouse uteri weights, tumor volumes, and protein band densities were analyzed using a two-way ANOVA (analysis of variance) with a post-hoc Tukey Honest Significant Difference test using MiniTab.

3. RESULTS

3.1. Tolerability

A dose of 4 mg/kg of AFPep was administered to three normal beagle dogs intravenously and to three normal beagle dogs subcutaneously. In all dogs this dose was well tolerated. Animals remained calm and still during the injections (indicating no immediate irritant effects) and maintained normal activity and behavior during the 24-hour observation period after injection. Dogs remained playful, showed no attention to the injection site, and exhibited no vocalization indicative of pain, stress or discomfort. Body posture was normal, eyes were bright and alert, and locomotion was normal. There were no changes in heart rate and respiration rate. Appetite/food consumption, urine and feces output remained normal throughout the observation period. Complete blood count with cell differential and full panel of blood chemistry values taken at 30 minutes before, 30 minutes after, and 24 hours after AFPep administration were unchanged, indicating that hematological, renal and hepatic systems were operating within the normal range before and after AFPep. Blood levels 30 minutes after administration of AFPep were in the range of 80 μg/ml (i.v.) and 6 μg/ml (s.c.) which were well above the range (0.1 μg/ml) that had previously shown anti-tumor activity in mice [3], indicating that more than sufficient levels of AFPep were circulating in these dogs throughout the observation period.

Oral bioavailability of AFPep had previously been found to be in the range of 2% in rodents [3], so in dogs, dose by the oral route [enteric capsules] was escalated to 12 mg/kg in 4 normal beagle dogs and then 25 mg/kg in 4 normal beagle dogs. Both the 12 mg/kg and 25 mg/kg oral doses of AFPep were well tolerated in these dogs as determined by the endpoints described above. In addition, there was no evidence of gastrointestinal distress as measured by the endpoints of drug palatability, reflux, food/water consumption, stool consistency/ frequency and overall playfulness of each dog throughout the 24-hour observation period after oral administration of AFPep. The 25 mg/kg dose consistently yielded blood levels of AFPep in the range of 0.1 μg/ml which had been associated with antitumor activity in rodents [3] indicating that the oral route is a viable option for AFPep administration during its clinical translation. The normal beagles studied at Albany Medical College were adopted by local families at the conclusion of the study and one year later were alive and well and leading normal lives.

In the canine mammary cancer study carried out at the Animal Medical Center in New York City, six tumor-bearing dogs ranging from 5 to 11 years in age were given 10 mg AFPep [~1 mg/kg], s.c., once daily for eight days between tumor biopsy and mastectomy for tumor removal. Dogs ranged in weight from 5-30 kg. Three dogs were ovariectomized and three were sexually intact. The only adverse events identified were Grade 1 [mild] pain on injection of AFPep in three of six dogs. Blood samples 30 min before and 30 min after the first and last administration of AFPep indicated no changes in any of the hematological and blood chemistry measures described above. AFPep blood levels 30 minutes post AFPep were in the range of 1-5 μg/ml which exceeded values that had previously been found to be associated with anti-tumor activity in rodents [3] suggesting that tumor response could safely be achieved with AFPep in dogs.

Five dogs were alive 18 to 22 months following mastectomy. One non-ovariectomized dog with an ER– simple tubular adenocarcinoma grade II died 11 months after treatment from the multiple masses in the lungs.

3.2. Efficacy and biomarkers

As shown in Figure 1, i.p. administration of 100 ug of AFPep to immature female mice significantly inhibited the estrogen-stimulated growth of the uterus in these animals. Biomarker analysis of excised uteri indicated that AFPep inhibited the activation of ER and tamped down expression of KLF-5 and TRIM 25 downstream from ER. Figure 2 shows tumor transplant studies in which immune-deficient mice with human MCF-7 breast cancer xenografts actively growing in their mammary fat pads were treated with 100 ug of AFPep given once daily p.o. for 14 days. AFPep initially slowed and then stopped the breast cancer growth in these mice [Figure 2]. Similar to the above studies of intact uteri in immature mice, tumor xenograft samples taken 1 hour after the last treatment with AFPep showed inhibition of ER activation [phosphorylation] as well as inhibition in the expression of KLF-5 and TRIM 25 [Figure 2]. This approach was extended to tumor-bearing dogs in the canine mammary cancer study. Six dogs were entered into the study. Each dog received 10 mg of AFPep s.c. once daily for 8 consecutive days between tumor biopsy and surgical resection of tumor. AFPep was well tolerated in all 6 dogs using endpoints described earlier for the 14 normal dogs. In this study, change in tumor growth could not be evaluated since standard treatment of these dogs was surgical removal of the tumor. However, biopsy of tumor samples for biomarker analysis could be obtained pre- and immediately post-AFPep

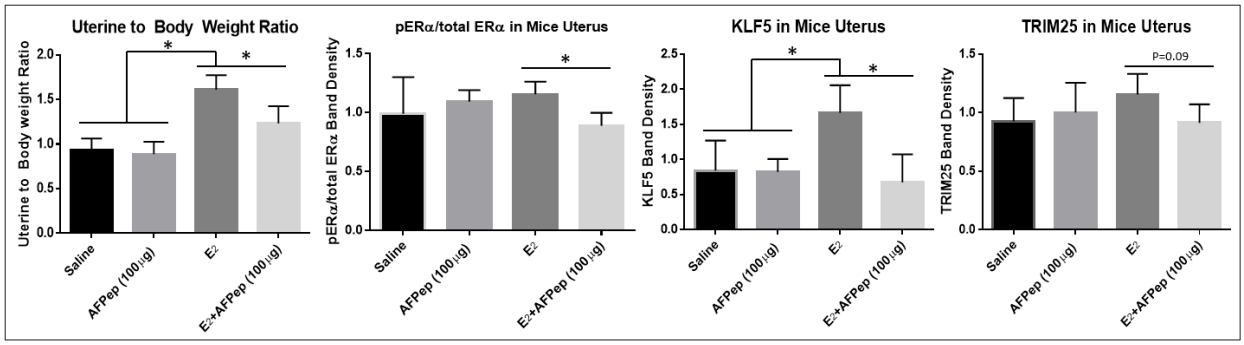

Figure 1. Antiuterotrophic activity of AFPep. Immature female mice were injected i.p. with either saline [0.2 mL] or 100 µg of AFPep in 0.2 mL saline. One hour later mice were injected i.p. with either saline or 0.5 µg E_2. Twenty-two hours later, uteri were harvested, weighed and processed for Western blotting. There were five replicate mice per group. Mean ± standard error of uterine weights and band densities were calculated. Differences were assessed using Tukey's tests. * $p < 0.05$.

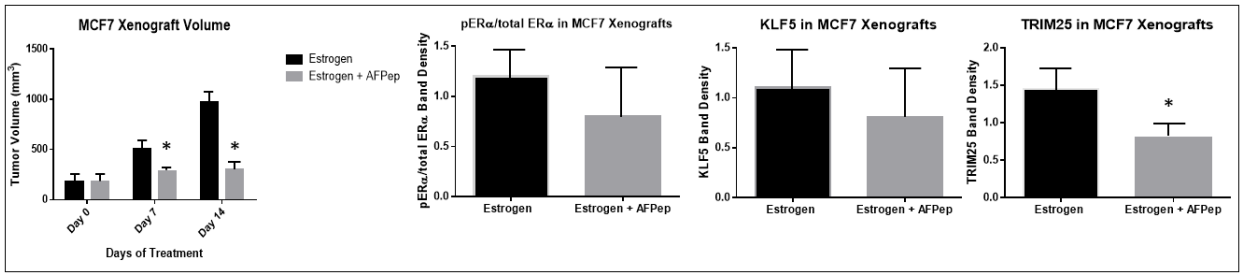

Figure 2. Anti-breast cancer activity of AFPep. Pieces of MCF-7 human breast cancer tissue were injected into the thoracic mammary fat pad of SCID mice. Mice were supplemented with E_2 implants. Tumors were on average 6.7 mm in diameter [156 mm^3] on day 22 after implantation [the day on which treatment was begun] and measured once daily thereafter. Either vehicle or AFPep [100 µg/mouse] was given in 0.5 mL once daily for 14 days by oral gavage, beginning 22 days after tumor implantation. Tumors were harvested for biomarker analysis on day 35 after implantation. Mean ± SE of tumor endpoint from 3 replicate mice/group are reported. * $p < 0.05$.

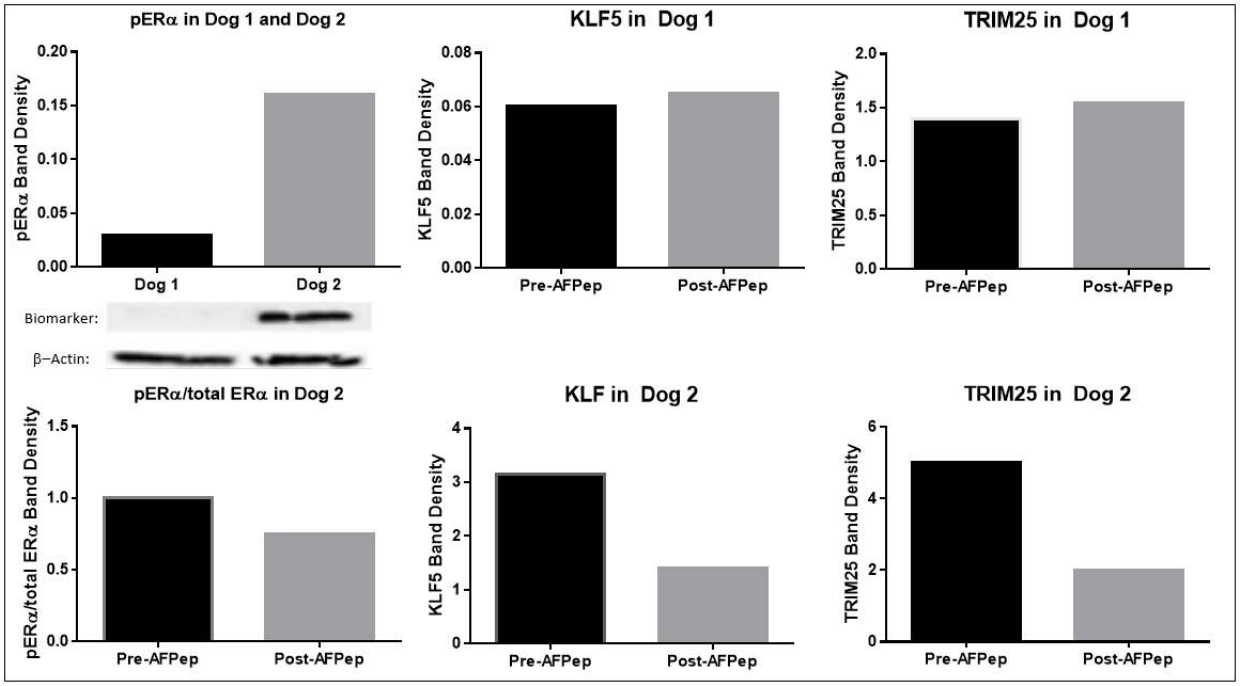

Figure 3. AFPep decreases expression of pER, KLF5, and TRIM25 in canine mammary tumors. Dogs presented with spontaneous mammary tumors as described in Methods. Following initial biopsy, AFPep [10 mg s.c.] was administered once daily for 8 days prior to surgical resection of the tumor. Treatment with AFPep decreased relative expression of pER/total ER by 30% and decreased expression of KLF5 and TRIM 25 by 70% relative to their pre-AFPep levels in the ER+ dog mammary tumor. Dog 1 is representative of the 5 dogs with ER– tumors. Dog 2 had the ER+ tumor.

administration to these dogs. The tumor in one of the six dogs was positive for ER. This was somewhat lower than what the literature has indicated, namely that approximately 40-50% of canine benign and malignant mammary tumors are considered to be ER+ [23], and a somewhat lower percentage of ER positivity is reported for advanced canine mammary cancers [24]. AFPep treatment of the dog bearing the ER+ tumor resulted in an expected inhibition of the activation of the ER in that dog's tumor and significantly reduced the expression of KLF-5 and TRIM 25 [Figure 3]. In contrast, AFPep treatment did not inhibit the expression of KLF-5 and TRIM25 in the 5 dogs that had ER- mammary tumors.

4. DISCUSSION

The importance of drug tolerability cannot be over emphasized as lack of tolerability is a major reason for failure in translation of potential pharmaceutical agents to human use [25]. The results of this study demonstrate that AFPep is well tolerated and inhibits intermediate biomarkers of estrogen-stimulated growth of normal and cancerous tissues in mice, dogs, and human tumor xenografts. The excellent tolerability of AFPep is likely due to its derivation from an endogenous growth regulatory mammalian protein, AFP.

Previous studies of AFPep in rodents had indicated that in mice, doses as low as 40 µg/kg were efficacious and dose escalation up to 40 mg/kg showed no adverse effects [2, 3, 8]. Similarly in rats, doses of AFPep as low as 250 µg/kg were efficacious and dose escalation up to 10 mg/kg showed no adverse effects [2, 6, 8]. This extremely large therapeutic index in rodents provided confidence that 4 mg/kg would be a safe starting dose of AFPep in dogs. Moreover, previous pharmacokinetic studies suggested that this dose would yield blood levels of AFPep in dogs that were associated with anti-breast cancer activity in rodents [3, 19]. The doses of AFPep used in the studies

described herein [4 mg/kg mice; 1-4 mg/kg dogs] led to blood levels well above the 0.1 µg/ml range [3] which is reported to have anti-estrogenic properties, but is well below the mg/ml quantities of its parent protein [AFP] normally found in both human fetal blood [26] and newborn puppies [27]. This is likely another reason for the excellent tolerability of this active site analog [AFPep]. The anti-estrogenic properties of AFP have been shown in multiple experimental studies [28-35], and AFPep has been carefully designed to contain only the anti-estrogenic portion of AFP [18, 32, 36-38]. AFPep has been shown to be well tolerated in mice and rats in several other studies, even when given in much higher doses and over a more prolonged duration than that used in this study [2]. In a separate study of the chemopreventive property of AFPep against estrogen-induced breast cancer in rats [8], we reported the effective dose versus the toxic dose of AFPep in comparison to other endocrine agents currently in use for breast cancer management and showed that AFPep has a therapeutic index several fold above all of those agents that are currently making an impact against breast cancer but have side effects which greatly decrease patient tolerability and acceptability during their long-term use. AFPep's apparent lack of side effects is a highly striking feature of this novel anti-cancer agent and bodes well for its successful clinical translation.

Another important reason for failure of drug translation is inability to maintain the efficacy seen in preclinical models [39]. Several reasons have been reported including the complexity of human disease in comparison to the simplicity of most preclinical models of disease. In this study, the focus was on the anti-estrogenic activity of AFPep across different species including the very complex situation of spontaneous canine mammary cancer in companion animals. It is well known that estrogen plays a role in the promotion of canine as well as human breast cancer. Dogs that experienced ovariectomy prior to their first estrus have a significantly reduced incidence of mammary cancer later in life [23]. In humans, lifetime exposure to estrogen is highly associated with subsequent breast cancer incidence [40]. In the study reported herein, three models of *in vivo* estrogen-stimulated growth were used, two with intact tissue [immature mouse uterus and biopsied spontaneous canine mammary gland tumors] and one with transplanted human breast cancer tissue [MCF-7 breast cancer xenograft]. In all three of these cases when AFPep was administered to the animals, anti-estrogenic effects were observed [growth inhibition for uterus and xenograft and relevant biomarker inhibition in all three models]. AFPep inhibited ER activation as measured by diminution of the phosphorylated ER to total ER ratio as well as relevant biomarkers downstream from ER [TRIM 25 and KLF 5]. TRIM 25 and KLF 5 have both been associated with breast cancer aggressiveness [15, 16] and add additional import to the ER activation profile. The consistency of the AFPep inhibitory effects in three diverse models bodes well for maintenance of its efficacy during its clinical translation.

It is important to note that blood levels of AFPep associated with growth inhibition of experimental breast cancers have already been established [3]. These blood levels were readily attained in all the models in this study and will be an important endpoint in future studies.

CONCLUSION

From this study, relevant biomarker and tolerability data have been added to the AFPep profile. All observations look promising for translation to human clinical trials and eventual human use for treatment of ER+ breast cancer. Furthermore, because of its exquisite tolerability profile, AFPep may be able to contribute to the acute need for an effective, well tolerated chemopreventive agent that stops the development of breast cancer in high risk patients [1].

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CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflict of interest to disclose.

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