Review

Cancer Prevention Research

Development of an Active Site Peptide Analog of α-Fetoprotein That Prevents Breast Cancer

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Abstract

Epidemiologic studies associate elevated maternal serum levels of α-fetoprotein (AFP) with reduced breast cancer risk for parous women. Laboratory studies demonstrate direct anti-breast cancer activity of AFP. Here, we review the development of a small cyclic peptide that is an active site analog of AFP, referred to as AFPep, which is composed exclusively of amino acids, is orally active, has no discernable toxicity, and is effective for the treatment and prevention of breast cancer in animal models. *Cancer Prev Res; 7(6); 565–73.* ©*2014 AACR.*

Introduction

A reasonable starting point to consider cancer prevention may be to review risk factors to identify opportunities for intervention. For breast cancer, major risk factors include gender and age, but neither offers obvious opportunity to intervene nor does the observation that close relatives have breast cancer. Other risk factors include exposure to radiation, carcinogenic chemicals, or viruses, and arguably the best intervention would be avoidance. Still, the majority of women who encounter a diagnosis of breast cancer have none of the known risk factors. Thus, a substantial challenge remains: Is it possible to intervene pharmacologically in a diverse set of processes to prevent breast cancer? Synthetic pharmacologic agents for chemoprevention of breast cancer would be successful if they were entirely safe and if they would interact with breast tissue to prevent progression down oncogenic pathways. We assert that these goals can be achieved.

Efficacy without toxicity may have been considered an unachievable goal, due likely to experience with xenobiotic molecules such as those derived from plants (taxol) or from a reliance on chemical entities foreign to the body, such as tamoxifen. These molecules are extrinsic entities that do not occur naturally in mammalian physiology. Virtually all xenobiotic molecules have some toxicity precluding their utility as preventive agents. An alternative approach to developing a preventive agent would be to utilize a homobiotic molecule that is present in normal human physiology. Such a molecule, or analog of homobiotic molecules, might be expected to be of low toxicity. If these analogs were composed exclusively of amino acids, even their metabolites would be nontoxic. Such an approach would reduce the major challenge to that of identifying homobiotic peptidic molecules that have the desired activity, in this case, the ability to suppress breast cancer.

Herein we review a homobiotic molecule (α-fetoprotein, AFP) that has activity against breast cancer and the development of a small, active site cyclic peptide analog of the active site of AFP (termed AFPep) composed exclusively of amino acids. We provide evidence that AFPep retains its parent molecule's efficacy against breast cancer and that it has no discernable toxicity. We describe the utilization of AFPep for treatment and prevention of breast cancer in animal models. In doing so, we suggest that efficacy without toxicity is an achievable goal.

The Case for AFP as an Anti-Breast Cancer Molecule

Epidemiologic data, clinical observations, and laboratory studies suggested that AFP participates in normal physiologic processes that prevent breast cancer.

Epidemiology: AFP and reduced risk of breast cancer

From the earliest collection of breast cancer incidence data (1), it had been seen that parous women were at lower risk of breast cancer than nulliparous women (2, 3). One of the consistently observed characteristics of breast cancer incidence was the inverse association between the number of children a woman has borne and her risk of developing breast cancer later in life (4). The interpretation of these observations was that a factor associated with pregnancy protects women against subsequent development of breast cancer and that the degree of protection was related to the number of pregnancies (2). Investigators probing this phenomena proposed various pregnancy-associated components such as estrogen, androstenedione, progesterone, chorionic gonadotropin, and others (reviewed in ref. 3) as possible causative agents responsible for reducing risk of breast cancer. Jacobson and Janerich proposed that AFP

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should be considered as the major determinant of breast cancer risk reduction wrought by parity (4).

Noting that AFP is a major protein component of amniotic fluid and of fetal plasma and that a fetus experiences an environment that contains high levels of both estradiol and AFP, it was reasoned (5) that AFP might function as an endocrine agent that allows fetal cells to avoid responding to estrogen. The concentration of these analytes in amniotic fluid is gestational age-dependent, normally attaining peak values of 600 pg/mL for estradiol (6) and 10 μg/mL for AFP (7). In fetal plasma, AFP concentration attains 1 to 4 mg/mL (7). Despite exposure to these high plasma levels of estrogen and the presence of estrogen receptors (8, 9), fetal mammary epithelial cells do not respond (10) to estrogen perhaps because of an anti-estrogenic property of AFP (the only feasible protein component of amniotic fluid that could provide such a property). AFP traverses the placenta and normally appears in maternal circulation during pregnancy, at more modest levels, up to 200 ng/mL (11). It was reasoned (4) that if AFP brought the same putative endocrinologic message to newly emerging estrogen-dependent tumors in the maternal breast as it does to normal fetal tissues, and thereby blocked the responsiveness of those tumors to estrogen, cells in those tumors would be expected to die, the tumor to diminish.

The AFP hypothesis (4) proposed that AFP acts in an endocrine manner to decrease risk of breast cancer. This was a useful concept in that it allowed the authors to predict that elevated AFP levels in maternal sera would be associated with greater subsequent reduction of maternal risk of breast cancer for such women. Maternal conditions that are associated with higher-than-average maternal serum levels of AFP provided opportunities to explore the AFP hypothesis. For example, Jacobson and colleagues (12) reasoned that if AFP were the agent of pregnancy responsible for decreased risk of breast cancer, then women who gave birth to twins and who therefore experienced higher maternal serum levels of AFP (produced by 2 fetal livers) should subsequently be at lower risk of breast cancer than women who experienced only singleton births. They noted that such a correlation would not be expected for the other agents (e.g., estrogen, progesterone, etc.) postulated as being the focus of risk reduction since their concentration is not dependent on the number of fetal livers *in utero*. When these investigators analyzed data from the Cancer and Steroid Hormone Study (13) on women newly diagnosed with breast cancer, they found that those who were parous and had experienced a twin pregnancy were at substantially lower risk than those who had experienced only singleton pregnancies (12). It became clear that an early full-term pregnancy significantly lowered a woman's risk of developing breast cancer and multiple-birth pregnancies further reduced that risk (12). This is especially significant because women who remain nulliparous or who have their first pregnancy late in life (i.e., after 30 years of age) are at a lifetime risk that is 3 times that of women who experienced term pregnancy by the age of 18 years (3, 4, 14). It was noted further (4) that the postpartum surge in estrogen receptor negative (ER^-) breast cancer that occurs in humans is dependent upon the mammary gland's burden of malignant microfoci at the time of pregnancy. That burden of malignant microfoci increases with maternal age. For women in their late 30's, the postpartum cancer surge exceeds the parity-wrought protection.

A number of epidemiologic studies support the concept that pregnancy-associated protection from breast cancer varies directly with the concentration of AFP in maternal serum. Bennett and colleagues (15) summarized several unrelated epidemiologic observations that together suggest that women who experience a high level of AFP during pregnancy are at lower risk of developing breast cancer later in life. They identified a collection of publications in which investigators reported maternal conditions (such as maternal race, weight, hypertension) that were associated with elevated levels of maternal serum AFP, information that was offered as being useful for monitoring pregnancies. Bennett and colleagues (15) also identified an unrelated collection of publications examining similar maternal conditions during pregnancy and relating those conditions to subsequent breast cancer risk. These observations suggest that several physiologic conditions that occur during pregnancy are associated with both an increase in maternal serum AFP and a decrease in subsequent risk of breast cancer.

We are aware of no sufficiently powered reports that are contradictory to the conclusion that elevated levels of AFP during pregnancy are associated with reduced risk of breast cancer later in life. However, a weakness of these observations is that they stemmed from different study cohorts. To meet this concern, Richardson and colleagues (16) analyzed AFP in stored sera that had been obtained from pregnant women who were enrolled in the Child Health and Development Studies initiated in 1959, and data from breast cancer registries from the 1990s to establish a single cohort of women for whom both maternal serum AFP levels and subsequent history of breast cancer were available. Their analysis was consistent with the concept that maternal serum AFP levels and subsequent risk of breast cancer are inversely correlated. Quoting the authors (16), "The results of this study agree with the protective effect reported by Jacobson and colleagues and Thompson and colleagues, using surrogate indicators (multiple births and hypertension) for a high level of AFP during the index pregnancy." In 1995, Albrektsen and colleagues (17) reported a lower risk of breast cancer among Norwegian women having had a multiple birth than among women with singletons only, and in 1997, Murphy and colleagues (18) reported similar results among a cohort of Swedish women. This observation was later confirmed by Melbye and colleagues (19) who substantially reproduced and extended the conclusions of Richardson, namely that "a high level of α-fetoprotein in maternal serum during any pregnancy is associated with a low overall incidence of breast cancer and, in particular, with a low incidence of advanced breast cancer at diagnosis. This association appears particularly strong for a pregnancy occurring at a young age." (19). Interestingly, Albrektsen and colleagues reported no protective effect of pregnancy against ovarian (20) or endometrial cancers (21).

Clinical observations

Clinical reports suggest that AFP interferes with estrogen-induced responses in normal and malignant tissues in humans. Clinically, it is known that fetal mammary tissues do not respond to estrogen even though receptors for estrogen are present in those tissues (8–10). It has long been postulated that AFP is the internal regulator which tamps down the response to estrogen (22). This becomes important for breast cancer since several studies have concluded that unchecked, or overexposure to, estrogen promotes the development of breast cancer (23). It has been reported that premenopausal women bearing an AFP-secreting hepatoma became amenorrheic (24, 25). After surgical resection of hepatoma, normal monthly menstruation resumed and serum estrogen levels returned to normal. Similarly, Nerad and Skaunic reported occurrence of pregnancy following hepatic lobectomy in hepatoma patients (26) and suggested that the estrogen effect was re-engaged after reduction of AFP. AFP in serum is used as a biomarker for hepatoma and gastric cancer (though it does not cause those cancers; refs. 27, 28).

Laboratory observations

AFP has been shown to inhibit the growth of experimental breast cancers (15, 22, 29–31) and to shrink estrogen-dependent tumors in rats (29) and to counteract many of the effects of estrogen (32–34).

Soto and colleagues (22, 31) and Sonnenschein and colleagues (30) implanted estrogen-dependent and -independent tumors into newborn or adult rats and measured tumor growth rate. They showed that estrogen-independent tumors grew without delay, and without respect to added estrogen, in both newborn and adult rats. Estrogen-dependent tumors grew promptly in adult rats but experienced a delay of several days when inoculated into newborn rats. These investigators showed that growth delay was not due to immunologic considerations and suggested clearly that AFP, present in newborn animals, was responsible for the inhibition of tumor growth (35). As the animals aged (i.e., after 20 days when AFP levels decrease; refs. 36, 37), tumors began to grow. They showed these effects for a variety of estrogen-dependent cancers, including pituitary (22) and mammary tumors (30), and they showed that purified AFP had growth-inhibiting effects against the estrogen-sensitive cell lines in culture (22).

Another observation relating to putative anti-estrogenic effects of AFP was provided by Pool and colleagues (38) who reported that in rats bearing a hepatoma that secreted AFP, uterine wet weight was significantly decreased and stimulation of uterine epithelial mucosa was diminished. In an elegant demonstration, Sonnenschein and colleagues (29) implanted into rats bearing estrogen receptor–positive breast cancers a rat hepatoma cell line which secreted large quantities of AFP, or as a control, hepatoma cell lines that did not secrete AFP. They showed that mammary tumors decreased in size after introduction of the AFP-secreting hepatoma cell line, whereas tumors continued to grow in the presence of a hepatoma cell line that did not secrete AFP. They concluded that AFP, alone, has substantial anticancer, anti-estrogenic effects (29). Soto and Sonnenschein (22) obtained similar results by injecting partially purified preparations of AFP, a step toward focusing on AFP as opposed to other agents that could be produced by AFP-secreting tumors. Continuing this focus, Bennett and colleagues (15) purified AFP from culture media of human hepatoma cells and used it to treat severe combined immunodeficient (SCID) mice bearing human breast cancer cell lines growing as xenografts under the kidney capsule. In a dose-dependent manner, AFP inhibited the growth of estrogen-dependent human xenografts (including MCF-7 and T47D lines). AFP did not inhibit xenografts of estrogen receptor–negative cell lines (such as MDA-MB-231 or BT-20).

In terms of focusing on AFP as the active, anti-oncogenic agent in these various approaches, studies progressed from using animals bearing AFP-secreting hepatomas (29, 38), to using preparations of AFP purified from rat fetuses (22), from human umbilical cord blood (39), from rat amniotic fluid (5), or from culture media of human hepatoma cells (15). In each of this sequence of studies, the starting material should have contained fewer contaminants than the material in the preceding citation. In 1997, recombinant AFP was expressed from an *Escherichia coli* expression system and shown to possess the anti-estrogenic and anticancer activities of native AFP (40). Any putative contaminants from this preparation should be unrelated to the mammalian systems used earlier, suggesting definitively that anticancer activity is associated with AFP, not with any putative contaminant molecule. Subsequent work involved parsing the AFP molecule to define the anticancer active site, thereby deleting any other active sites such as those related to immunosuppression (41).

Central role of AFP as an anticancer protein. The pregnancy-based epidemiologic considerations of the effect of AFP in breast cancer, together with the laboratory and clinical observations that AFP has activity against cancer (and especially breast cancer), were brought together to call attention to the centrality of AFP as an anticancer protein (42). Over the years, investigators had proposed various hormones of pregnancy (e.g., estradiol, estriol, androstendione, testosterone, progesterone, chorionic gonadotropins) as the agent responsible for the pregnancy-associated reduction in risk of breast cancer later in life. Many of these agents had been shown to decrease incidence of breast cancer when administered to carcinogen-exposed rats. Serial injections into carcinogen-exposed animals of either estriol (43–45), estrogen together with progesterone (46), or human chorionic gonadotropin (47) decreased the incidence of mammary cancer compared with carcinogen-exposed animals not treated with those hormones of pregnancy. Seemingly, a number of different pregnancy-associated endocrine substances are effective surrogates for parity in terms of reducing mammary cancer risk, but there had been no reasonable explanation of why these diverse treatments should lead to the same outcome. Jacobson and colleagues (42) repeated the studies of the earlier investigators, duplicating their

methods of treating carcinogen-exposed rats with estradiol plus progesterone, estriol plus progesterone, estriol alone, or chorionic gonadotropin. In each case, serum levels of AFP were elevated as a result of exposure to the various hormones of pregnancy while tumor incidence decreased. In addition, they employed an *in vitro* system to challenge cultures of human liver cells with these hormonal agents. Each hormone treatment increased the level of AFP in the culture medium of HepG2 cells. Medium from HepG2 cell cultures challenged with chorionic gonadotropin or vehicle was added to MCF-7 cell cultures. Media from gonadotropin-challenged liver cells inhibited proliferation of cultured human MCF-7 breast cancer cells while that from vehicle-treated cells did not. Addition of antibodies to AFP (but not to gonadotropin) neutralized the proliferation-inhibiting effect of AFP-containing media. Jacobson therefore proposed a unified mechanism for the reduction of breast cancer risk by the hormones of pregnancy (42), namely that neither estradiol, estriol, progesterone nor chorionic gonadotropin is the proximal inhibitor of breast cancer as had been asserted (43–47) but rather that these agents act on the liver to elicit production of AFP and that AFP is the proximal mediator of reduction of breast cancer risk. The conclusion is that in the treatment of carcinogen-exposed rats with the hormones of pregnancy and by inference in women who have experienced pregnancy, AFP is the proximal agent that inhibits breast cancer. What is particularly appealing about this unifying hypothesis is that it presents an obvious pharmacologic strategy for harnessing the pregnancy-based, anticancer observations and the anti-breast cancer potential of AFP so as to translate it to the clinic. Whereas there may be strictures on possible long-term treatment of women with estradiol or the other hormones of pregnancy, treatment with an analog of AFP that is orally active and nontoxic would offer substantial possibilities.

If agents such as estrogen, estriol, and others elicit AFP from the adult liver, the question arises whether other drugs or toxins might do the same. A large-scale study by Mayes and colleagues (48) examined the toxicity of polychlorinated biphenyls in male and female rats and reported that individual arochlors or mixtures of those molecules exhibited substantial carcinogenicity for multiple organs. Interestingly, breast cancer incidence in females was significantly and substantially decreased relative to control, unexposed rats. It is reasonable to suggest that arochlors exhibited liver toxicity leading to the production of AFP and that AFP suppressed carcinogenesis in the breast but not other tissues.

Development of AFPep

Strong epidemiologic suggestions relating to AFP, together with laboratory findings that AFP is inherently an anti-estrogenic, anticancer protein engendered the concept that a drug developed from this natural protein could be successful if it were to find its way to the clinic. AFP itself, however, would make a very poor drug, so it became necessary to identify the anti-oncogenic active site within that 69,000 MW protein. That such a site existed was supported by difference spectroscopy data (49) which indicated that a conformational change in the structure of the molecule occurred in the presence of estrogen and that change actually generated increased anti-estrogenicity of AFP (an increase that could not be explained by binding and sequestration of estrogen by AFP; refs. 5, 49).

Rather than working with full-length AFP (39), Festin and colleagues parsed the molecule by expressing domains and subdomains (49) and showed that the anti-estrotrophic activity of AFP was contained in the C-terminal third of the protein, a 23,000 MW fragment known as domain III (50). Domain III yielded anti-uterotrophic dose–response curves similar to full-length AFP (49), whereas the N-terminal half of the protein had little or no anti-uterotrophic activity. Continued efforts to parse the active fragment employed peptide synthesis, beginning with the synthesis of large peptide fragments (51). Extensive studies of a 34-mer peptide that contained the biologic activity (52, 53) showed that the large peptide aggregated over time, forming trimers and other disadvantageous aggregates which compromised anti-estrogenic activity. Therefore, it was necessary to identify yet smaller, stable peptides, and Mesfin identified an 8-amino acid fragment of AFP as the smallest peptide that retained full activity (54). Peptides smaller than 8 amino acids showed substantially less activity than did the 8-mer (54, 55), while the 8-mer yielded dose–response curves similar to intact AFP or to domain III. This octapeptide is the anti-estrogenic/anti-breast cancer active site of the AFP molecule; it has none of the other active sites and has none of the other side effects associated with AFP, for example, immunosuppression (41, 56) or liver growth (57). The anticancer site of human AFP, using the one-letter code for amino acids, is EMTPVNPG.

The 8-mer peptide was developed further through modeling and rational design approaches (54, 58). Modeling suggested that the octapeptide existed in a horseshoe shape (54, 55, 58–60), probably stabilized by hydrogen bonding between amino acids 1 and 8 that would not be available to 7-mer or shorter peptides. This suggested the approach of cyclizing the peptide to lock in the putative active shape, as well as to enhance its conformational stability. While there are many synthetic peptides that are cyclic by virtue of a disulfide bond, that option was less attractive for the AFP 8-mer because of its lack of cysteine residues and arguably less desirable for any peptide drug due to the reactivity of the sulfhydryl/disulfide moiety. Mesfin chose to avoid disulfide bonds and other approaches and cyclized the peptide using a head-to-tail peptide bond (58). Rather than adding 2 cysteine residues, Mesfin added one asparagine residue to facilitate cyclization and generated a 9-mer analog (58). This cyclic peptide retained full biologic activity, both in anti-estrogenic uterine growth inhibition assays and in anticancer assays. It arrests growth of human breast cancer xenografts. This cyclic peptide is stable on storage and does not interact with the estrogen receptor (58). DeFreest and colleagues (59) studied optimization of synthetic routes

and investigated the pharmacophore within the 9-mer. They found that replacement of the naturally occurring Met with Lys improved synthetic yield and biologic activity, and they identified which amino acids in the cyclic 9-mer are essential for biologic activity and which can be modified or substituted without loss of activity. They reported that substitution of proline by hydroxyproline (O) improved solubility, decreased aggregation, and did not compromise the biologic activity. Joseph and colleagues (55, 60) used modeling and synthesis of analogs to explore the optimal compound. Smaller cyclic analogs (8-mers, 7-mers, 6-mers, etc.) were investigated and shown to be biologically active but not as active as the 9-mer analog. Taken together, these studies resulted in the identification of *cyclo*[EKTOVNOGN] as the optimal ligand; this molecule is called AFPep (see Fig. 1).

Design and synthesis of anticancer drugs

The development of AFPep featured several concepts of peptide design that may be useful in the development of other peptide drugs. Notably, the process began with a naturally occurring biologically active protein, as opposed to identification of lead compounds via high-throughput screening of libraries of natural or synthetic molecules. Many useful drugs (e.g., tamoxifen) have been developed through synthesis of small organic molecules and others had their origin in natural products (e.g., taxol). However, drawbacks are often associated with the development of xenobiotic drugs that have no counterpart in mammalian physiology, especially toxicity associated with the introduction of a foreign molecule. Toxicity may be unavoidable for cytotoxic drugs intended for use as anticancer therapeutics but for drugs intended to serve as preventive agents, efficacy without toxicity is required. Peptides may be one of only a few ways to achieve efficacy without toxicity.

That AFPep is a peptide rather than a small organic, xenobiotic molecule is an important design feature that offers substantial benefit for a potential drug. One recent review cataloged 60 different peptide drugs (61) that are in clinical use. Peptides are advantageous in that they can be exquisitely specific for their intended receptor (see below) more so perhaps than analogs of xenobiotic organic molecules. This specificity would be expected to manifest itself in mitigation of side effects since only a very limited number of targets would be activated by a well-designed peptide ligand and the activation would re-engage self-promoting regulatory circuits. Peptides, when metabolized, yield simple amino acids which are not toxic, unlike xenobiotic molecules that may become even more toxic when they undergo oxidation by liver enzymes.

Another design feature of AFPep is that, intentionally, it is a cyclized peptide that contains no moieties other than natural amino acids (i.e., it is not a peptidomimetic). The peptide is cyclized by means of a head-to-tail peptide bond (not entirely unprecedented but arguably greatly underutilized in drug development), rather than a side chain disulfide bond or other non-amide bond (which may be non-metabolizable). That it is cyclized is an important design feature resulting in drug stability, both in terms of shelf-life (58) and in the sense that the cyclized peptide is not subject to exopeptidases; it is active after oral administration (62). The cyclized 9-mer is hydrodynamically smaller than a linear 9-mer peptide and is not immunogenic. This molecule is the basis of subsequent studies described in this review except where noted; second-generation analogs are described below to

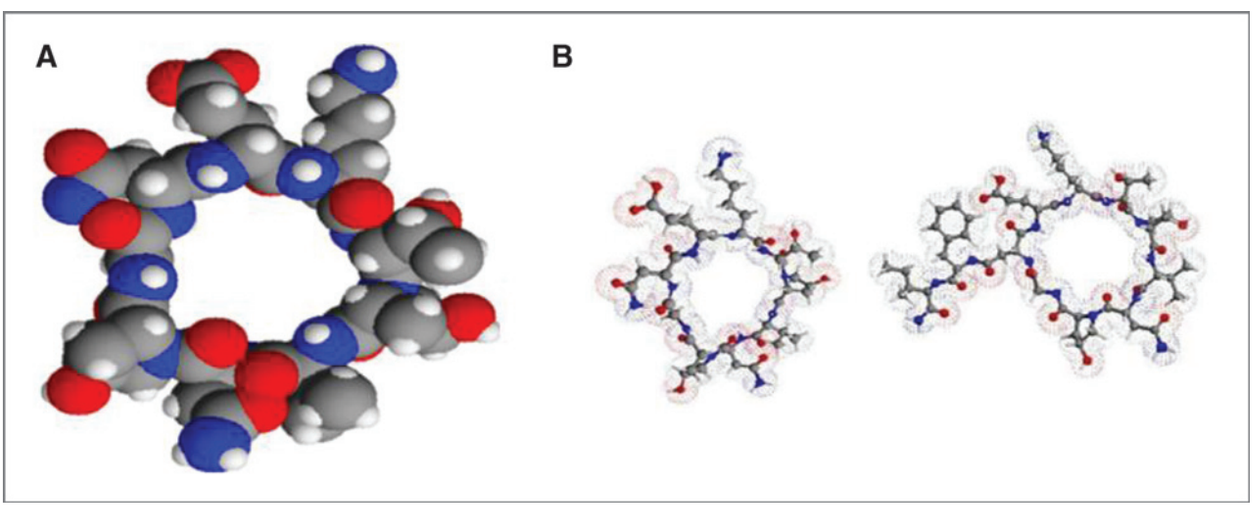

Figure 1. AFPep and analogs. A, space-filling model of AFPep, a cyclic 9-amino acid peptide fragment of AFP, the sequence of which is *cyclo*[EKTOVNOGN]. The Glu (E) side chain is in the 12 o'clock position with the remaining amino acids in clockwise order. As described in the text, hydroxyproline (O) was substituted for proline (found in human AFP) to enhance stability and solubility; lysine (K) was substituted for the naturally occurring methionine to enhance synthesis and activity (59). B, ball-and-stick model of AFPep (left) and a ring-and-tail analog (right). The molecules have the same orientation as shown in A. The ring-and-tail analog is *cyclo*[EKTOVNOGN]FI, in which the FI (Phe-Ile) side chains point to the left, just under the glutamate side chain. These amino acids are attached to the side chain of asparagine and are not part of the ring of peptide bonds.

illustrate the exquisite specificity that is achievable with peptide drugs.

Anticancer Activity of AFPep

As the anti-oncogenic, anti-estrotrophic active site of AFP, the cyclic 9-mer peptide AFPep has substantial biologic activity that suggests it may be useful in the fight against breast cancer. AFPep is efficacious *in vitro* and *in vivo* for therapy and prevention of breast cancer, is well tolerated, and has a unique mechanism of action.

AFPep is efficacious

AFPep is active as a therapeutic agent. Bennett and colleagues (63) compared the activity of AFPep to tamoxifen for activity against growth of human breast cancer xenografts in immunodeficient mice. Either agent (AFPep or tamoxifen) completely stopped the growth of estrogen-dependent breast cancer xenografts; both MCF-7 and T47D cell lines stopped growing when treated with either agent. Neither agent slowed the growth of estrogen-independent MDA-MB-231 human breast cancer xenografts (63). Importantly, AFPep completely stopped the growth of a subline of MCF-7 that had been made resistant to tamoxifen by repeated culture passage in the presence of tamoxifen. This subline showed substantial growth in the presence of tamoxifen but could not grow in the presence of AFPep (63). This observation may portend utility for AFPep among women who have discontinued, or have rejected, tamoxifen use. A clinically worrisome side effect of tamoxifen is its hypertrophic effect on the uterus. In this study (63), tamoxifen stimulated the growth of the immature mouse uterus *in vivo*, whereas AFPep did not exhibit this uterotrophic effect and, in fact, inhibited the uterotrophic effect due to tamoxifen which suggests a clinical opportunity for combination of AFPep with tamoxifen.

AFPep is active as a preventive agent. Parikh and colleagues (64) used the MNU model for carcinogen-induced mammary cancer to explore the preventive potential of AFPep. Methyl nitroso urea (MNU) was given to 50-day-old female rats, followed by administration of AFPep for 23 days (a duration chosen to mimic pregnancy). AFPep resulted in a reduction (40%) in cancer incidence and in tumor burden (60%), similar to results seen for tamoxifen. Significantly longer mean tumor-free days and lower tumor multiplicity were observed for AFPep-treated groups (64). AFPep prevented breast cancer in a dose-dependent manner and was effective after treatment durations as short as 10 days or as long as 30 days (65). These studies suggest that AFPep can be used as a chemopreventive agent to reduce the incidence of breast cancer in individuals at high risk for development of this disease. The high safety index of AFPep (see below) further supports its use as a chemopreventive agent, especially since it appears to be gravidomimetic in that it co-opts natural mechanisms to reduce the incidence of breast cancer. Further study of the chemoprevention capabilities of AFPep, using more relevant models, are needed because the impact of the opportunity to prevent breast cancer could be enormous.

AFPep can be used in combination with tamoxifen. Although tamoxifen is effective for either treatment or prevention of breast cancer, it has toxic drawbacks, and cells sometimes become refractory to its actions, resulting in chemotherapeutic failure. It was therefore of interest to determine whether combining AFPep with tamoxifen would increase efficacy and reduce toxicity of tamoxifen in experimental models of breast cancer. Suboptimal doses of AFPep and of tamoxifen were shown to be more effective when used in combination than when either agents' suboptimal dose was used alone, in 3 assays: inhibition of T47D cell proliferation *in vitro*, inhibition of human tumor xenograft (MCF-7) growth *in vivo* (i.e., therapy mode), or prevention of carcinogen (MNU)-induced tumors in rats (i.e., prevention mode; ref. 65).

AFPep is active after oral administration. Chronic oral administration of AFPep is effective for the treatment or prevention of breast cancer in 3 different animal models, in 2 species, for both immature and adult animals (62). Orally administered AFPep stopped the growth of human tumor xenografts in mice (when used in therapy mode), decreased the incidence and multiplicity of breast cancers in carcinogen-exposed rats (when used in prevention mode), and inhibited estrogen-stimulated growth of mouse uteri (when screened for anti-estrotrophic activity). In each of these models, orally administered AFPep produced effects similar to those obtained for AFPep administered by either intraperitoneal or subcutaneous routes and at the same doses.

AFPep is well tolerated

Existing drugs for treatment of breast cancer are tolerable but are not without significant toxicity, especially if used for long periods of time. Advantages of a homobiotic agent such as AFPep are that it may be designed to be less toxic to have fewer side effects than do xenobiotic molecules. AFPep has shown no toxicity or side effects in any model tested to date (62, 64–66). In female rats exposed to therapeutic doses of AFPep (ranging between 100 and 300 μg/rat/d) for various durations (between 23 and 45 days), toxicity endpoints were assessed 65 days after the onset of treatment (64). There was no effect on animal body weight at any time during the study, no effect found at necropsy on weight of organs, including liver, heart, or uterus. Using standard toxicity screens in mice, animals were treated with a single bolus injection of 10 mg/mouse, i.v. (1,000 times the effective dose of AFPep, which is 10 μg/mouse), or with daily i.v. injections of 2 mg AFPep/mouse for 5 days. The experiment was terminated 10 days after treatment with 10 mg of AFPep or 1 day after the final treatment with 2 mg AFPep. There was no effect on body weight, cage activity, or fur texture of animals during the study, and at necropsy, it was seen that there was no effect on weights of liver, heart, spleen, kidney, or uterus (64). Similar results were seen in studies in which AFPep was administered by oral routes (62).

Since AFPep is anti-estrogenic, it was essential to assess its effects on the female reproductive cycle and fertility (66). Ten cycling female Sprague-Dawley rats (age, 81 days) were given 100 μg AFPep in saline s.c. daily for 20 days. A second

group of 10 rats was given 50 μg tamoxifen s.c. daily and a third group received saline only. Vaginal smears were obtained twice per day and stained to assess estrous cycle phase. After completion of estrous cycle assessment (5 cycles, 21 days), rats were maintained on drug and allowed to mate. Effects on birth of offspring and maternal body weights were assessed. AFPep had no significant effect on the incidence or duration of any estrous cycle phase and no effect on reproductive potential or maternal body mass. AFPep did not affect the number of pups per litter or the mass of the pups. Tamoxifen had pronounced effects on the estrous cycle and fertility in rats. It significantly increased the length of diestrus, locking the cycle in this phase for most animals. Only half of the tamoxifen-treated rats mated, and none became pregnant. Tamoxifen significantly slowed the rate of body mass increase relative to the other groups. The results of this study (66) demonstrate that the estrous cycle, fertility, and fecundity of the rat are not disrupted by AFPep. This is critical for the development of AFPep as an acceptable chemopreventive, as well as a therapeutic, agent for breast cancer in women. Ideally in chemoprevention, one would require efficacy without toxicity (including no threat to reproductive potential) for an agent that is being given to women who are presumably healthy.

AFPep has a unique mechanism of action

AFPep is different mechanistically from the drugs currently in use to treat breast cancer. Cytotoxic drugs inhibit DNA synthesis and replication, whereas endocrine agents interfere with the production of estrogen or the binding of estrogen to its receptor. AFPep does neither of these but does interfere with the phosphorylation of the ER, particularly at Ser 118 (64) but not at Ser 167 or Ser 104 (62). Unlike tamoxifen, AFPep does not alter the number of binding sites or their affinity for radiolabled estrogen (65, 67, 68). AFPep likely binds to receptors on the plasma membrane of target cells, presumably the same receptors to which AFP binds. Sierralta and Torres and colleagues studied cellular responses and mechanism in a variety of ER^+ and ER^- cells, notably including canine mammary cancer cells, which respond like human mammary cancer cells (67–70). They found that AFPep inhibits estrogen-induced proliferation of MCF-7, T47D, and ZR75-1 breast cancer cells, but not of ER^- cells. In human ER^+ breast cancer cells (MCF-7 and ZR75-1), increased expression of p21Cip1 suggested that AFPep slows cell proliferation via that regulator (67). AFPep interfered markedly with the regulation of mitogen-activated protein kinase (MAPK) by activated c-erbB2; it showed no effect on estrogen-stimulated release of matrix metalloproteinases 2 and 9 (69). Estrogen induced the proliferation of canine mammary cancer cells as well as phosphorylation of extracellular signal–regulated kinase (ERK)1/2 and HER2 immunoreactivity, whereas AFPep inhibited all of these functions (70).

Drug Development: Efficacy without Toxicity

Studies on AFPep have been focused on development of a nontoxic agent that is useful for the prevention or treatment of breast cancer. The impact of these studies is best articulated by the phrase "efficacy without toxicity." Major limitations for most therapeutic agents include dose-limiting toxicity and chemotherapeutic failure, whereas for preventive agents, the almost insurmountable barrier has always been toxicity. While few believe that efficacy without toxicity is achievable, we posit that it is too soon to give up on that goal. That goal is essential, and one way to achieve it is to develop peptide drugs that (i) provide exquisite specificity necessary to limit side effects, (ii) eventually get metabolized solely to byproducts (amino acids) that are nontoxic, and (iii) deliver sustained efficacy because they mimic the action of naturally occurring proteins while regulating biologic response. An orally active peptide that is truly nontoxic, one that is derived from a natural protein and which co-opts the biologic mechanisms of that protein, one that is so efficacious that it can be used at concentrations that are *lower* than those of the natural parent protein from which it was derived, and one that does not succumb to chemotherapeutic or chemopreventive failure, may have a transformative impact on prevention and therapy.

By way of demonstrating the potential for exquisite specificity of peptide analogs, we designed analogs of the cyclic AFPep molecule that bore a short (one or two amino acids) "tail" extending out from the ring, distal to the pharmacophore (Fig. 1; ref. 71). Analogs that bore a tail of either 1 or 2 amino acids, either of which had a hydrophilic moiety in the side chain (e.g., *cyclo*[EKTOVNOGN]FS) exhibited greatly diminished biologic activity (inhibition of estrogen-stimulated uterine growth) relative to AFPep. Analogs that bore a tail of either 1 or 2 amino acids which had exclusively hydrophobic (aliphatic or aromatic) side chains (e.g., *cyclo*[EKTOVNOGN]FI) retained (or had enhanced) growth inhibition activity. These results were useful to explore the concept of a 2-receptor model for binding of AFPep and ring-and-tail analogs (71) but also serve to illustrate that it is possible, using amino acids exclusively, to target homologous receptors such that a single, desired target may be activated. It is thought unlikely that xenobiotic molecules could achieve this level of specificity while simultaneously exhibiting lack of toxicity. We suggested (71) that tails on cyclic peptides may comprise a generally useful method to enhance diversity of peptide design and specificity of ligand–receptor interactions.

AFPep is not a cytotoxic agent designed to kill cells, nor does it shut down hormonal control as agents such as tamoxifen or aromatase inhibitors are designed to do. AFPep does not use an all-or-none approach to modulation of cell growth. AFPep modulates the biologic responses to estrogen in a natural manner, tamping down the growth response much as AFP does for the fetus *in utero*. Because AFPep utilizes a natural regulatory pathway, it may be less likely than are other drugs such as tamoxifen to activate alternate, parallel signaling pathways and thus engender chemotherapeutic or chemopreventive failure. AFPep employs an innovative strategy: it should not be necessary always and everywhere to kill cancer cells or shut down their metabolism completely;

it should be possible to use natural pathways to induce quiescence and maintenance of nonmalignant, nonmetastatic states. The idea is to diminish growth response of tumors or breast cancer stem cells such that their progression to clinical cancer is delayed by 10, 20, or 30 years. This would be analogous to the increased latency seen in the carcinogen-exposed rat cancer prevention data: delay of tumor appearance by several weeks in that short-duration model may be similar to delay by several years in women using an optimized drug administration schedule. This would constitute effective prevention of breast cancer and would contribute importantly to eradication of the disease. The paradigm is to use preventive interventions before establishment of cancer as propensity toward disease may be more responsive to pharmacologic intervention than is established disease. AFPep has substantial potential to reengage intrinsic pathways of growth control and stop the development of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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