AWARD NUMBER: W81XWH-15-1-0242

TITLE: Prevention and Treatment of Breast Cancer

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REPORT DATE: OCT 2019

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
OCT 2019

2. REPORT TYPE
Final

3. DATES COVERED
15 July 2015-14 July 2019

4. TITLE AND SUBTITLE
Prevention and Treatment of Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0242

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland. 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this project was to test the hypothesis that AFPep is safe and effective for prevention and treatment of breast cancer. Aim 1 was to document the preventive efficacy and safety of AFPep. The data reported herein allow us to conclude that AFPep prevents estrogen-induced breast cancer in ACI rats, just as it prevented carcinogen-induced breast cancer in Sprague Dawley rats. Data from rats, mice, monkeys and dogs allow us to conclude that AFPep is extraordinarily safe. We have reported a therapeutic index of greater than 1000. Aim 2 was to measure and maintain blood levels of AFPep at effective doses in mice, dogs, and non-human primates. Assessing pharmacokinetic data in mice, dogs, and primates, we conclude that an efficacious blood level of AFPep is approximately 0.1 µg/ml, and this blood level can easily be achieved in higher mammals with no evidence of toxicity. Based on a proof-of-concept clinical trial in dogs, we conclude that blood levels of AFPep 10 to 50 times the effective blood level are easily achieved, well tolerated, and effective (as monitored by biomarker response to AFPep). We conclude from the data of Years 1 through 3 of this project that AFPep is safe and effective for the prevention and treatment of breast cancer, and should proceed to clinical trials for the prevention and treatment of breast cancer as soon as possible. We outline our efforts to secure funding toward that end.

15. SUBJECT TERMS
Breast Cancer; Prevention; Therapy; Therapeutic Index; tolerability; acceptability; biomarkers; pharmacokinetics

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified		**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified			

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1. INTRODUCTION

This project offers solutions for two overarching challenges in that it demonstrates: *a.*) primary chemoprevention of breast cancer and *b.*) revolutionize treatment regimens for ER+ breast cancer that are safe. The Breakthrough that is needed to realize the CDMRP's Vision "to eliminate breast cancer" is a chemopreventive agent that is as safe as it is effective. The subject of this project is just such a molecule: AFPep is a first-in-class, well tolerated, growth regulatory synthetic peptide that stops development and growth of breast cancer in rodents. AFPep is a small molecule mimic of the active site of a naturally occurring protein of pregnancy (α-fetoprotein, AFP) which is largely responsible for the lifetime reduction in risk of breast cancer that occurs as a result of pregnancy. The scope of this translational project entails essential work necessary to move AFPep beyond discovery and position it to enter clinical trials. The purpose of the research was to test **the hypothesis that AFPep is safe and effective for prevention and treatment of breast cancer.** There were two specific aims being studied simultaneously: 1) Use an innovative cancer prevention model in rats to document the preventive efficacy and safety of AFPep. We showed that AFPep can offer primary prevention of estrogen-induced rat mammary cancer, that there is substantial flexibility in scheduling the administration of AFPep, and that AFPep is extraordinarily well tolerated. 2) Use a veterinary clinical trial in dogs to document tolerability and efficacy of AFPep against spontaneous heterogeneous mammary cancer. We showed that we can measure and maintain blood levels of AFPep at effective doses in dogs and that spontaneously arising breast cancer responds to treatment with AFPep as documented by modulation of various biomarkers in the tumor tissues.

2. KEYWORDS

Breast Cancer; Prevention; Therapy; Therapeutic Index; Tolerability; Biomarkers; Canine clinical trial; pharmacokinetics; FDA Type B meeting; dose fractionation;

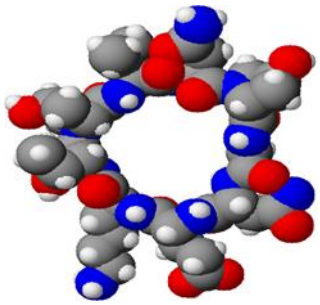


Figure 1. AFPep is *cyclo(EKTOVNOGN)*, the active site of AFP. Its metabolites are simple amino acids. *E* is at the six o'clock position; the sequence runs clockwise; *O* is hydroxyproline. MW = 970

3. ACCOMPLISHMENTS

3.a. What were the major goals of the project?

As we did in previous progress reports, we reproduce from the original Statement of Work the goals of the project. These two tables indicate graphically that all tasks were completed, that all objectives were achieved successfully. In fact, we accomplished far more than we planned originally (with the help of funding beyond that of BC 132567). None of the usual hindrances in drug development pathways were encountered. There are no observations that would suggest a concern for moving AFPep to clinical trials and eventual utility for the treatment and prevention of breast cancer.

As a more readable summary, we include the Outcomes Statement that we prepared for inclusion with our recent Expansion Award proposal. This brief, two-page statement serves as an Executive Summary and highlights the accomplishments of BC 132567. The Expansion Award application (BC 191174) and an application for FL3 support (BC 190375) are described below as the logical outgrowth of BC 132567.

Following the Outcomes Statement, we provide a brief discussion of the overall Impact that we see accruing from these studies. We then enter the narrative description portion of this Final Progress Report, detailing the major accomplishments and elaborating on their importance to the mission of the BCRP.

3.a.1 This is the approved Statement of Work for Specific Aim 1

Specific Aim 1 Demonstrate that AFPep will interdict breast cancer at any stage of progression	**Proposed Timeline**	**Progress**	**Details**
Major Task 1 Start-up tasks	Months		
Subtask 1 IACUC approval	-3	Complete	Y1PR
Subtask 2 Pre-engage CRO for drug synthesis	-2	Complete	Y1PR
Subtask 3 Pre-engage animal supplier for rat acquisition	-3	Complete	Y1PR
Subtask 4 Hire technician	1	Complete	Y1PR
Subtask 5 Test quality of drug	2	Complete	Y2PR
Major Task 2 Determine dose-response curve for prevention in ACI rats			
Subtask 1 Acquire rats, implant with estrogen pellets	2	Complete	Y1PR
Subtask 2 Monitor rats for tumors	3 - 13	Complete	Y1PR
Subtask 3 Necropsy of animals; pathology analysis	12-13	Complete	Y1PR
Subtask 4 Ongoing quality analyses of commercial AFPep	1 - 36	Complete	Y3PR
Milestone(s) Achieved: Determination of dose-response curve of AFPep for prevention of cancer; determination of safety of AFPep	13	Complete	Y1PR
Major Task 3 Demonstrate interdiction of cancer progression			
Subtask 1 Acquire animals, implant with estrogen pellets	13	Complete	Y3PR
Subtask 2 Monitor animals for tumors	14 - 24	Complete	Y3PR
Subtask 3 Necropsy of animals; pathology analysis	23-24	Complete	Y3PR
Subtask 4 Preparation of manuscript for publication	23-25	Complete	Y3PR
Milestone(s) Achieved: Interdiction of progression of cancer; schedule flexibility.	2 years	Complete	Y3PR
Major Task 4 Determine minimal duration of treatment sufficient to produce life-long prevention			
Subtask 1 Acquire animals, implant with estrogen pellets	25	Complete	Y2PR
Subtask 2 Monitor animals for tumors and side effects	25-36	Complete	Y2PR
Subtask 3 Necropsy of animals; pathology analysis	35-36	Complete	Y2PR
Subtask 4 Preparation of manuscript for publication	35-36	Complete	
Milestone(s) Achieved: Determine optimal schedule for AFPep use	Year 3	Complete	Y2PR
Summary of Year 1 through Year 3 Prevention Studies			
Associated Studies		Complete	Y2PR

3.a.2 This is the approved Statement of Work for Specific Aim 2:

Specific Aim 2 Demonstrate that AFPep has efficacy against spontaneous, heterogeneous mammary cancer in higher mammals	**Proposed Timeline**	**Progress**	**Details**
Major Task 1 Establish in normal dogs blood levels of AFPep known to be efficacious against human breast cancer xenografts	Months	Complete	Y2PR
Subtask 1 PK of AFPep in mice	1-3	Complete	Y2PR
Subtask 2 PD – Time to onset and durability of efficacy of AFPep against human breast cancer xenografts as measured by biomarker analyses	2 - 8	Ongoing	
Subtask 3 PK of AFPep in dogs	6 - 18	Complete	Y2PR
Milestone(s) Achieved: Half-life, bioavailability and effective blood levels of AFPep in dogs *Comparison of Canine and murine PK parameters*		Complete	Y2PR
Major Task 2 Demonstrate in dogs with spontaneous mammary cancer that AFPep given systemically induces an anti-proliferative phenotype in the autochthonous cancer prior to its surgical resection			
Subtask 1. In multiple dogs with ER+ mammary cancer, assess biomarker levels in pre- and post-AFPep tumor biopsies	12 - 36	Complete	Y3PR
Subtask 2 Present data at national meetings; publish data in peer reviewed journals	24-36	Complete	Y3PR
Milestone(s) Achieved: AFPep proof of efficacy and validation of safety against spontaneous mammary cancer in higher mammals *Summary assessment of canine clinical trials*		Complete	Y3PR
Major Task 3 Organize data to revise design of preclinical toxicology and Phase I/Ib clinical trial for future grant proposals			

Details can be found in the indicated progress reports (Y1PR: Year 1 Progress Report; Y2PR is Year Two Progress Report, etc.)

3.a.3 Outcomes Statement taken from Expansion proposal entitled *Expansion: Companion Diagnostic Signature for AFPep facilitates Personalized Treatment of Breast Cancer*
Outcomes from: BC 132567 Prevention and Treatment of Breast Cancer

(a) *Summary of the research funded by* BC 132567

The purpose of BC 132567 was to test the hypothesis that AFPep is safe and effective for prevention and treatment of breast cancer. Aim 1 documented the preventive efficacy and safety of AFPep. The data allowed us to conclude that AFPep prevents estrogen-induced breast cancer in ACI rats (just as it had prevented carcinogen-induced breast cancer in Sprague Dawley rats in earlier studies). From the ACI rat model, together with data from mice, dogs, and primates, we concluded that AFPep is extraordinarily safe: we reported a therapeutic index of > 1000, substantially above agents currently in use for treatment and prevention of breast cancer. In Aim 2 we measured blood levels of AFPep at effective doses in mice, dogs, and non-human primates. (Primate studies were supported by Albany Medical College, not by BC 132567.) Assessing PK data in mice, dogs, and primates, we concluded that an efficacious blood level of AFPep is approximately 0.1 µg/ml, and this blood level can easily be achieved in higher mammals with no evidence of toxicity. Based on a proof-of-concept clinical trial in dogs, we concluded that blood levels of AFPep 10 to 50 times efficacious levels in mice are easily achieved, well tolerated, and effective. We concluded that AFPep is safe and effective for the prevention and treatment of breast cancer.

(b) The Expansion Proposal is an <u>expansion</u> of the original project.

Historically, BCRP provided support in the form of 6 grants while support from other agencies included 7 additional grants. That support facilitated development of AFPep to the point that it is a candidate for advancement to clinical trials. In an FL3 proposal, we are competing for funding so that AFPep can enter FDA-mandated pre-clinical assessments under GLP conditions *en route* to obtaining an IND. That avenue constitutes the major line of work on this project. The Expansion proposal enlarges those efforts so that we will be able to define robust proteomic and genomic responses to AFPep; later we will develop an *in vitro* companion diagnostic tool that will navigate the FDA approval process simultaneously with AFPep. Given the funding constraints of the Expansion Award program, it is not likely that we can provide a fully validated companion diagnostic methodology sufficient to meet the specifications of the FDA; rather, the Expansion project is essential to define the signature that will be developed into the companion diagnostic.

(c) Research (i.) accomplishments and (ii.) outcomes from BC 132567

(i.) Accomplishments.

Prevention assessment: AFPep is effective for prevention of breast cancer in two independent rat models

- Low dose (25 µg/rat or 0.1 mg/kg s.c. or p.o.); short duration of treatment (one month, to mimic one pregnancy) is fully effective. AFPep is similar to tamoxifen in preventive efficacy

Therapy assessments: AFPep is effective for treatment of human ER+ breast cancer in two xenograft models

- Xenografted human MCF7 breast cancer tumors in mammary fat pad or sub-renal capsule. AFPep effective after 0.04 mg/kg i.p. administration. Fully active after s.c., p.o. administration

Quality and Stability of AFPep: AFPep has excellent drug-like physical and biological characteristics

- Accurate and reproducible synthesis of AFPep (commercial supplier ready for GMP synthesis)
- *In* vitro stability: Full activity following storage of lyophilized drug at -80 ^{o}C, -20 ^{o}C, +27 ^{o}C; and following storage for extensive time periods (weeks, months, years). Full activity following storage in saline or serum at room temperature for days
- *In vivo* stability: AFPep is not metabolized by liver cells or liver slices; intact AFPep but very little metabolite found during PK studies in mice, primates, dogs

Safety assessments of AFPep: AFPep is remarkably safe. Therapeutic Index of 1000 in rats.

- Extensive assessments including body mass (mouse, rat; short and long term), organ mass (rats, long term) following full body necropsy, blood chemistry, complete blood count, organ histological assessments (rats, long term), behavioral aspects. No liver toxicity, no effects on reproductive system
- Long term (months) administration to rats, intermediate (weeks) administration in mice and dogs, short term (hours or days) administration to mice, rats, dogs, primates
    - Extensive studies on liver: AFPep does not affect liver or liver cancer. No hepatic effects of AFPep using multiple models including long term administration to whole animals (rats), cell culture (two hepatic cell lines, one AFPep-secreting and one not secreting), and two cell lines in xenograft models

Pharmacokinetic assessments: Easy and safe to exceed efficacious blood levels in all species tested

- Developed mass spec assay for AFPep and metabolites to assess PK in mice, dogs, primates after administration by s.c., i.p., i.v., and p.o. routes. Efficacious blood level established as 0.1 µg/ml in mice, a level that is easily achieved (and exceeded) in higher animals (dogs and primates). $T_{1/2}$ in rodents, dogs, and monkeys: 11, 37, 86 minutes respectively
- Dose fractionation studies demonstrated once-daily treatment is sufficient to stop human breast cancer xenograft growth.
- Pharmacodynamic studies demonstrated that durability of effect of AFPep on biomarkers in breast cancer tissue lasts for at least 48 hours.

Biomarkers: Identified several proteomic biomarkers by western blotting that respond to AFPep, but also demonstrated that time course of response would pose challenges for a robust companion diagnostic. This led us to propose more robust genomic and proteomic approaches in this Expansion proposal.

Canine clinical trial demonstrated effective blood levels after s.c. administration, safety of AFPep, tumor penetration and biomarker response in 6/6 dogs. All dogs alive and healthy 5 to 10 months after trial.

Team members: Recruited 3 Consumer Advocates, 6 professional collaborators, 3 consultants for FDA interactions, and trained 8 students

Technology transfer: Entrepreneurship training at AMC's Biomedical Acceleration and Commercialization Center (BACC), won BACC award, developing Preventide (spin-out company to take AFPep and the companion diagnostic toward commercial development). Two patents issued.

Effect on Other Disciplines: Demonstrated therapeutic effect of AFPep against leiomyomata

(ii.) Outcomes.

FDA Type B meeting. The data amassed under BC 132567 allowed us to submit to the FDA a pre-IND meeting request to seek guidance on the clinical development of AFPep for the treatment of patients with ER+ breast cancer and for the prevention of breast cancer. The FDA convened a Type B meeting and provided detailed advice as to how to proceed with pre-clinical toxicology assessments, advised that we should accomplish assessments focused on therapy first, and invited us to return for a second Type B meeting to plan for prevention clinical trials when we have the data from the first assessments.

Publications: **Five publications** (listed elsewhere in the proposal) (plus one in preparation) were generated with support of BC 132567.

Patents: **Two** of the 9 patents protecting intellectual property associated with AFPep issued during this period.

3.a.4 Overall Impact of BC 132567 and associated efforts to develop AFPep.

AFPep accelerates progress toward "ending breast cancer": Preventative use of AFPep for a brief time has the very real potential to result in a dramatic reduction in incidence of breast cancer, and to do so without side effects. Primary prevention is essential to "end breast cancer." Therapeutic use of AFPep will not "end breast cancer" but will make treatment of the disease much more tolerable, more acceptable to patients, much more durable, and will not lead to uterine cancer. Two Overarching Challenges are met.

Moves beyond minor advance: These studies are beyond incremental in a drug development pathway. Studies in BC 132567 lead directly to FDA-required pre-clinical toxicology studies in a GLP-certified laboratory and IND preparation, which are major and essential steps to move AFPep to clinical trials. The next steps include pre-clinical assessments that are beyond the capabilities of most academic laboratories. Our non-GLP studies have de-risked AFPep such that positive outcomes of GLP studies are virtually assured to generate compelling data and justify investment in clinical trials. Having already conferred with the FDA through a Type B meeting, we can complete the necessary pre-IND GLP studies within one year. Emphasizing as it does the concept of *efficacy without toxicity*, this project is beyond incremental in both concept and content and represents a substantial and critical milestone. While some believe that all drugs are toxic, our data show that efficacy without toxicity is possible and can be applied to the effort to end breast cancer because AFPep is based on a molecule that is indigenous to the normal function of the human body, may be dosed at levels that are effective and yet below the physiological levels, and therefore safe. The next major steps (*i.e.*, FDA-required preclinical assessments and application for an IND, followed by Phase I trials) are highly likely to be positive: AFPep at expected effective doses is unlikely to be toxic in humans. If the mission is to develop safer, more effective therapeutics, AFPep must be considered a leading candidate.

Fundamentally better: For therapy of ER+ breast cancer, AFPep should be fundamentally better than use of current pharmacological agents such as tamoxifen or exemestane because: a.) AFPep is effective against tamoxifen-resistant ER+ cancers; b.) used in combination with tamoxifen, AFPep could mitigate tamoxifen-induced uterine hyperplasia and possible uterine cancer and could permit use of lower doses of tamoxifen without loss of efficacy; c.) used instead of tamoxifen, AFPep could eliminate all tamoxifen-induced side effects such as disruption of reproduction capabilities; d.) AFPep is likely to prevent the post-partum surge of breast cancer diagnoses. For prevention of ER+ breast cancer, no other agents to date have found appreciable acceptance for prevention of breast cancer. AFPep provides a substantial reduction in incidence of ER+ breast cancer in animal models.

In all likelihood, use of AFPep among various cohorts will follow this chronology:

Cohort	**Utility**	**Rationale** (based on our preclinical studies)	**Benefit** (over existing options)
Women whose ER+ cancer is, or has become, resistant to tamoxifen	Therapy	AFPep stops the growth of tamoxifen-resistant xenografts (MCF-7), as well as others (T47D, ZR75-1).	Prolonged healthy survival.
Women who are using tamoxifen	Therapy	a.) AFPep is at least as effective as tamoxifen in rats . b.) In combination with AFPep, lower doses of tamoxifen maintain efficacy, obviate toxicity, mitigate side effects of tamoxifen in rats and mice	Eliminate increased incidence of uterine hyperplasia and uterine cancer. Reduction in unpleasant side effects due to tamoxifen; mitigate tam resistance
Post-partum women	Prevention	The post-partum surge in breast cancer appearance may be prevented by AFPep	Brief use of AFPep after parturition may tip the balance of the post-partum growth environment toward reduction in breast cancer incidence
At-Risk women i) Young women ii) Women whose relatives have breast cancer	Prevention	Our animal studies indicate that the earlier AFPep is used, the greater is its prevention potential and that it is NOT necessary to maintain AFPep for extended duration in order to prevent breast cancer.	Reduction in incidence of breast cancer among the group of women currently experiencing the greatest increase in diagnoses. Empower women to reduce their diagnosed high risk of breast cancer. Better than 'watchful waiting'

3.b. What was accomplished under these goals?

3.b.1 Availability, quality and stability of commercial AFPep

For a molecule that may become a useful drug, it is essential to document the availability of a commercial source that can reproducibly synthesize the molecule, to show that the molecule produced by that supplier is pure and of the desired composition, that the molecule has the desired biological properties, and that the molecule is stable during storage. We documented that a commercial supplier, AmbioPharm, Inc. can produce large quantities of pure AFPep and can do so with repeated syntheses. We showed that AFPep produced by AmbioPharm is pure (using HPLC and mass spectrometry), and that it is identical to AFPep produced in our own laboratory. We documented repeatedly that commercial AFPep has the same bioactivity as that produced in our laboratory, and that AFPep is stable during storage for as long as we have monitored it (2 to 3 years). In addition, AmbioPharm can produce GMP-quality AFPep that we will use for pre-clinical assessments and for clinical trials. We have documented that commercial production of AFPep is fully satisfactory for continued progress on the drug development pathway.

3.b.2 Prevention of breast cancer

Prior to BC 132567 we had showed the efficacy of AFPep for prevention of carcinogen-induced mammary cancer. With support of BC 132567 we used a model of estrogen-induced mammary cancer, more similar to that found in humans. The August Copenhagen Irish (ACI) rat is a cross between the August and Copenhagen-Irish strains of rat. A number of investigators have used this strain of rat to assess breast cancer preventive potential of various agents. In Y1PR we completed a dose-response assessment and showed that very low doses of AFPep are sufficient for maximal preventive efficacy. That study indicated that 25 µg/rat yields optimal results.

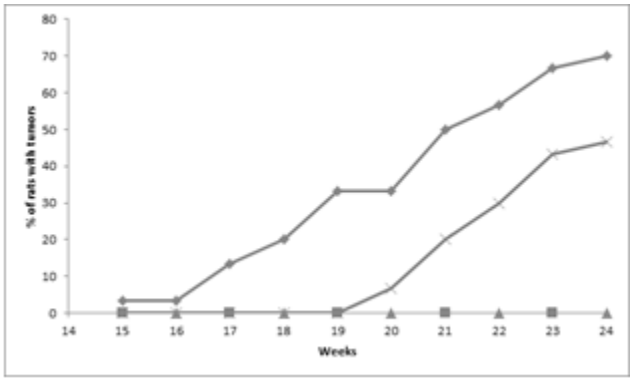
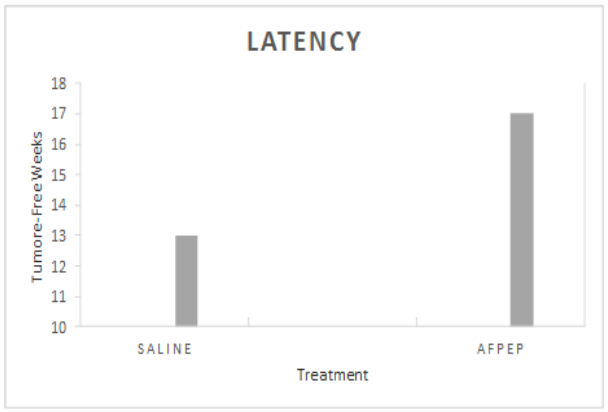


Figure 2. AFPep prevents estrogen-induced breast cancer. ACI rats received Silastic tubing implants containing 9 mg estrogen at Week 0. Untreated control animals (n = 30, diamonds) received s.c. injection of 0.2 ml saline, once daily for 4 weeks (5 days on, 2 days off) beginning on the day of estrogen 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. The difference in incidence between Untreated and AFPep-treated groups at 24 weeks is significant (p = 0.024, Fisher's exact) as is the impressive increase in latency.

We noted that AFPep did not provide full protection against breast cancer, but always provides an impressive increase in latency. Cancer delayed is cancer prevented, but it is also likely that some tumors do not respond to AFPep. This observation led us to the studies proposed in our Expansion Award application (see **3.b.9.*ii***).

In Y2PR we reported that brief duration of AFPep treatment is fully sufficient to achieve maximal protection. We showed that AFPep exposure for one month, mimicking the duration of a single pregnancy in rats (23 days), provides as much prevention as does AFPep administration for 3 months duration (representing 3 pregnancies). We documented further the powerful effect on latency as well as on reduction in tumor incidence (as shown in Figures 3 and 4).



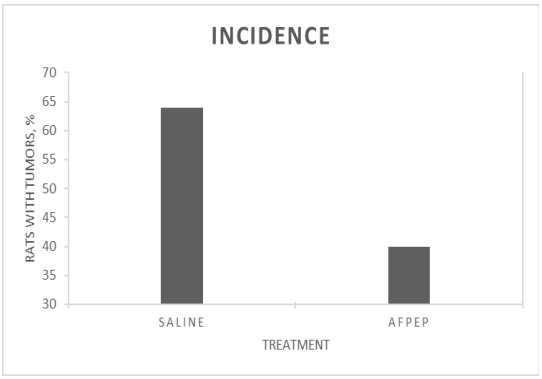


Figure 3. AFPep greatly enhances latency. **Figure 4. AFPep greatly decreases incidence.**
Data in the ACI rat model of estrogen-induced breast cancer show that AFPep is effective at reducing but not eliminating tumor incidence, and that AFPep is greatly effective at delaying onset of tumor appearance.

In Year 3, we showed that a one-month delay of onset of AFPep treatment results in prevention capability similar to the optimal treatment group. However, a two-month delay resulted in tumor incidence similar to the No Treatment group. This would suggest that there is a window in which prevention will occur, but that delayed use of AFPep may preclude maximal effectiveness. While it is difficult and probably inappropriate to extrapolate from animals to humans, the data suggest that earlier treatment is likely to be more impactful. The excellent tolerability of AFPep favors acceptability by patients as an early prevention strategy for breast cancer.

AFPep prevents breast cancer more effectively when treatment is started early

		Treatment				
Group #	**Purpose**	**Month 1**	**Month 2**	**Month 3**	**Months 5 - 7**	**% of rats with tumors**
1	Maximal tumors	Saline	Saline	Saline	Saline	68
2	Optimal treatment	**AFPep**	Saline	Saline	Saline	38
3	One-month delay	Saline	**AFPep**	Saline	Saline	42
4	Two-month delay	Saline	Saline	**AFPep**	Saline	64

It is our conclusion that AFPep is effective for the prevention of breast cancer. We have repeatedly pointed out that the data in support of AFPep is at least as strong as were the data that supported efforts to assess the prevention capability of tamoxifen when that drug was in pre-clinical assessments. In addition, we have reported and stress that AFPep is better tolerated than is tamoxifen (see **3.b.4**), and that AFPep mitigates some of the toxicities associated with tamoxifen when the two molecules are used in combination. It is our contention that AFPep should proceed to FDA-specified assessments and then to clinical trials for the treatment and prevention of breast cancer.

3.b.3 Therapeutic evaluation of AFPep

AFPep is designed to be a therapeutic agent as well as a preventive agent. We have documented the therapeutic capability of AFPep in several of our publications. **Figure 5** shows the efficacy of AFPep (100 ug/mouse) against human tumor xenografts. AFPep stopped the growth of human MCF-7 breast cancer xenografts growing orthotopically in the mammary fat pad of SCID mice. AFPep is not designed to be a cytotoxic agent that kills cancer and non-cancer cells. Rather, it is intended to be a stasis-inducing agent that keeps cancer in a suspended state indefinitely. The data show that AFPep is very effective at stopping growth of tumors.

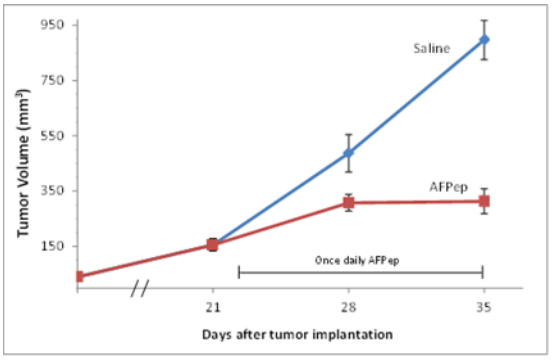


Figure 5: AFPep stops growth of palpable MCF-7 human breast cancer xenografts. MCF-7 human breast cancer cells (5 x 10^6) were implanted into the mammary fat pad of SCID mice. Tumors were on average 6.7 mm in diameter (156 mm^3) on day 21 after implantation. Either vehicle or AFPep (100 µg/mouse) was 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 (see Table 2) on day 35 after implantation. Mean ± SE of 3 replicate mice/group are shown.

Using the human xenograft models growing in mice, we had shown that AFPep is effective at stopping the growth of tamoxifen-resistant tumors, that it is effective after oral administration (as well as i.p., or s.c. routes), and that it can be used effectively in combination with tamoxifen or as a stand-alone agent. See also section **3.b.7** for efficacy in a dog model.

3.b.4 Tolerability of AFPep

It is of interest to compare the therapeutic index of AFPep and other agents, as shown in the accompanying table. Based on effective dose and lethal doses in rodent studies, it is clear that AFPep enjoys a very broad therapeutic index, certainly broader than agents currently in use for breast cancer. Efficacy without toxicity may be a major challenge, but it is possible, it is the demand of cancer survivors, and it is embodied in AFPep.

Therapeutic Index of Breast Cancer agents, based on rodent studies	
Agent	**Therapeutic Index** (reference)
AFPep	**>1000**
Tamoxifen	16
Exemestane	25
Faslodex	72
Herceptin	54
Gefitinib	15
Cyclophosphamide	3
Paclitaxel	4
Adriamycin	3

During BC 132567 and in earlier studies, animals of several species were treated with AFPep including a range of doses, durations, and routes of administration. The table below summarized a number of those studies, none of which had been intended as formal toxicity analyses. In none of those studies was there any indication that AFPep induced toxicity as monitored by any of the wide variety of parameters monitored. Although none of those studies were conducted as formal Irwin studies in rats such as would be utilized in the pre-clinical assessments required by the FDA, the sum of the earlier studies suggest that Irwin studies are highly likely to confirm that AFPep is well-tolerated.

Summary of Exposure studies

Species	**Number of Animals**	**Treatment Duration** Days	**Dose, Route** ug/animal/day	**Autopsy Date** Days after Treatment
Mouse	4000	1	1 – 10,000 i.p. or p.o	1
Mouse	300	30	10 – 100 i.p. or p.o.	1
Rat	1000	23	3 – 300 sc or p.o.	200
Rat	170	200	10 – 1000 sc	1
Dog	7	1	32,000 i.v. sc p.o.	None
Dog	6	7	10,000 s.c.	None
Mouse	5	5	2,000 i.v.	1
Mouse	5	1	10,000 i.v.	5
Primate	11	1	8000 - 100,000 i.v. sc p.o.	None

3.b.4.a AFPep does not affect liver.

AFP (alpha-fetoprotein, the parent protein of AFPep) is used as a biomarker for many birth defects. It has also been used as a biomarker for hepatocellular carcinoma despite reports [1, 2] that it is of very low sensitivity and very limited prognostic value in this regard. There are reports, relying primarily on a single cell line, of *intracellular* AFP being related to hepatocellular carcinoma (HCC). These observations may sometimes be conflated to suggest that AFP *causes* HCC, despite the notable observation that all infants are exposed to high levels of AFP *in utero* and all pregnant women are exposed to varying concentrations of AFP during pregnancy, and yet the prevalence of HCC is not pandemic. Whatever may prove to be the status of AFP as a biomarker for HCC, it became necessary to assess the potential of its active site analog (AFPep) on carcinogenesis of liver.

We reported that direct measurements of the effects of AFPep on intact liver, and on two hepatic cell lines growing *in vitro* or as xenografts *in vivo* indicated no toxicity due to AFPep. AFPep did not promote growth of normal liver; indeed, AFPep had no detectable effect on liver at all. In tissue culture, AFPep did not stimulate growth of HepG2 hepatic carcinoma cell line and only modestly affected SNU-182 cells. These same cell lines, growing as xenografts under the renal kidney capsule were not stimulated (or inhibited) by AFPep. The data suggest that AFPep does not affect hepatocellular carcinoma, regardless of what the parent protein may or may not do. The intent of the drug design process which led from AFP to AFPep was to eliminate all of the biological functions of AFP that were not related to inhibition of breast cancer growth. This appears to have been successful.

3.b.4.b AFPep does not affect estrous cycle.

We had documented that AFPep does not disrupt the estrous cycle of rats, does not reduce fertility, and does not engender birth defects in this species. The advantages over tamoxifen offered by these observations suggest that AFPep might find wide acceptance as a therapeutic agent, addressing the Overarching Challenge of "revolutionary treatment regimen that is safe, effective, non-toxic and durable".

3.b.5 Pharmacokinetics of AFPep

3.b.5.a PK in mice

Having completed the pharmacokinetic studies on mice in Year 1, and in dogs in Year 2, it is now possible to compare the outcomes of these studies. They bring forward an important concept.

AFPep was administered to mice and dogs at 3 mg/kg using the subcutaneous route. It is important to note that 3 mg/kg is 100 times above the minimum dose that inhibited growth of human breast cancer xenografts in mice. This dose was well tolerated in dogs as well as mice. The table indicates that the C_{max} in both mice and dogs is 60-fold above the minimum blood level that provided anti-tumor activity in mice. The $T_{1/2}$ and AUC are higher in dogs than in mice, suggesting that efficacious blood levels can be obtained and sustained in a higher mammalian species such as dog without any evidence of toxicity. These data gave us confidence to proceed to our canine clinical trial.

Pharmacokinetic parameters for AFPep in mice and dogs.

Parameter	Mouse	Dog
C_{max} (µg/µl)	6.4 ± 1.2	5.8 ± 0.3
T_{max} (min)	10	31 ± 5
$T_{1/2}$ (min)	19 ± 4	69 ± 17
AUC (min) (µg/µl)	207 ± 17	806 ± 133

3.b.5.a.*i*. Dose fractionation in mice

Data indicate that a C_{max} of 0.1 µg/ml and an AUC of 4.9 (min) (µg/ml) are needed for intended maximum biological effect. These data also provided target blood levels to be achieved as we translated AFPep into dogs. From our early studies, it was not clear whether C_{max} or AUC is the driver of the anti-proliferative effect of AFPep. To address this question, dose fractionation studies were carried out using the immature mouse uterine growth inhibition assay. The minimum dose of AFPep with maximum effect in the uterine growth assay was 1 µg/mouse as described above. This dose (D) was fractionated to $\frac{1}{3}$D or $\frac{1}{3}$D given at 5 hours, 2 hours, and 1 hour before stimulation with estradiol. As shown in **Figure 6**, the partial dose 0.33 D was less inhibitory than D as expected, whereas the fractionated dose 0.33 D x 3 was equivalent to a full dose (D) of AFPep. This suggests that AUC is at least as important as C_{max} in obtaining the maximum anti-proliferative effect of AFPep. This was important as we moved into dog studies since the half-life of AFPep is longer in dogs than in mice, leading to larger AUCs in dogs compared to mice.

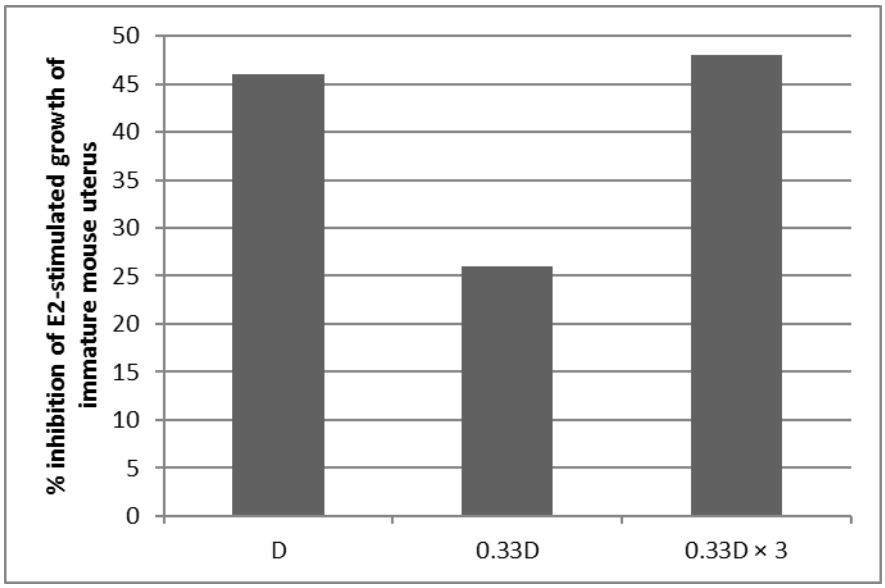


Figure 6. Fractionation of the full dose of AFPep yields full-dose anti-estrogenic activity. Immature female mice were injected i.p. with: **D**, the minimum dose of AFPep that yields maximum anti-uterotrophic activity when given 1 hour before stimulation with estrogen, or with **0.33D**, one-third of that dose, or with **0.33D × 3**, one-third of that dose given 5 hours, three hours and 1 hour before stimulation with estrogen.

3.b.5.b PK and PD in dogs.

For PK studies, normal beagle dogs weighing approximately 10 kg each were fasted overnight and injected with various doses of AFPep. For blood draws, their cephalic vein was cannulated with a 20-gauge catheter by the veterinary staff at Albany Medical College. AFPep (3 mg/kg, parenteral) (20 mg/kg oral via enteric capsules) was administered i.v., s.c., or p.o. Blood samples were collected at 5, 15, 30, 60, 120, 240 and 1440 minutes after AFPep administration. The experiment was repeated 3 times for each of the parenteral routes and four times for the oral route.

PK parameters of AFPep given to Dogs by the various routes of administration			
	I.V.	**S.C.**	**P.O.**
C_{max} (µg/mL)	77.7 ± 27.3	5.8 ± 0.27	0.085 ± 0.02
T_{max} (min)	5	31 ± 5	60 ± 16
$T_{1/2}$ (min)	37 ± 7	69 ± 17	39 ± 9
V_d (L)	1.4 ± 0.1	3.9 ± 0.5	1256 ± 440
AUC (min)(µg/mL)	1223.3 ± 202	806.2 ± 132.9	12.9 ± 2.5
Bioavailability %	--	65.6 %	1.05 %

In dose range finding studies in dogs, it was determined that 3 mg/kg of AFPep was needed by parenteral routes to achieve blood levels of AFPep comparable to those that were obtained in mice. The blood concentrations and PK parameters of AFPep in dogs following administration of AFPep by various routes are shown in the table above. The dose of AFPep given by parenteral routes readily yielded blood concentrations of AFPep that were more than enough to have anti-estrogenic and anti-breast cancer activity in mice (in the range of C_{max} at 7 µg/ml). The half-life of AFPep was longer in dogs than in mice (37 minutes versus 11 minutes). The bioavailability of AFPep by the subcutaneous route was quite high and comparable to what was found in mice. However, the bioavailability by the oral route was lower than expected compared to mouse studies. The p.o. data in the table above represent values obtained following administration of a dose of AFPep that was escalated to 20 mg/kg and given in enteric capsules. Nevertheless, at this 20 mg/kg p.o. dose in dogs, C_{max} (0.085 µg/mL) and AUC (12.9 min ug/mL) approximated those that were obtained p.o. in mice (0.14 µg/mL; 4.9 min µg/mL), suggesting that effective levels of AFPep can be achieved safely by the oral route in dogs.

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 Index 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 autoplatelets, 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, ALP, ALT, AST, and GGT). A few of those parameters are tabulated here; there were no changes due to AFPep in any of the parameters measured.

Typical Renal-related Blood Chemistry Values in Dogs treated with AFPep

Marker	Reference Value	Pre-Treatment	Post-Treatment
	IU/L	IU/L	IU/L
Alanine Transaminase	18 - 121	43 ± 7	36 + 6
Aspartate Aminotransferase	16 - 55	30.2 ± 2.6	19.3 ± 1.9
Creatine Kinase	10 - 200	234 ± 48	141 ± 21
Gamma-glutamyl transferase	0 - 13	4 ± 0.4	7 ± 2
Albumin	2.7 - 3.9	3.30± 0.2	3.3 ± 0.2
Total Bilirubin	0 - 0.3	0.1 ± 0	0.09 ± 0

The PK studies in dogs enabled us to establish a rational starting dose of AFPep at 1 mg/kg, s.c., for the clinical study in dogs presenting with mammary cancer (see **3.b.7** below). Due to the excellent tolerability of AFPep, this 1 mg/kg starting dose could likely be utilized in planned future human studies once it is validated in FDA-sanctioned preclinical toxicology studies.

3.b.5.c PK in primates

As described in Y1PR, we had a unique time-sensitive opportunity to study AFPep in monkeys at no cost to the DOD-BCRP grant. The monkey work was funded intramurally. We started with the high end of effective mouse doses (4 mg/kg) and converted this to equivalent monkey dose (1 mg/kg). This dose was administered using the intravenous or oral routes. Monkeys (*Macaca mulatta*) were sedated and anesthetized using atropine, ketamine, Midazolam, isoflurane and nitrous oxide. The left cephalic vein was catheterized and used for fluid balance, i.v. administration of AFPep, and for obtainment of blood samples. Oral administration of AFPep was through a gavage tube inserted through the mouth, esophagus, and into the stomach. Blood was obtained at multiple time points after AFPep administration. Plasma was derived and assessed for AFPep levels. Data represent the mean AFPep level $\pm$ standard error in three replicate studies.

The i.v. route yielded an average C_{max} of 7 µg/mL and an area under the curve of 254 min*µg/mL. These values are within the efficacious blood level range that we reported earlier for immature mice. There was no evidence of toxicity for the primates. The $t_{1/2}$ of AFPep was 68 min which is substantially longer than that found in mice. Very low and inconsistent blood levels of AFPep were found following oral administration of AFPep to monkeys (less than 0.1 ug/mL).

The dose of AFPep was escalated to 4 mg/kg in monkeys. The C_{max} from this dose i.v. was 14 µg/ml and the AUC was 1574 min*µg/ml. Again, no evidence of toxicity was detected in the monkey after this dose of AFPep. We then tried the s.c. route at 4 mg/kg and found effective blood levels and very good bioavailability (~90 %). When this 4 mg/kg dose was administered orally to monkeys, consistent but low blood levels of AFPep were observed; they were well below the expected efficacious blood level of 0.9 µg/ml. However, the persistence of orally administered AFPep in the monkey circulation is quite impressive.

Table 11: PK parameters of AFPep (4 mg/kg) given to Monkeys through various routes of administration

	i.v.	***s.c.***	***p.o.***
C_{max} (ug/mL)	*13.7*	*8.2*	*0.03*
T_{max} (min)	*5*	*38*	*214*
$T_{1/2}$ (min)	*107*	*27*	*810*
V_d (L)	*3.7*	*2.9*	*3.9*
AUC (min)(ug/mL)	*1574*	*1407*	*30.9*
Bioavailability %	*---*	*89*	*1.9*

3.b.6 Biomarkers and pharmacodynamics of AFPep

3.b.6.a Biomarkers

We invested substantial time and effort to establish the ability to monitor biomarkers of response to AFPep. When AFPep goes to clinical trials, it will be necessary to measure biomarkers of response, as it is not feasible to wait for tumors to grow, shrink, or develop in therapy or prevention studies. Blood levels of the drug known to be efficacious is an important parameter and has been monitored and reported in our recent publications. Molecules that respond to treatment with AFPep can be assessed by western blot analysis and used as reporters to show that AFPep is hitting its intended target (*i.e.*, tissues such as breast or uterus that respond to AFPep) and having the intended effect of growth inhibition. These studies proved to be quite complex and we found it necessary to begin studies with tissues that are simpler and easier to procure than are xenograft tissue. We developed biomarker response data in *i*.) normal estrogen-responsive tissue (mouse uterine tissue) responding to AFPep, *ii*.) human tumor xenografts (MCF-7) growing in mice, and *iii*.) spontaneous tumors from a canine clinical trial.

3.b.6.a.*i* Anti-uterotrophic activity of AFPep in normal tissue. We have frequently reported on the use of the immature mouse uterine growth inhibition assay that we use to monitor the anti-estrogenic activity of AFPep. The anti-estrogenic activity closely parallels the anti-cancer activity, but the uterine assay is much quicker and easier to perform than are mammary gland-based assays. 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 ± uterine weights and western blot band densities were calculated. Differences were assessed using Tukey's tests. * $p < 0.05$.

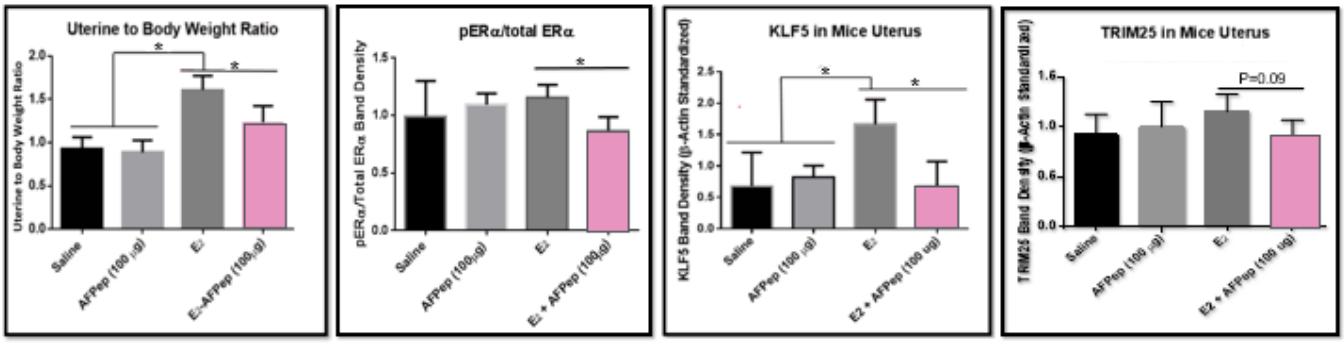


Figure 7. AFPep attenuates estrogen-induced responses in mouse uterine tissue.

As shown in the first panel of the figure above, AFPep has no effect on basal mouse uterus, whereas estrogen markedly stimulates growth of mouse uterus. AFPep mitigates the estrogen-stimulated growth of the uterus. This is the first time we have reported that AFPep has no effect on basal uterus; it speaks to the targeted effects of AFPep and to the tolerability and probably acceptability of this agent.

Tissues from these studies were used in western blot analysis to monitor several biomarkers, including ER, pER, KLF5, and TRIM25 among others. The next panels in the figure report results for the biomarkers. Monitoring these intracellular biomarkers yields the same result as does monitoring the gross effect on the intact uterus: AFPep does not affect biomarkers from the basal uterus, estrogen stimulates the uterine biomarkers, and AFPep mitigates the biomarker response to estrogen stimulation.

3.b.6.a.*ii* Anti-breast cancer activity of AFPep in human xenografts. Pieces of MCF-7 human breast cancer tissue were injected into the thoracic mammary fat pad of SCID mice. Mice were supplemented with estrogen implants. Tumors were on average 6.7 mm in diameter (156 mm^3) on day 21 after implantation 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 three replicate mice/group are reported. * $p < 0.05$

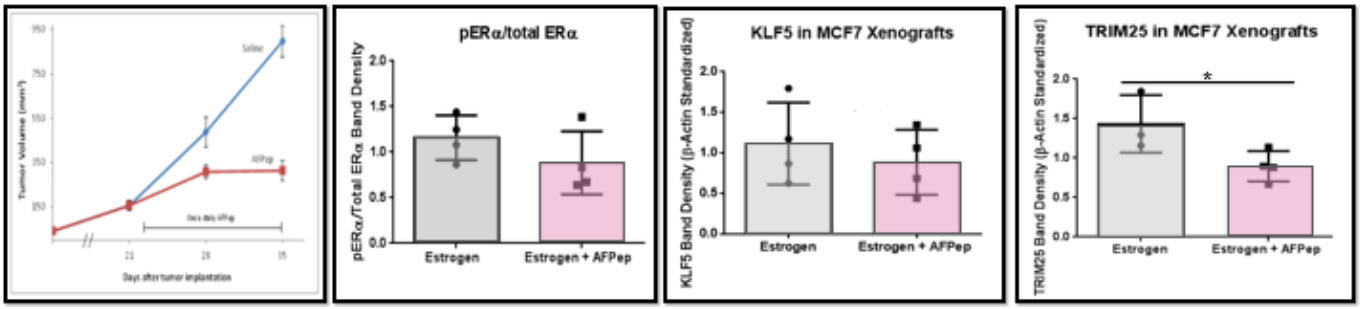


Figure 8. AFPep attenuates estrogen-induced responses in human breast cancer xenografts.

As we moved into the more complex *in vivo* models of human breast cancer xenografts, we continued to see an AFPep-induced inhibition of proliferation in MCF-7 human breast cancer xenografts. The first panel in the figure above indicated that AFPep arrested the growth of MCF-7 human tumor xenografts growing in mice. At the end of the experiment tumor tissue was harvested for biomarker analysis. The biomarker results indicate that AFPep inhibited phosphorylation of ER α at Ser 118, inhibited KLF5, and inhibited TRIM25, just as it did in the normal estrogen-responsive (uterine) tissue.

3.b.6.a.*iii* Anti-breast cancer activity of AFPep in spontaneous canine mammary tumors. Biopsy samples from canine breast cancers (see **3.b.7 Canine Clinical Trial**, below) were processed for western blot analysis to yield the data shown in the figure below. Just as in normal estrogen-responsive tissue and in human estrogen-responsive xenografts, canine tumors that are estrogen-receptor positive responded to AFPep with decreased expression of pER, KLF5, and TRIM25. In this study, Dog 1 was not ER+ (first panel) and did not respond to AFPep (panels 2 and 3). Dog 2 was ER+ (first panel), and responded to AFPep as did the normal tissue and ER+ human breast cancer xenografts (panels 3 – 6). The other four dogs in this trial exhibited ER- cancer, similar to Dog 1.

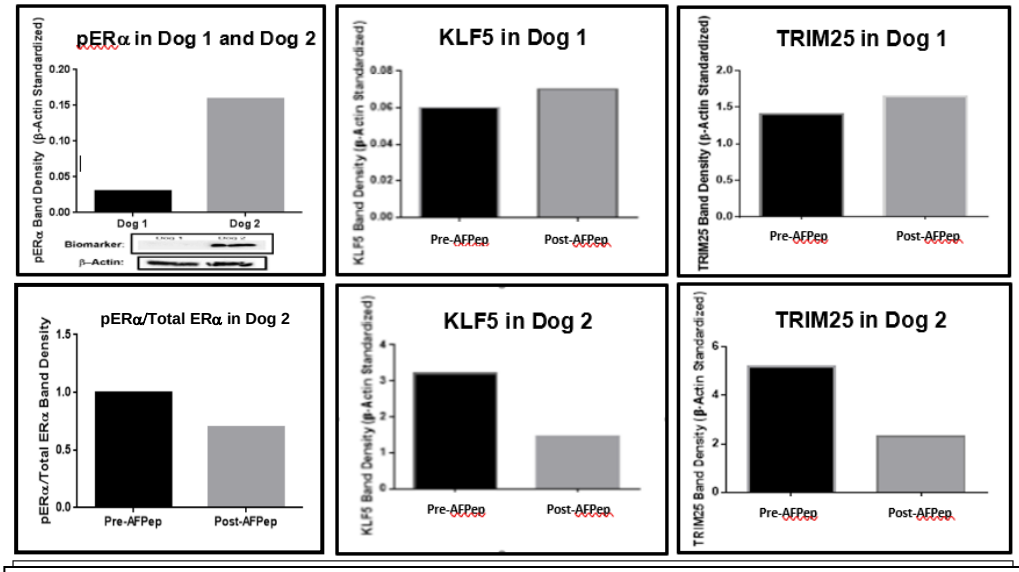


Figure 9. AFPep attenuates estrogen-induced responses in ER+ canine breast cancer.

3.b.6.b Pharmacodynamics

To address concerns about the relatively short half-life of AFPep in mice, which contrasts the time-insensitive pharmacodynamics of AFPep in mice, we undertook the study whose experimental design is evident from the table below. To assess the pharmacodynamics of AFPep, we identified a panel of biomarkers which are useful for assessing the time course of effects of AFPep, since it is not feasible to wait for macroscopic evidence of change in tumor size (therapeutic interventions) or incidence of tumors (prevention interventions). Once-daily treatment with AFPep of orthotopically established human MCF-7 breast cancer xenografts over a 14-day interval initially slowed and then completely stopped breast cancer growth. Tumors were biopsied at various times after cessation of AFPep treatment and assessed for biomarkers shown here. Data were compared to MCF-7 breast cancer xenograft biopsies harvested concomitantly from tumor-bearing mice treated once daily with injections of saline. One hour after the last AFPep treatment, the biomarkers indicated that AFPep induced a reciprocal ERα/ERβ decrease/increase, a decrease in PCNA, and increases in Rb and p21. This suggests that the AFPep-induced ER perturbation signaled a delay in progression of tumor cells through the cell cycle leading to a cessation of further tumor growth. Two major aspects can be deduced from this study: ***a.)*** The study shows definitively that once-daily treatment with AFPep is more than sufficient to stop tumor growth or prevent tumor appearance. In fact, the data suggest that even less-frequent dosing intervals (as indicated by pharmacodynamic studies), rather than more frequent intervals (as suggested by pharmacokinetic studies) would be rational to explore in future studies. ***b.)*** The time-dependency of the biomarkers suggests that they would serve nicely to assess mechanism of action of AFPep (which we have not defined in detail), but that these biomarkers are NOT a good choice for a companion diagnostic tool. For such purposes, it would be better to have a stable, robust response that would be independent of time after onset or cessation of treatment with AFPep. For this reason, the Expansion proposal that we submitted (see **3.b.9.*ii***) focuses on stable, robust genomic and proteomic responses. In addition, genomic and proteomic responses should be easier to disseminate across multiple laboratories than western blot approaches.

Durability of Biomarker Response after AFPep Treatment

	Time after last AFPep Treatment				
	1 hr	24 hr	48 hr	72 hr	96 hr
Biomarker	**% Change compared to No AFPep level**				
ERα	-61	-38	-49	-11	-2
PCNA	-62	-40	-24	-18	-12
ERβ	+170	+48	+58	+23	+24
P21	+40	+40	+66	+21	+21
Rb	+137	+88	+113	+42	+59

Mice bearing orthotopic MCF-7 human breast cancer xenografts were treated with AFPep as described in the legend to Fig. 4. After 14 days of once-daily treatment, tumor tissue was biopsied at 1 hr, 48 hr, 72 hr, and 96 hr after cessation of AFPep treatment. Biomarkers were assayed by Western blot. Change in band density in tumor tissue from AFPep-treated mice relative to control (saline-treated) mice is reported.

3.b.7 Canine clinical trial

Progress in Years 1 and 2 were crucial in our design of the canine clinical trial. In those years we learned:

- How to measure AFPep blood levels in mice, rats, dogs, and monkeys
- That AFPep was well-tolerated in 14 normal dogs in dose escalation studies that yielded AFPep blood levels 100-fold above the minimum level needed for anti-estrogenic activity
- That AFPep is orally bioavailable in dogs. However, to achieve effective blood levels by the oral route in dogs would have required high doses of AFPep that were not consistent with the budget constraints of the grant. Therefore, the subcutaneous route of administration was used for AFPep in the canine clinical trial. This route enabled lower dose; for convenience we used 10 mg/day regardless of animal weight.

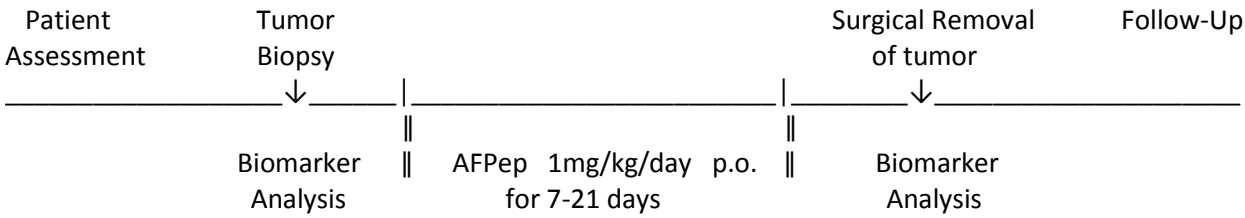
In Year 3, we had sufficient funding for only 6 animals but this was sufficient for us to accomplish our objectives for the canine clinical trials, and we learned a great deal more than we had anticipated. The purposes of the canine trial were to demonstrate that we could measure AFPep blood levels, tolerability, and biological response in a model of spontaneously arising breast cancer tumors. Few other models of spontaneously arising tumors exist, so the dog model seemed quite appropriate. In collaboration with investigators at the Animal Medical Center in New York City, we conducted a proof-of-concept clinical trial of AFPep. Our intent was to establish blood levels of AFPep, monitor tumor biomarkers in response to AFPep treatment, and assess tolerability of AFPep. The intent of our New York City colleagues was to assess AFPep as a potential treatment for canine mammary cancer. Patient accrual was not problematic; dogs seem to be available to the Animal

Medical Center in New York City in numbers sufficient to accommodate clinical trials larger than this one. Patient compliance was convenient in that dog owners, after training by veterinarians at the Animal Medical Center, had the means to inject their own animal. All enrolled dogs completed the trial. All dogs tolerated AFPep (and notably, cannot tolerate tamoxifen). All dogs obtained blood levels of AFPep that were in the intended range. All dogs yielded detectable biomarker responses.

The following is a summary of the protocol of the clinical trial, diagrammed below:

1. Canine patient presents with mammary cancer. Benign or malignant, tumor > 2 cm; animal life expectancy > 3 months. (Exclude for major organ dysfunction, respiratory compromise)
2. Blood sample for baseline CBC and blood chemistry. One sample taken prior to AFPep; one sample taken 30 min after administration of AFPep
3. Obtain informed consent from owner
4. Tumor is biopsied and analyzed for appropriate biomarkers
5. Patient is treated once daily with 10 mg/day of AFPep s.c. for a minimum of 1 week up to time of surgery.
6. Tumor is surgically removed. Blood sample obtained for AFPep blood level, CBC, chemistry
7. Tumor is analyzed for biomarkers

Clinical parameters were analyzed throughout to assess well-being of patients. Pre- and post-AFPep tumor samples were compared to determine whether AFPep was hitting its intended targets and changing the proliferative status of the tumor.



Protocol for clinical trial of AFPep in dogs. This protocol was used to guide clinical trials at the Animal Medical Center in New York city. This is the model proposed for future human studies.

Six dogs were enrolled in the canine clinical study. This table shows their breeds, spay status, body weights and tumor pathology status. The mix of dogs and tumor pathologies were quite heterogeneous, which was desirable for this proof-of-concept clinical trial and was to be expected in a busy veterinary medical center.

Animals enrolled in canine clinical trial, together with tumor pathology status

Dog	Breed	Sexually Intact[1]	Body Weight (kg)	Tumor Pathology
1	Pomeranian	Y	7.98	Simple tubular adenocarcinoma**, grade II**
2	Yorkie	Y	5.68	Simple tubular adenoma
3	Poodle Mix	N	5.8	Adenoma
4	Pit bull	N	30.2	Complex adenocarcinoma, grade I
5	Shih tzu	Y	11.02	Tubulopapillary adenocarcinoma, grade II
6	Chihuahua mix	N	7.53	Mixed carcinoma, grade II

[1] "Sexually intact" refers to whether the animal had been spayed or not. Spayed animals are not (N) sexually intact.

Dogs with mammary gland tumors larger than 2 cm in diameter were eligible to enroll. All dogs underwent complete clinical staging. Tumors were biopsied prior to administration of AFPep (*i.e.*, on Day 0) and after repeated daily dosing of AFPep (*i.e.*, on Day 8). Dogs were administered AFPep 10 mg subcutaneously once daily for 8 consecutive days. Animals were injected on Days 1 and 8 by a veterinarian at the Animal Medical Center in New York City and by their owner on Days 2-7. Blood levels of AFPep were measured before and 30 min following AFPep administration on Days 1 and 8. Note that we had shown in Y2PR that 30 minutes is T_{max} for s.c.-administered AFPep.

This table shows that C_{max} blood levels of AFPep in all dogs were found to be in the range of 2 µg/ml which was well above the minimum blood level (0.1 µg/ml) needed for biological activity in rodents. In separate studies in rodents, 2 µg/ml AFPep significantly inhibited the development, outgrowth and progression of mammary gland tumors. These C_{max} blood levels in the canine clinical study were 20-fold above the minimum blood levels needed for biological activity and were levels that we expected from studies reported in Y2PR. We intentionally used a dose of AFPep in this canine study that would achieve these high blood levels in order to produce results that emphasize the safety of AFPep and to be sure that there were more than sufficient blood levels on board in the canine patients to produce a change in biomarkers in the tumor tissue itself.

AFPep Concentration in Dog Blood

	Day 0		Day 8	
Dog	Pretreatment	Posttreatment	Pretreatment	Posttreatment
	ng/ml	ng/ml	ng/ml	ng/ml
1	1.2	2400	0	2100
2	0	2200	0	1900
3	0	4400	8	5000
4	2.8	9000	0	1200
5	0	800	0	1200
6	0	2500	0	3300
Mean $\pm$ S.E.	0.7 $\pm$ 0.5	3600 $\pm$ 1200	1.3 $\pm$ 1.3	2500 $\pm$ 600

Histopathology identified two adenomas and four carcinomas. Eight-day repeat dosing of AFPep was well tolerated in 6/6 dogs as measured by Complete Blood Count (including white blood cells, red blood cells, platelets, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and % neutrophils, lymphocytes, monocytes, eosinophils, and basophils), all blood chemistry parameters and clinical observations (including heart rate, respiration rate, O_2 saturation, rectal temperature, capillary refill time, and mucous membrane color).

Since there were only six animals in this proof-of-concept study, and because the tumors in the dogs were of various types and pathologies, the values reported here are not statistically significant, but they do indicate that AFPep is sufficiently penetration the tumor tissue and that it is possible for us to monitor response to treatment

with AFPep using these biomarkers. This is considered an enabling observation for the Phase I trials contemplated in the near future.

In summary, our proof-of-concept clinical study of AFPep in dogs presenting with mammary tumors was highly informative:

- We confirmed previous data that AFPep is an extremely well-tolerated drug in higher mammals. Its therapeutic index appears to be unprecedented.
- New from this study was the tolerability of AFPep after repeat dosing. This study included 8-day repeat dosing of high levels of AFPep administered subcutaneously, which resulted in no adverse effects.
- Effective blood levels of AFPep can be achieved following parenteral administration and can also be achieved after oral administration with appropriate dose escalation.
- AFPep changes the biomarker status of a heterogenous array of canine mammary tumors.
- These studies will serve as a guide when it comes time for the FDA-required preclinical toxicology studies and the first-in-human dosing studies.

There are several confounding issues that became apparent in this canine clinical trial, such as:

- Whether and when the dog had been spayed
- A smaller percentage of canine mammary tumors are likely to be ER+ when compared to human breast cancer tumors
- There is a high level of cellular heterogeneity within canine tumors including benign, malignant, fibrous, fatty and necrotic elements (all in one tumor)
- The endocrine status of dogs is obviously quite different from humans, which leads to very different responses to endocrine therapy as evidenced by the poor tolerability of tamoxifen in dogs.

This study achieved its intended objectives and will inform human clinical trials in the manner intended when we plan that work. It became clear to us that the canine model is not really a useful model for detailed studies of breast cancer treatment or prevention. The observation that half of the animals were not sexually intact was an unexpected confounder; the literature suggests that dogs that get spayed early do not develop mammary cancer. The heterogeneity of tumor types, although desired, was greater than expected. The estrogen receptor status of the tumors was difficult to assess. We conclude that it would be too costly to use dogs in future studies, and that too few of them would be ER+.

Milestones achieved

- Efficacy of AFPep demonstrated by biomarker response in uterine bioassay, human tumor xenografts, and canine spontaneous tumors
- Tolerability of AFPep demonstrated in canine clinical trial with 8-day repeat dosing
- Pharmacokinetic analysis demonstrates that canine blood levels surpass the levels shown in mouse models to provide anti-cancer activity through all routes of administration including the oral route.

3.b.8 Meeting with the FDA.

All of the studies related to AFPep indicate that the molecule should proceed toward clinical assessment. The next steps in the drug development pathway (after discovery and characterization) entail FDA-mandated pre-clinical toxicology assessments, continue with application for an Investigational New Drug (IND), and culminate with entry into clinical trials. Preparing for that process, we requested of the FDA a Type B meeting to refine their guidance with respect to what would be required during pre-clinical studies for both prevention and therapy indications.

To prepare for the meeting with the FDA, we gathered our Consumer Advocates, Albany Medical College Investigators, Consultants from Compliance Resources, Inc., technical staff, and our students in training. We developed a detailed package of data together with an extensive list of questions for the FDA in order to gather their advice as to how to structure pre-clinical studies and Phase I trials. The FDA The Type B meeting (PIND # 135846) was very helpful, and we used that guidance to prepare an FL3 proposal.

The FDA is guiding us toward pre-clinical toxicology assessments and a Phase I clinical trial for the therapy indication for AFPep. We have established that the FDA-mandated pre-clinical assessments necessary to lead to an IND for therapy are manageable and could be accommodated by the allowable budget of an FL3 proposal. In a rarely seen occurrence, the FDA actually invited us to return for a second Type B meeting for the prevention indication (which could include healthy human volunteers) after we accumulate data in human cancer patients in an initial Phase I clinical trial developed for the therapy indication. A motivating factor in this discussion was the attractive tolerability of AFPep which could translate to excellent acceptability and use in at-risk patients. There are no regulatory barriers to bringing AFPep forward for clinical trials, and eventual clinical utility if it proves efficacious in Phase II and III studies. The robust Team that we have assembled (see **3.b.10** below) will work to achieve the pre-clinical studies (hopefully, to be funded by FL3) and the clinical trials (hopefully, to be funded by FL4). We note that there are not many academic research teams that could take a molecule all the way from discovery through to clinical trials, but we are very well positioned to do so.

3.b.9 Submission of additional grant applications.

All of the data amassed to date relative to AFPep indicate that the molecule should proceed to pre-clinical toxicology assessment and clinical testing. The next logical step beyond the current funding is to apply for an FL3 opportunity to obtain the FDA-mandated preclinical assessments. We submitted an FL3 application highlighting the prevention prospects and the therapy prospects for AFPep. We also submitted an Expansion Award proposal to develop a companion diagnostic tool to accompany AFPep through clinical trials and report on its efficacy. Overviews of both the FL3 proposal and the Expansion Award proposal are included here.

3.b.9.*i* FL3 Application entitled: ***FDA-prescribed pre-clinical assessment and IND for AFPep: Prevention and Treatment of Breast Cancer***

Overarching Challenges. This proposal offers Breakthrough solutions for two overarching challenges, namely (a) a revolutionary treatment regimen that is safe, effective, non-toxic and durable, and (b) primary prevention of breast cancer. AFPep is a first-in-class growth regulatory molecule shown, in rodents, to stop growth of human breast cancer xenografts and to prevent development of estrogen-induced or carcinogen-induced mammary cancers. Well tolerated even after escalation to 1000 times its effective dose, AFPep stops growth of multiple xenografted human breast cancer cell lines, including tamoxifen-resistant cell lines. These outcomes were achieved because AFPep is a mimic of the active site of α-fetoprotein (AFP), a naturally occurring protein of pregnancy that contributes importantly to the lifetime reduction in risk of breast cancer that occurs as a result of pregnancy and that has demonstrable anti-estrogenic activity in multiple experimental models.

Therapy: Pharmacological treatment of ER+ breast cancer has been very effective but is accompanied by side effects and substantial toxicities, and frequently fails due to acquired resistance. Patients with ER+ tumors have a consistent long-term risk of death and a greater risk after 7 years (*Landscape*), and most breast cancer deaths occur in ER+ women. It is important to prioritize development of an orally available, well tolerated therapeutic agent that would benefit the patients who account for 75% of breast cancer cases. Used alone, AFPep could obviate the toxicities associated with tamoxifen altogether; used in combination with tamoxifen, AFPep could allow for lower doses of tamoxifen and could mitigate the uterotrophic side effect induced by tamoxifen.

Prevention: Prevention of breast cancer is possible, but it is fraught with substantial challenges. Bilateral mastectomy prevents breast cancer, but we need to develop better options. Multiple pregnancies can prevent breast cancer, but only with lifestyle-altering consequences. Pharmacological prevention occurs with tamoxifen or other agents, but only with worrisome levels of toxicity and concerns about the attendant risk of engendering acquired resistance. Toxicity and acquired resistance have been the restrictive risks associated with chemoprevention of cancer. The Breakthrough that is needed to realize the CDMRP's mission "to end breast cancer" is the development and translation of a chemopreventive agent that is potent, safe, effective, and does not engender resistance. This proposal offers such a molecule.

Objective. The BCRP of the CDMRP has supported development of AFPep from its conception and has invested in AFPep through several funding opportunities, including current Breakthrough support. The research program has produced steady progress, and none of the usual indicators of concern for drug development have been encountered. AFPep is now ready to move toward clinical trials, dependent only on obtaining a license for an Investigational New Drug (IND) from the Food and Drug Administration (FDA). **The objective of this FL3 proposal is to carry out extensive GLP preclinical toxicology studies** required for an IND application**.** Based on the data discussed above, AFPep has been thoroughly de-risked, and it is highly likely that the GLP preclinical toxicology data proposed herein will support an IND.

Specific Aim. There is one Specific Aim.

- Aim 1: Employ an array of FDA-prescribed pre-clinical GLP studies in rats and dogs to define the tolerability of AFPep and provide a safe first-in-human starting dose
    - Obtain GMP-quality AFPep in kilogram quantities from a commercial supplier.
    - Work with a Contract Research Organization (CRO), employing Good Laboratory Practices (GLP), to complete the battery of studies required by the FDA.
    - Develop a briefing package to support the IND application at the FDA

We have assembled a robust Team of investigators, clinicians, regulatory consultants, consumer advocates and contract research organizations (CROs) to accomplish this work. Everything is in place to achieve the stated outcomes of this project which will complete FDA-prescribed preclinical toxicology assessments and allow us to obtain a license for an Investigational New Drug (IND), leading thereafter to first-in-human Phase I trials.

3.b.9.*ii* Expansion Award proposal entitled: *Expansion: Companion Diagnostic Signature for AFPep facilitates Personalized Treatment of Breast Cancer*

Overarching Challenge. This proposal focuses on the Overarching Challenge of conquering the problem of over-treatment of breast cancer. Patients do not wish to use drugs when effectiveness is uncertain or when side effects occur. For many drugs there are individuals who respond better than others, individuals that do not respond to treatment at all. Many drugs lose their effectiveness over time, and it is wasteful and perhaps harmful to continue to use such agents. AFPep is a first-in-class growth regulatory molecule shown, in rodents, to stop growth of human breast cancer xenografts and to prevent development of estrogen-induced or carcinogen-induced mammary cancers. Well-tolerated even after escalation to 1000 times its effective dose, AFPep stops growth of multiple xenografted human breast cancer cell lines, including tamoxifen-resistant cell lines. Nevertheless, there are indications in our data, and suggestions from reviewers of our proposals, that perhaps some of the animals in our studies were not responding to AFPep. If there are women who do not respond to AFPep, it should not be used, even though it is extraordinarily safe. This pre-clinical project focuses on defining a diagnostic signature of genomic and proteomic responses to treatment with AFPep. This proposal is an Expansion of BC 132567 for which the Overarching Challenges were a.) treatment regimens that are safer and more effective, and b.) primary prevention of breast cancer. Those challenges are met effectively by AFPep.

Objective and Hypothesis. The **objective** is to define the companion diagnostic signature of AFPep by identifying proteomic and genomic responses of ER+ breast cancers to AFPep. This proposal will not develop the actual companion diagnostic tool because the effort to validate the actual tool will require extensive

investment and time. By defining the signature (*i.e.*, a sensitive, specific and robust array of genomic and proteomic signals), we lay the groundwork that will lead to validation of the tool (which will occur under FL4 support in the future). **The hypothesis of the Expansion Award proposal is that AFPep stably induces specific genomic and proteomic responses in ER+ breast cancer models**. We will test this hypothesis with two specific aims, employing increasingly complex models of breast cancer including:

a. A panel of cell types in culture will allow us to assess a broad *diversity* of breast cancers

b. Xenografts of selected cell types (including PDX) will offer the challenge of greater *heterogeneity* of tumor specimens (compared to cell culture work), essential for development of a robust companion diagnostic

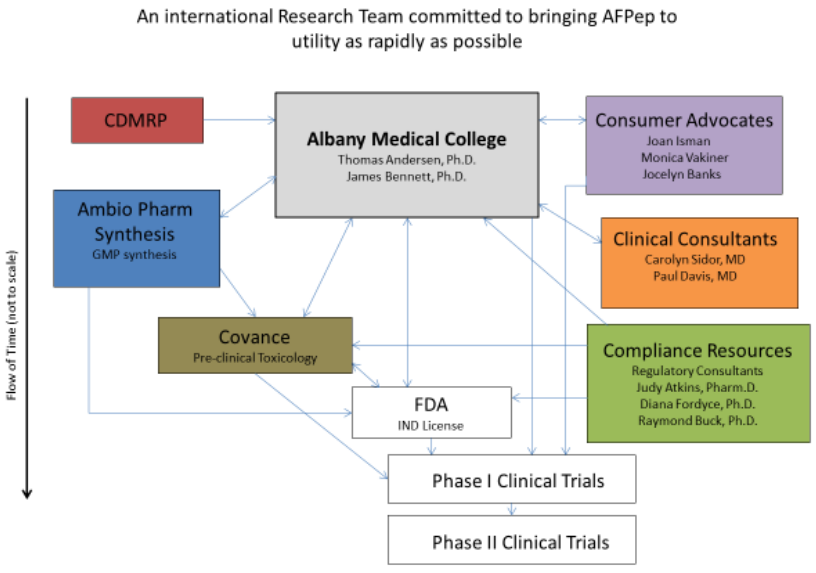
c. Whole animal models of spontaneously arising breast cancer offer *heterogeneity* of sample comparable to clinical specimens.

Aim 1 is to assess genomic responses to AFPep using array-based techniques. **Aim 2** is to assess proteomic responses to AFPep using mass spectrometry approaches.

3.b.10 Research Team.

During Years 1 through 3 of BC 132567 we assembled a robust, international research team with expertise in all areas necessary to achieve the goals of this project and to prepare for the next projects (the FL3 project and the Expansion Award project). With support from **CDMRP**, the AMC scientists developed AFPep, beginning with the early epidemiological studies of **Herbert Jacobson** who proffered the idea that increased levels of AFP during gestation correlate with subsequent reduction in risk of breast cancer. That epidemiological basis, coupled with drug design (**Thomas Andersen**) and pharmacology (**James Bennett**) expertise, led to the development of AFPep. In recent years, Dr. Bennett championed the therapeutic avenue while Dr. Andersen led the prevention avenue of investigations. Additionally, both Dr. Bennett and Dr. Andersen have extensive experience in overseeing diverse, inter-institutional collaborative and administrative activities and are well qualified to champion the continued development of AFPep. The **Albany Medical College** is fully supportive of this research program; the College constitutes an outstanding base from which to interact with our collaborators from around the world as we endeavor to bring AFPep through the final stages of drug development. Several students have trained and worked with Drs. Bennett and Andersen (see Personnel list in section 7). In addition, we have recruited our colleagues from the University at Albany core facilities to collaborate on our published pharmacokinetic studies and on genomic and proteomic work. **Qishan Lin, Ph.D**. developed mass spec approaches to quantitate AFPep is blood and thus enabled our PK work. **Sridar Chittur, Ph.D**. is the head of the genomic institute and collaborated with us to show the effects of AFPep on the genome of responsive cells/

The **AFPep Team** members, including the **Consumer Advocates**, are committed to the rapid development of AFPep. All three **Consumer Advocates (Joan Isman, Monica Vakiner, Jocelyn Banks)** are keenly interested and contribute importantly to the progress of our research. These Advocates bring a diverse set of views and priorities, all focused on rapid development of a new pharmaceutical entity that could contribute importantly to the **CDMRP** mission.



The consultant firm **Compliance Resources** encompasses impressive prior experience in navigating all aspects of the process of bringing drugs to clinical utility, notably including statistical analysis (**Raymond Buck, Ph.D.**). Experts (**Judy Atkins, Pharm. D.** and **Diana Fordyce,**

Ph.D.) from **Compliance Resources** worked with us to prepare our data in a suitable package so as to obtain the guidance of the FDA in our Type B meeting. Their experience with drug development in the pharmaceutical industry facilitated interactions with the FDA, and they will be instrumental in the interface with both CROs.

We have recruited experienced **clinical colleagues** for development of the IND and future Phase I studies: **Carolyn Sidor, M.D.** is a physician with extensive experience in the pharmaceutical industry who successfully brought several agents to clinical utility. **Paul Davis, M.D.** is an Albany Medical Center physician scientist who has been interacting with us for many years. He worked with Covance to bring to clinical trials a drug which **Dr. Davis** invented for treatment of thyroid cancer.

AmbioPharm, Inc. has been synthesizing AFPep for us for several years, and their extensive experience with bringing peptides to clinical trials and clinical utility is of enormous benefit to this program. **Covance** has brought many drugs through preclinical assessments and into clinical trial. Working with **Covance**, we intend to accomplish the pre-clinical work (this proposal) and the phase I clinical trial (next proposal).

3.b.11 Related studies.

3.b.11.*i* Primate study

As described in detail in this and earlier reports, our primate work was funded by the Albany Medical College and yielded important information about tolerability and pharmacokinetics in higher mammals. Few potential pharmaceutical agents emerging from academia have data in primates. We expect these data to be enabling when formulating our Phase I trials and in negotiating an IND with the FDA.

3.b.11.*ii* Leiomyomata studies

Funded by the Department of Obstetrics and Gynecology at Albany Medical College, we have gathered some observations about the efficacy of AFPep against uterine fibroids. Although these observations are preliminary, they are encouraging enough to suggest that it may be worthwhile to pursue studies, largely because there are no effective pharmacological interventions for leiomyomata and because the incidence among women is very high. AFPep may have some potential to stop the growth of human uterine fibroids. After safety of AFPep is demonstrated in Phase I trials, it may be possible to engineer a Phase II trial in leiomyomata patients; those patients could be followed afterwards to detect a decrease in breast cancer incidence. That is to say, uterine fibroid trials or treatments may provide a segue to prevention of breast cancer trials. See also **4.b**

3.c. What opportunities for training and professional development have been provided by the project?

Although it was not the intention of this award to promote training, we have, in fact trained several individuals and enhanced their professional skills and development.

- Ms. Marcela Desemone, B.A. 2013 (Marist), M.A., Ed.M. 2015 (Columbia) joined our team to gain experience with the scientific methods of modern biological sciences and to enhance her professional skills. Her career path has led her to medical school and to a career as a physician scientist. She entered medical school in the fall of 2018.
- Ms. Wasila Mansouri, B.S. 2015 (Rensselaer Polytechnic Institute) joined our team to gain experience with the scientific methods of modern biological sciences and to enhance her professional skills. Her career path has led her to medical school and to a career as a physician scientist. She entered medical school in the fall of 2017.

- Four medical students have been training in the scientific method by working on this project and will continue their training to the point that they will earn their M.D. with Distinction in Research. Each has contributed substantially to the data contained in this Progress Report.
    - Mr. Kanthi Bommareddy is a student in the Rensselaer Polytechnic Institute-Albany Medical College Physician Scientist program. He gathered data on various biomarkers of response to AFPep. He will be the lead author on our paper reporting the effects of AFPep in the canine clinical trial.
    - Ms. Priya Shivraj completed her first year of medical school here at AMC. She contributed importantly to the identification of biomarkers of response to AFPep.
    - Mr. Samuel Fordyce is a student in the Rensselaer Polytechnic Institute-Albany Medical College Physician Scientist program. He contributed importantly to the study of the effects of AFPep on liver, using both xenograft and cell culture approaches. He is co-author on one published paper.
    - Ms. Anusri Kadakuntla is a student in the Rensselaer Polytechnic Institute-Albany Medical College Physician Scientist program. She contributed importantly to the prevention studies and to the biomarker studies in ER+ and ER- breast cancer cell lines.
- One undergraduate student, Mr. Christopher Sullivan (University of Buffalo, NY) worked with us on the prevention studies. He matriculated at the University at Buffalo medical school in 2019.

The PI has been involved with substantial training in entrepreneurial activities, preparatory to bringing AFPep to commercial utility. While we intend to compete for CDMRP funding to bring AFPep through pre-clinical toxicology assessments (FL 3 proposal) and Phase I clinical trials (a future FL4 proposal), it may be expedient to form a spin-out company so as to be able to compete for SBIR funding and to raise capital in the venture markets. The PI has completed:

- BACC Academy at Albany Medical College. The Biomedical Acceleration and Commercialization Center at AMC offered training in entrepreneurship and awarded $17,500 to the PI as the *2017 Albany Med and Innovate518 Entrepreneurship Award* for the purposes of advancing AFPep.
- I-Site training at Rensselaer Polytechnic Institute (RPI). I-Site is sponsored by a grant from the National Science Foundation and exists for the purpose of developing start-up companies and equipping them to be competitive for NSF- and NIH-sponsored Small Business Innovation Research (SBIR) funding opportunities.
    - In association with the RPI I-Site, the PI met with entrepreneurs at two biotech start-up companies in Boston to gather insights on how to move from spin-out to start-up to ongoing concern status.
- University at Albany sponsored workshops on how to prepare competitive SBIR proposals. The PIs took advantage of University at Albany funding and met with experts from a local consulting agency preparatory to competing for SBIR support.

Funded by the BACC Academy, the RPI-I-Site grant, and the Albany Medical College (but not by this grant), the PI attended BIO 2018 in Boston and met with a dozen concerns in an effort to publicize the effectiveness of AFPep and to seek commercial partners to bring AFPep through pre-clinical toxicology assessments and Phase I clinical trials and beyond.

3.d. How were results disseminated to communities of interest?

Four papers were published, one is in preparation: See listing in Section 6 below.

Three abstracts were presented at professional meetings and two are in preparation. See listing in Section 6 below

In addition to meeting with our Consumer Advocates, we met with an activist in the Komen Foundation. Deborah E. Moore provided a letter of support for our recent grant proposal. She is a member of the Susan G. Komen Foundation's Advocates in Science and a graduate of the Breast Cancer Coalition's Project LEAD. Ms. Moore helped disseminate information about our research program to the local Komen community.

The PI met with local interest groups to discuss the research and our efforts to prevent breast cancer. The PI continues to entertain invitations to small local groups with interest in breast cancer.

News media reported on the research and the entrepreneurial activities. Local media and Facebook listings generated interest and comments from diverse sources.

3.e. What do you plan to do during the next reporting period to accomplish the goals?

Not applicable. This is the Final report for BC 132567. Obviously, we are competing for additional funding

4. IMPACT. Distinctive contributions; major accomplishments.

Every day, 105 American women die from breast cancer. A pharmaceutical therapeutic intervention more effective than current modalities could reduce that number. An intervention that could prevent the disease altogether could shrink that number substantially. That is the mission of the CDMRP BCRP.

This project speaks to the Overarching Challenge of achieving primary prevention of breast cancer. AFPep very effectively prevents breast cancer in rats: in the face of a potent carcinogen (MNU), or in the presence of the biological carcinogen estrogen (estrogen), AFPep prevented breast cancer more effectively than did tamoxifen. When AFPep comes to clinical utility, the impact will be a reduction in incidence and deaths due to breast cancer.

This project speaks to the Overarching Challenge of replacing drugs that have toxicities with safe, more effective interventions. AFPep is more effective than tamoxifen, is effective when tamoxifen fails, and mitigates some of the toxicities that accompany tamoxifen use. Blood levels of AFPep can be escalated 1000-fold beyond what is needed for efficacy with no evidence of toxicity. Safer than xenobiotic molecules, AFPep consists of simple amino acids, and neither it nor its metabolites exhibit toxicity. When AFPep comes to clinical utility in a therapeutic mode, the impact will be fewer toxicities and side effects than are caused by existing SERMs, at least as much protection as provided by SERMs (when used alone), mitigation of the toxicity of tamoxifen when used in combination with that drug, prolonged utility of the pharmaceutical regimen (and increased compliance), prolonged life and prolonged quality of life of the patient.

There are no aspects of our data that suggest anything other than that AFPep should proceed to clinical trials as expeditiously as possible.

4.a What was the impact on the development of the principal discipline of the project?

As we noted earlier, many people believe that all drugs have some toxicity, but this project is demonstrating clearly that *efficacy without toxicity* is possible and can be achieved for the prevention and treatment of breast cancer. We are demonstrating this by using molecules that are indigenous to the human biome, natural, safe,

and effective, and whose metabolites are also non-toxic. Peptides fit that description; AFPep demonstrates those ideals. Peptide-based drugs are increasingly being used in the clinic with excellent results.

4.b What was the impact on other disciplines?

Obstetrics and Gynecology. Cassandra Denefrio, M.D., Senior Resident and now Attending Physician showed activity of AFPep against freshly resected first generation human uterine fibroid xenografts. Funded by the Albany Medical College Department of Obstetrics and Gynecology, Dr. Denefrio presented her work at two professional meetings (see Section 6 below). Currently, the only treatment for severe leiomyomata is hysterectomy. If AFPep were shown to be effective against fibroids, it would make an important contribution to women's health. We note, further, that leiomyomata could play an important role in advancing AFPep to clinical trials and utility for prevention of breast cancer. The most likely scenario for AFPep is that it would first be approved for use in the treatment (not prevention) of breast cancer. When that happens, the drug would be available for off-label usage, and due to its tolerability profile, would probably be prescribed for women suffering from leiomyomata. This may include a large number of women, and those women would likely experience fewer subsequent diagnoses of breast cancer. This observation would compel clinical trials for prevention of breast cancer by AFPep.

Our preliminary studies with canine mammary cancer suggest that AFPep may be useful in the veterinary space. We have had initial discussions with three veterinary pharmaceutical companies regarding the possibility of assessing AFPep for use against mammary cancer in dogs. Tamoxifen is toxic to dogs, and AFPep might be used as adjuvant therapy after surgical removal of tumors in companion animals.

4.c What was the impact on technology transfer?

Ten patents have issued for the protection of the intellectual property related to AFPep and its analogs. It is the intent of the PIs to bring AFPep to clinical utility and toward that end, we have identified the following strategies:

- It is possible that the Albany Medical College could offer a license to one or more commercial entities to facilitate the development and commercialization of AFPep for use against breast cancer or other diseases.
- It is possible that the PIs will establish a start-up company for the purposes of competing for SBIR funding and collaborating with pharmaceutical partners to facilitate the development and commercialization of AFPep for use against breast cancer or other diseases.
- The PIs intend to continue to compete for CDMRP funding, FL3 for pre-clinical toxicology assessments and FL4 for clinical trials, with the intent to continue the development and commercialization of AFPep for use against breast cancer.

Note also the description of professional development listed in Section 3 above, related to entrepreneurial activities to facilitate the commercialization of AFPep.

4.d What was the impact on society beyond science and technology?

We have interacted with multiple breast cancer patients and advocates for improved breast cancer management. A unanimous theme is that better agents are desperately needed to improve breast cancer management. To date, all of our data point to the conclusion that AFPep is a novel, well tolerated agent that may address that need. Although we speak in this report, of "tolerability" of AFPep, we encounter a more useful concept of

"acceptability" of agents when we talk with survivors and advocates. Drugs that may be "well tolerated" in the minds of clinicians are often not 'accepted' by patients. We believe that AFPep will be acceptable to patients and clinicians alike.

5. CHANGES/PROBLEMS

5.a Changes in approach and reasons for change.

As reported briefly in Y2PR, there was a change in leadership at the University of Pennsylvania School of Veterinary Sciences (UPenn Vet) that impacted our project. We had planned to collaborate with UPenn Vet for the clinical trial in dogs that was a part of this project. A disruption in that institution led us (last year) to begin to identify alternate (or additional) institutions, and we were fortunate to identify the Animal Medical Center in New York City. As noted in Y2PR, we prepared animal care and use protocols for both institutions and worked with the DoD-BCRP to have those protocols approved. Eventually, it was not possible to continue to collaborate with UPenn Vet, and we discontinued that interaction. We collaborated with Dr. Ann E. Hohenhaus at the New York Animal Medical Center to complete the canine clinical trial described elsewhere in this report.

5.b Actual or anticipated problems or delays and actions or plans to resolve them.

All problems were resolved in a timely fashion.

5.c Changes that had a significant impact on expenditures.

The cost of ACI rats increased dramatically during the three years of this project. Animals were originally available at a cost of $69/rat; by Year 3, the cost had risen to $189/rat (and now are reportedly in excess of $200/rat). Fortunately, we were able to conserve elsewhere in the supply budget so as to be able to complete the planned studies.

5.d Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to report.

6. PRODUCTS

6.a Journal Publications

Bennett JA, Mansouri W, Lin Q, Feustel P, Andersen T: P**harmacodynamic and Pharmacokinetic Properties of AFPep, a Novel Peptide for the Treatment of Breast Cancer**. *International Journal of Peptide Research and Therapeutics 24: 431-439,* 2018.

Mansouri W, Sullivan, C., Desemone, M., Bennett, JA., Andersen, TT: **AFPep prevents estrogen receptor-positive breast cancer in ACI rats**. *Trends in Cancer Research **12**: 87-95,* 2017.

Mansouri W, Fordyce, S.B., Wu, M., Jones, D., Cohn, D., Lin, Q., Feustel, P., Sharma, T., Bennett, J.A., Andersen, T.T.: **Efficacy and Tolerability of AFPep, a cyclic peptide with anti-breast cnacer properties**. *Toxicol Appl Pharmacol **345**: 10-18,* 2018.

Zhu J. MW, Andersen T.T., Bennett J.A., Cohn D., Feustel P., Lin Q.: **Quantification of a novel anti-breast cancer cyclic peptide (AFPep) in serum using liquid chromatography coupled with mass spectrometry and its application in a pharmacokinetic study**. *Current Topics in Peptide and Protein Research* **18**:59-70, 2017.

Bomareddy, K., Ginnan, R., Hohenhaus, A., Bennett, J.A., and Andersen, T.T. **Tolerability and Efficacy of AFPep in multiple models of breast cancer including spontaneous canine mammary cancer.** *In preparation for submission to British Journal of Cancer.*

6.b Conference presentation

Ann Hohenhaus, James A. Bennett, and Thomas T. Andersen, **Proof of concept study of AFPep as a new treatment for canine mammary gland cancer**. Veterinary Cancer Society Annual Conference, Louisville, KY 2018.

Cassandra Denefrio, Kevin J. Kiley, and James A. Bennett. **The Effect of AFPep on the Growth of Human Fibroid Xenografts.** Presented at the American College of Obstetricians and Gynecologists Armed Forces District, San Antonio, Texas, September 26, 2017

Cassandra Denefrio, Kevin J. Kiley, and James A. Bennett. **The Effect of AFPep on the Growth of Human Fibroid Xenografts**. Presented at the American College of Obstetricians and Gynecologists, Austin, Texas, April 27, 2018

Bommareddy,KJ, Kadakunta,A, Kuna,M, Shivraj,P, Ginnan,R, Hohenhaus,A, Bennett,JA, Andersen,TT. **Trim 25 and KLF 5 expression predict AFPep-mediated inhibition of estrogen-dependent growth**. Proc. Amer. Assoc. Cancer Research Abstract # 2968 Atlanta, Georgia. April 2, 2019

Samuel Fordyce, Thomas T. Andersen, and James A. Bennett. **AFPep for treatment and prevention of breast cancer.** In preparation for presentation at the 2020 meeting of the American Association for Cancer Research, San Diego.

6.c Books.

Bennett, J.A., Jacobson, H.I., Andersen, T.T. **Anti-Breast Cancer Drug Derived from Alpha-Fetoprotein.** In Alpha-Fetoprotein: Functions and Clinical Applications. N. Lakhi and M. Moretti, eds. Nova Science Publishers, Inc. New York. 2016

6.d Technologies or techniques
Nothing to report.

6.e Inventions, patent applications, and/or licenses.

In addition to the eight patents protecting AFPep and related technology (listed in Y2PR), two additional patents issued during the tenure of BC 132567:

9,249,189 T Alpha-fetoprotein "ring and tail" peptides
10,167,326 T Alpha-fetoprotein "ring and tail" peptides

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

7.a What individuals have worked on the project?

A

Name:	Thomas Andersen, Ph.D.
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-6476-856X
Nearest person month worked:	3
Contribution to Project:	Dr. Andersen takes the lead on prevention studies and administers all aspects of the award.
Funding Support:	Funding from this award, and from the Albany Medical College

B

Name:	James A. Bennett, Ph.D.
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-8466-5995
Nearest person month worked:	3
Contribution to Project:	Dr. Bennett takes the lead on PK studies
Funding Support:	Funding from this award, and from the Albany Medical College

C

Name:	Herbert I. Jacobson, Ph.D.
Project Role:	Co-Investigator
Researcher Identifier	
Nearest person month worked:	0
Contribution to Project:	Dr. Jacobson consults and advises on endocrinology-related aspects of the project.
Funding Support:	None

D

Name:	Paul Feustel, Ph.D.
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5
Contribution to Project:	Dr. Feustel is the statistician on this project.
Funding Support:	Funding from this award, and from the Albany Medical College

E

Name:	Qishan Lin, Ph.D.
Project Role:	Consultant (not paid)
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.3
Contribution to Project:	Dr. Lin manages a core facility for mass spectroscopy analysis of PK samples. He is lead author on one of the manuscripts generated under this award
Funding Support:	Employed at University at Albany

F

Name:	Samuel Fordyce, B.S.
Project Role:	Student in training
Researcher Identifier	
Nearest person month worked	0.5
Contribution to project	Mr. Fordyce generated data related to the effects of AFPep on liver, liver cell xenografts, and liver cells in culture
Funding support:	Funding from the Albany Medical College

G

Name:	Marcela Desemone, M.S.
Project Role:	Technician
Researcher Identifier	
Nearest person month worked	10
Contribution to project	Ms. Desemone began working with us in June of 2017 and has subsequently matriculated in medical school
Funding support:	This grant

H

Name:	Kanthi Bommareddy, B.S.
Project Role:	Student in training
Researcher Identifier	
Nearest person month worked	0.5
Contribution to project	Mr. Bommareddy is the lead author on our canine clinical trial and biomarker paper.
Funding support:	Albany Medical College

I

Name:	Anusri Kadakuntla, B.S.
Project Role:	Student in training
Researcher Identifier	
Nearest person month worked	0.5
Contribution to project	Ms. Kadakuntla contributed data related to biomarkers, using Western blot and qPCR approaches
Funding support:	Albany Medical College

J

Name:	Ann E. Hohenhaus, DVM
Project Role:	Collaborator
Researcher Identifier	
Nearest person month worked:	1
Contribution to Project:	Dr. Hohenhaus takes the lead on canine clinical trials
Funding Support:	Dr. Hohenhaus is employed at the Animal Medical Center in New York

K

Name:	Joseph Wiegartner, B.S.
Project Role:	Technician
Researcher Identifier	
Nearest person month worked	7
Contribution to project	Mr. Wiegartner began working with us in November of 2017; he left for a different job opportunity.
Funding support:	This grant

L

Name:	Wasila Mansouri, B.S.
Project Role:	Technician
Researcher Identifier	
Nearest person month worked	7
Contribution to project	Ms. Mansouri worked with us on prevention and PK studies. She has since matriculated in medical school
Funding support:	This grant

M

Name:	Diana Fordyce, Ph.D.
Project Role:	Consultant
Researcher Identifier	
Nearest person month worked	1
Contribution to project	Dr. Fordyce helped us package the data from this project so as to facilitate our meeting with the FDA
Funding support:	None

N

Name:	Judy Atkins, Pharm.D.
Project Role:	Consultant
Researcher Identifier	
Nearest person month worked	1
Contribution to project	Dr. Atkins helped us package the data from this project so as to facilitate our meeting with the FDA
Funding support:	None

O

Name:	Raymond Buck, Ph.D.
Project Role:	Statistician
Researcher Identifier	
Nearest person month worked	0.1
Contribution to project	Dr. Buck contributed to the statistical package for the data we presented to the FDA
Funding support:	This grant

P

Name:	Monica Vakiner
Project Role:	Consumer Advocate
Researcher Identifier	
Nearest person month worked	1
Contribution to project	Ms. Vakiner contributes to all aspects of our project and met with us as we developed our package for the FDA
Funding support:	None

Q

Name:	Joan Isman
Project Role:	Consumer Advocate
Researcher Identifier	
Nearest person month worked	0.2
Contribution to project	Ms. Isman contributes to all aspects of the project, especially with proposal writing
Funding support:	None

R

Name:	Jocelyn Banks
Project Role:	Consumer Advocate
Researcher Identifier	
Nearest person month worked	0.1
Contribution to project	Ms. Banks contributes to all aspects of the project
Funding support:	None

S

Name:	Paul Davis, M.D.
Project Role:	Consultant
Researcher Identifier	
Nearest person month worked	0.1
Contribution to project	Dr. Davis advises us as we package our data for presentation to the FDA
Funding support:	

T

Name:	Carolyn Sidor, M.D.
Project Role:	Consultant
Researcher Identifier	
Nearest person month worked	0.1
Contribution to project	Dr. Sidor advises us as we package our data for presentation to the FDA
Funding support:	None

U

Name:	Christopher Sullivan
Project Role:	Student in training
Researcher Identifier	
Nearest person month worked	3
Contribution to project	Mr. Sullivan worked on the prevention studies
Funding support:	Albany Medical College

V

Name:	Douglas Cohn, DVM
Project Role:	Consultant
Researcher Identifier	
Nearest person month worked	0.1
Contribution to project	Dr. Cohn advised us as we performed PK studies in primates
Funding support:	Albany Medical College

7.b Has there been a change in the active support of the PI or other senior personnel?

Nothing to report.

7.c What other organizations were involved as partners?

Name: Animal Medical Center
Location: New York, NY
Contribution to project: Conducted canine clinical trial
Personnel exchanges: Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS
Not applicable

9. APPENDIX. Manuscript in preparation: *Tolerability and Efficacy of AFPep in multiple models of breast cancer, including spontaneous canine mammary cancer.*

Tolerability and Efficacy of AFPep in Multiple Models of Breast Cancer Including Spontaneous Canine Mammary Cancer.

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Abstract:

Alpha-fetoprotein (AFP) functions *in utero* to inhibit estrogen-mediated growth in fetal tissue. AFPep is a 9-amino acid cyclized form of the active site of AFP that impedes phosphorylation and activation of ERα. Previous studies have demonstrated that AFPep is well tolerated in mice and rats, inhibits estrogen-dependent growth of human breast cancer xenografts (MCF-7 and T47D) in mice, and decreases mammary tumor incidence and tumor burden in carcinogen-exposed rats. The purpose of this study was to determine the tolerability of AFPep in dogs and assess its effect on biomarkers of estrogen-promoted growth in several models of breast cancer. Methods: Expression of ERα, pERα, TRIM25, and KLF5 were assessed in three estrogen-sensitive models: immature mouse uterus, MCF7 xenografts, and spontaneous canine mammary tumors. Immature mice were treated with saline alone, AFPep (100 µg) alone, estrogen alone, or estrogen plus AFPep (100 µg). Twenty-four hours post treatment the uteri were removed, weighed, and blotted for biomarkers. Mice bearing MCF7 xenografts were treated with estrogen alone or estrogen plus AFPep and monitored for tumor growth. Tumors were biopsied after 14 days and analyzed for tumor specific biomarkers. Twelve normal beagle dogs were treated with AFPep through I.V., S.C. and P.O. routes. Blood levels of AFPep and clinical signs of tolerability were monitored. Finally, six dogs presenting with mammary tumors were treated with AFPep S.C. once daily for seven days. Biopsies of these tumors were analyzed for tumor specific biomarkers pre- and post-AFPep treatment. Results: Immature mice treated with estrogen had greater uterine-to-body weight ratio, increased ratio of pERα to total ERα, and increased KLF5 and TRIM25 expression compared to mice treated with saline alone or AFPep alone. Uteri from mice treated with estrogen and AFPep showed decreased uterine-to-body weight ratio, decreased ratio of pERα to total ERα, and decreased KLF5 and TRIM25 expression compared to mice treated with estrogen alone. MCF7 xenografts from mice treated with estrogen and AFPep showed decreased ratio of pERα to total ERα and decreased expression of TRIM25 and KLF5 compared to mice treated with estrogen alone. In dogs, AFPep was well tolerated at all doses and through all routes. In the clinical canine mammary tumor study, one of the six dogs had tumors expressing ERα. In that animal, treatment with AFPep resulted in a decrease of pERα to total ERα and decreased KLF5 and TRIM25

expression compared to pre-treatment levels. Tumors from the other dogs did not express ERα and treatment of those dogs with AFPep had little impact on the expression of TRIM25 and KLF5 in the tumor. Conclusion: We conclude that AFPep is well tolerated in multiple animal models, inhibits estrogen-mediated growth, and decreases pERα / ERα, KLF5 and TRIM25 expression. These responses may serve as markers for efficacy of AFPep in uterus and breast tissue.

Introduction:

AFPep is a first-in-class agent being developed for the treatment and prevention of breast cancer (3). 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 AFP (4). AFPep is well tolerated in rodent models (4) and has a unique mechanism of action (4, 5). It is a multikinase inhibitor that blocks the activation of ERα, FAK, and c-kit (6). AFPep is additive in combination with tamoxifen and mitigates some of the toxicity (uterine hypertrophy) of tamoxifen (7). AFPep blocks the growth of ER+ human breast cancer xenografts including those resistant to tamoxifen (7) and prevents the development of carcinogen-induced mammary cancer in rats (8-10). As an orally active peptide (6) that is non-toxic in rodents, whose metabolites are simple amino acids, and with efficacy against ER+ breast cancer, AFPep should be considered for development for treatment and prevention of breast cancer. Efficacy and tolerability in higher animals are necessary precursors to further development.

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 (4, 10), does not affect the estrous cycle or fertility in rats (11), and has no effect on liver (4). Prior to extensive studies *en route* to clinical trials in humans, it is essential to assess tolerability in additional models.

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 highly ER positive tumor cell lines (5, 6, 12-15). 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 correlated with inhibition of breast cancer tumor growth, invasion, and metastasis (16, 17). It seemed logical to probe the ability of ERα, TRIM25, and KLF5 to serve as reporters for AFPep efficacy.

Often, as tumor models become more complex and tumors become more heterogenous, biomarkers which change in the simpler models no longer change in the more complex models (18). The purpose of this study was to identify biomarkers that reliably report anti-estrogenic and anti-breast cancer activities of AFPep across rodent, canine, and human 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 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.

Methods:

AFPep: AFPep, sequence *cyclo*(EKTOVNOGN) where O is hydroxyproline, was synthesized using solid phase peptide synthesis approaches as described earlier (19). The agent was synthesized by AmbioPharm, Inc. (Augusta, SC) and assessed by mass spectroscopy (20). 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 an immature mouse (7, 20).

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, N.Y. 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 the Albany Medical College Animal Care and Use Committee.

Assays for Estrogen-dependent growth. Efficacy of AFPep was demonstrated in a uterine growth-inhibition assay in immature Swiss Webster female mice as described earlier (7). AFPep is defined as biologically active (anti-estrogenic) when it significantly inhibits the estrogen-stimulated growth of mouse uteri using the following calculation:

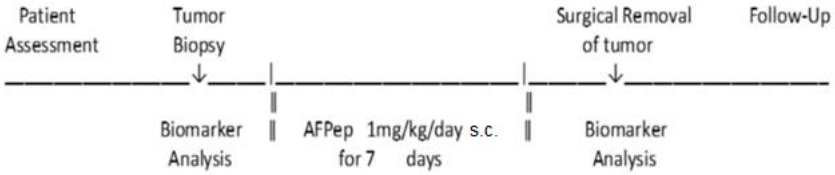
% Growth Inhibition = 100 x (Full E_2 Stimulation – E_2 Stimulation in test group)/(Full E_2 Stimulation – No E_2 Stimulation)

Efficacy of AFPep was also assessed in mouse/human breast cancer xenograft studies. MCF-7 human breast cancer cells were adapted for growth in SCID mice as previously described (5, 7). Briefly, five tumor pieces (each 1.5 mm in diameter) were loaded into a 16-guage 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 p.o. administration of agents once daily. 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.

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 (4, 8, 9). Normal beagle dogs weighing approximately 10kg 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 Index 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, autoplatelets, 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, ALP, ALT, AST, and GGT). Blood levels of AFPep were assessed by LC/MS/MS in our laboratories as previously described (20). Each assessment in normal dogs was repeated at least three times. At the end of these studies, dogs were adopted by local families.

Clinical Studies in Dogs with Mammary Cancer: Dogs with mammary gland tumors greater than 2 cm in diameter presenting to the Oncology Service at the Animal Medical Center in New York City were considered for inclusion in the study. All dogs underwent complete clinical staging. The clinical design of AFPep administration to dogs with spontaneous mammary cancer is shown below.



Tumors were biopsied prior to administration of AFPep and on Day 7 one hour after the last injection of AFPep. Dogs were administered AFPep 10 mg s.c. once daily for 7 consecutive days. Blood levels of AFPep were measured before and 30 min following administration on Days 1 and 7. Histopathology was obtained on all mammary gland tumors. Tolerability was graded according to

Biomarker Analysis: Uterus, xenograft, and canine biopsy tissues were homogenized with standard RIPA 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

14,000g 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.

Statistical Analysis. Mouse uteri weights and protein band densities were analyzed using a two-way ANOVA with a post-hoc Tukey HSD test using MiniTab.

Results

Tolerability

A dose of 4 mg/kg of AFPep was administered to three dogs intravenously and to three 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 of AFPep were well above the range that had previously shown anti-tumor activity in mice (5), indicating that 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 (5), so in dogs, dose by the oral route was escalated to 12 mg/kg in 4 dogs and then 25 mg/kg in 4 dogs. These doses were well tolerated by all of these dogs using endpoints described above and including gastrointestinal endpoints of normal palatability, no reflux, no vomiting and no change in food, water and treat consumption

throughout the observation period. The 200 mg/kg dose consistently yielded blood levels of AFPep that had been associated with antitumor activity in rodents (5) indicating that the oral route is a viable option for AFPep administration during its clinical translation. The dogs 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, tumor-bearing dogs were given 10 mg AFPep (~ 1 mg/kg), s.c., once daily for seven days between tumor biopsy and complete surgical removal of tumor. Dogs ranged in weight from 5-30 kg. 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. AFPep blood levels exceeded values that had previously been found to be associated with anti-tumor activity in rodents (5) suggesting that tumor response could safely be achieved with AFPep in dogs.

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 phosphorylation 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 previous 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. 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 administration to these dogs. The tumor in one of the six dogs was positive for ER. This was somewhat lower than the literature indication that approximately 40-50%

of canine mammary cancers are positive for ER (23). Nevertheless, in the ER+ mammary cancer in the study reported herein, AFPep treatment of the ER+ dog inhibited the activation of the ER in that dog's tumor and significantly reduced the expression of KLF-5 and TRIM 25 (Figure 3).

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 (24). The results of this study demonstrate that AFPep is well tolerated and inhibits intermediate biomarkers of estrogen-stimulated growth of normal and cancerous tissues across three different species. The excellent tolerability of AFPep is likely due to its derivation from an endogenous growth regulatory human 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 (4, 5, 10). 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 (4, 8, 10). 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 (5, 20). The doses of AFPep used in the studies described herein (4 mg/kg mice; 1-4 mg/kg dogs) led to blood levels in the 1 ug/ml range (5) which is well below the quantities of its parent protein (AFP) normally found in in fetal blood (25) where AFPep is reported to have anti-estrogenic properties (26, 27). This is likely another reason for the excellent tolerability of the 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 (19, 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 (4). In a separate study of the chemopreventive property of AFPep against estrogen-induced breast cancer in rats (10), 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 do have side effects that are quite difficult for patients.

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 three 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. In dogs, undergoing ovariectomy prior to their first estrus significantly reduces the incidence of mammary cancer later in life (23), and 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 tissue) 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 (16, 17) and add additional import to the ER activation profile. The consistency of the AFPep inhibitory effects in all three 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 (5). These blood levels were readily attained in all the models in this study and will be an important endpoint in future studies. From this study, relevant biomarker and tolerability data have been added to the AFPep profile, and all observations look promising for translation to human clinical trials and eventual human use for treatment of ER+ breast cancer. Further, because of its exquisite tolerability profile, AFPep may be able to contribute to the much-needed space of an effective, well tolerated preventive agent that stops the development of neoplastic tissue into breast cancer (3).

Acknowledgements

This project was supported by grant BC 132567 from the Breast Cancer Research Program of the CDMRP.

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 ± uterine weights and band densities were calculated. Differences were assessed using Tukey's tests. * $p < 0.05$.

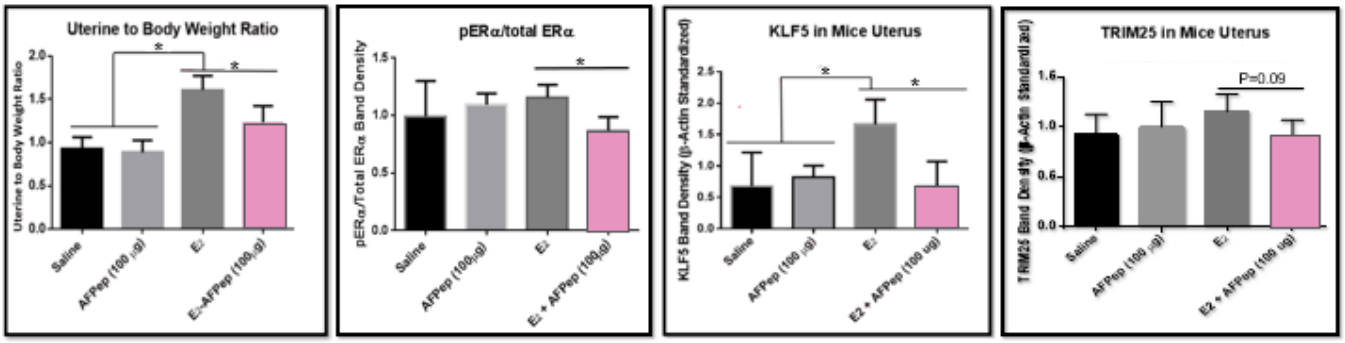


Figure 2. **Anti-breast cancer activity of AFPep.** Pieces of MCF-7 human breast cancer tissue were injected into the thoracic mammary fat pad of SCID mice. Mice were supplemented with E_2 implants. Tumors were on average 6.7 mm in diameter (156 mm^3) on day 21 after implantation and measured once daily thereafter. Either vehicle of 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. * $p < 0.05$

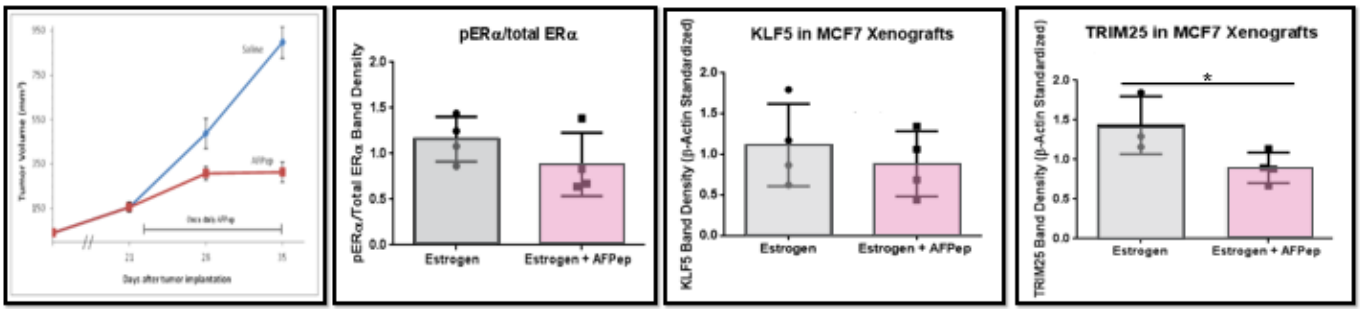
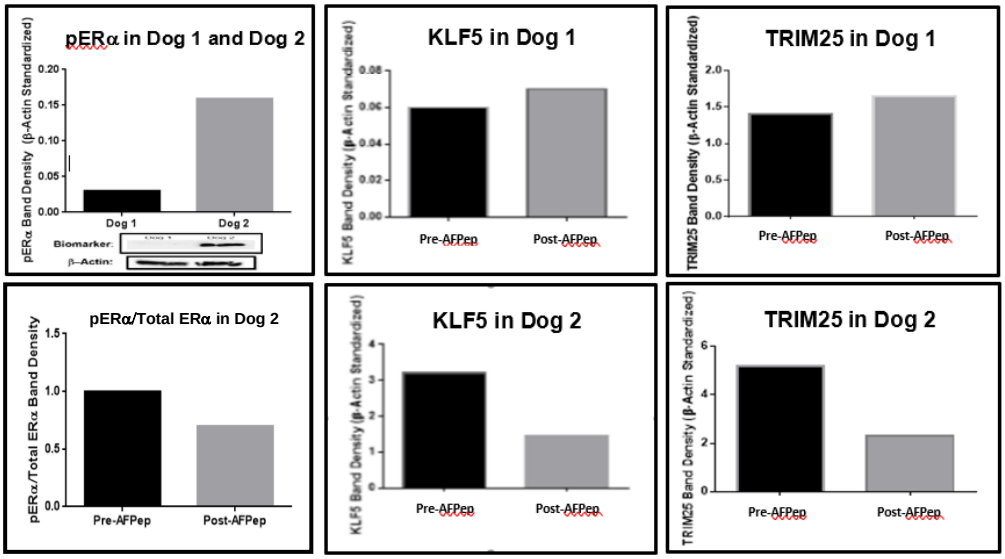


Figure 3. AFPep decreases expression of pER, KLF5, and TRIM25. Following initial biopsy, AFPep was administered once daily for 7 days prior to surgical resection of the tumor. Treatment with AFPep decreased relative expression of pER/total ER by 30 % and decreased expression of KLF5 and TRIM 25 by **X %** relative to their pre-AFPep levels.



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