Published in final edited form as:

Int J Pept Res Ther. 2010 ; 16(1): 23–30. doi:10.1007/s10989-010-9198-8.

Anti-Tumoral Activity of a Short Decapeptide Fragment of the Alzheimer's A β Peptide

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Abstract

The inhibition of angiogenesis is regarded as a promising avenue for cancer treatment. Although some antiangiogenic compounds are in the process of development and testing, these often prove ineffective *in vivo*, therefore the search for new inhibitors is critical. We have recently identified a ten amino acid fragment of the Alzheimer A β peptide that is anti-angiogenic both *in vitro* and *in vivo*. In the present study, we investigated the antitumoral potential of this decapeptide using human MCF-7 breast carcinoma xenografts nude mice. We observed that this decapeptide was able to suppress MCF-7 tumor growth more potently than the antiestrogen tamoxifen. Inhibition of tumor vascularization as determined by PECAM-1 immunostaining and decreased tumor cell proliferation as determined by Ki67 immunostaining were observed following treatment with the A β fragment. *In vitro*, this peptide had no direct impact on MCF-7 tumor cell proliferation and survival suggesting that the inhibition of tumor growth and tumor cell proliferation observed *in vivo* is related to the antiangiogenic activity of the peptide. Taken together these data suggest that this short A β derivative peptide may constitute a new antitumoral agent.

Keywords

Breast cancer; A β peptides; Alzheimer; Angiogenesis; Tumor xenograft; MCF-7

Introduction

Despite multiple advances in the treatment for breast cancer, the mortality rate remains high. The American Cancer Society estimates that over 182,000 new cases of invasive breast cancer are expected to occur among women in the US every year. An estimated 40,500 breast cancer deaths are expected yearly in the US. Breast cancer is therefore the most common female cancer in the United States and the second most common cause of cancer death in women. Surgical removal of the tumor is usually the first step in treating localized breast cancer unless the breast tumor is large or locally invasive. After surgery, systemic therapy is often recommended to decrease the likelihood that the cancer will return. Approximately 60–80% of these cancers are positive for estrogen receptors and therefore are candidates for hormone therapy based on estrogen antagonists and aromatase inhibitors.

The selective estrogen receptor modulator tamoxifen and its active metabolite 4-hydroxy-tamoxifen have been shown to be an effective adjuvant therapy for estrogen dependent tumors

by reducing the incidence of contralateral breast cancer by 50% (Early Breast Cancer Trialists' Collaborative Group 1992). In addition, tamoxifen has been introduced as a prophylactic drug for the prevention of breast cancer in high-risk women (Fisher et al. 2005). Approximately 50% of patients benefit from this therapy but almost all responsive tumors eventually relapse due to the development of tamoxifen resistance (Howell et al. 1990). Although the net benefits of tamoxifen have been proven, the risk of tamoxifen-resistant recurrent cancer is problematic (Rutqvist et al. 1995). Approximately 40% of patients with estrogen-responsive breast cancer die due to the recurrence of breast cancer after hormone therapy (Osborne 1998). Acquired resistance to tamoxifen is a serious problem, and major efforts are now being made to understand the underlying mechanisms responsible (Clarke et al. 2001).

New approaches for the treatment of breast cancer are therefore urgently needed. Recent advances in cancer research have elucidated new treatment strategies based on the use of angiogenesis inhibitors and have gained considerable attention in the cancer field. This is primarily due to several advantages that antiangiogenic therapy has over conventional chemotherapy which include selective targeting of tumor-associated vasculature resulting in relatively few adverse side effects; endothelial cells forming the tumor vasculature are easily accessible to antiangiogenic agents delivered via the blood; and resistance to antiangiogenic agents is unlikely to occur because endothelial cells are genetically stable and homogeneous. Unfortunately, angiogenesis inhibitors often show poor efficacy in vivo underscoring the need for the development of better antiangiogenic compounds.

Extracellular fibrillar β -amyloid ($A\beta$) deposits constituting senile plaques are prominent and universal Alzheimer's disease (AD) pathological features (Haass and Selkoe 2007). $A\beta$ peptides also form some deposits in the vessel walls of most AD patients and may exert a pathologic effect on the brain vasculature by destroying smooth muscle cells, decreasing capillary density and creating a cascade of negative impacts on cerebral blood flow contributing to the pathophysiology of AD (Paris et al. 2003, 2004a; Meyer et al. 2008; Matsuda et al. 2007). We have shown previously that pathological levels of soluble forms of $A\beta$ peptides are antiangiogenic in a multitude of in vitro and in vivo experimental models and antagonize both VEGF and bFGF induced angiogenesis (Paris et al. 2004b, 2005; Patel et al. 2008). Others have also replicated such findings (Drago et al. 2007; Donnini et al. 2006; Solito et al. 2009) and the hypothesis that an aberrant angiogenesis process may contribute to AD dementia is now emerging (Zlokovic 2005; Watson et al. 2005; Wu et al. 2005; Deane and Zlokovic 2007).

We have recently identified that a short fragment of the $A\beta$ peptide is antiangiogenic both in vitro and in vivo in the rat cornea model of angiogenesis (Patel et al. 2008). In the present report, we investigated the potential antitumoral activity of this short decapeptide derivative (EVHHQKL VFF) of $A\beta$ on the growth and vascularization of estrogen-dependent human breast tumor xenografts in nude immunodeficient mice.

Methods

Peptides

The $A\beta$ decapeptide derivative EVHHQKL VFF (M.W = 1,283) and a scrambled control peptide FQVHLFKH (deprived of antiangiogenic activity, data not shown) were obtained by custom synthesis from Quality Controlled Bio-chemicals (MA, USA) with a purity greater than 95%.

Cell Proliferation and Cell Toxicity Assays

For cell proliferation assays, MCF-7 cells (5×10^4 cells per well) were purchased from ATCC (MD, USA) and seeded in a clear sterile cell culture 96 well plate (Corning, MA) and allowed to attach for 18 h in EMEM supplemented with 10% fetal bovine serum (ATCC, VA). Cells were then washed once with PBS and medium replaced with EMEM 1% serum. Cells were incubated for 24 h with control (DMSO), a dose range of the peptide EVHHQKL VFF, or 4-hydroxy tamoxifen (positive control). Following 24 h of treatment, the medium surrounding the cells was collected and used to assess cytotoxicity using a Lactate Dehydrogenase (LDH) assay (Roche Diagnostics, Germany) according to the manufacturer's protocol. Cellular proliferation was estimated by monitoring the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenase using the Quick Cell Proliferation Assay Kit and the recommendations of the manufacturer (Biovision Inc., CA).

Capillary Morphogenesis Assay

Human Brain Microvascular Endothelial Cells (HBMEC) (Sciencell, CA) were cultured in Endothelial Cell Growth Medium (Cell Applications, CA) containing 5% fetal bovine serum, 1% penicillin/streptomycin and 1% Endothelial Cell Growth Supplement (Sigma-Aldrich, MO). HBMEC (7.5×10^4 cells/ml) in 500 μ l of medium were seeded in 24-well plates, on top of a layer of Matrigel basement membrane matrix (Invitrogen, CA) in F12K medium (ATCC, VA) containing 4% serum (Invitrogen, CA), 0.1 mg/ml Heparin and 0.03 mg/ml endothelial cell growth supplement. Cells were incubated with treatment (dose range of 4-hydroxy tamoxifen) or control conditions for 24 h. Capillary network formation experiments were performed in quadruplicate, and at least two randomly chosen fields were photographed for each well using a 4 $\times$ objective. Capillary length was measured using the Image Pro Plus software (Media Cybernetic, Inc., MD).

MCF-7 Tumor Xenograft Model

All experimental procedures involving animals were approved by the IACUC committee of the Roskamp Institute. MCF-7 tumor cells were purchased from ATCC (MD, USA) and cultured under standard conditions (5% CO₂, 95% humidity, 37°C) in EMEM supplemented with 10% fetal bovine serum, 1 mM glutamine and 1 $\times$ penicillin–streptomycin–fungizone mixture. Following trypsinisation, tumor cells were resuspended in 50% Matrigel/DMEM and injected (3 million/injection site) subcutaneously into the right and left flank of nude immunodeficient female mice that were previously implanted with a 90 days release subcutaneous biodegradable pellet (1.7 mg/pellet) of 17 β -estradiol (Innovative Research of America, FL, USA) in order to sustain the growth of MCF-7 tumors. Tumor volumes were evaluated using an electronic caliper and the following formulae: tumor volume (mm³) = tumor width² (mm²) $\times$ tumor length (mm)/2. When tumors reached approximately 150 mm³, mice were treated daily with an intraperitoneal injection of 100 μ L scrambled A β fragment (Placebo) at 50 mg/kg of body weight dissolved in PBS or with 100 μ L of EVHHQKL VFF (A β fragment) dissolved in PBS (50 mg/kg of body weight) or were implanted subcutaneously with a 60 days release biodegradable pellet (Innovative Research of America, FL, USA) containing 5 mg of tamoxifen, until the completion of the study.

Immunohistochemistry

After being collected, tumor xenografts were fixed in 4% paraformaldehyde in PBS and were infiltrated with paraffin using a Tissue-Tek instrument (Sakura, USA). The specimens were then cut into 6- μ m-thick sections for immunostaining. Sections were deparaffinized in xylene, rehydrated in an ethanol to water gradient, and then incubated in blocking buffer (Protein Block Serum-free, DakoCytomation) for 20 min. Diluted antibodies were applied overnight on the sections at 4°C. The PECAM-1 antibody (clone MEC 13.3 from BD Biosciences, NJ) was

diluted 1:40, and Ki-67 antibody (Epitomics, CA) was diluted 1:200. Endogenous peroxidase activity was quenched with a 20-min-H₂O₂ treatment (0.3% in PBS), sections were rinsed and antibodies were detected using the avidin-peroxidase complex from Vectastain ABC Elite kit (Vector Laboratories). Labeling was revealed with 3,3-diaminobenzidine and sections were counterstained with hematoxylin. Each staining was performed on 4–5 sections and captured with a digital camera. The area occupied by PECAM-1 staining was evaluated using Image-Pro Plus software (Media Cybernetics, MD). The number of Ki-67-positive cells was counted in seven 40×-magnified fields randomly selected within each section, and an average value was calculated. The mitosis count was performed similarly to the Ki-67-positive cells count using hematoxylin-eosin stained sections.

Statistics

Statistical analyses were carried out using SPSS software, version 12.0.1. Differences with a *P* value <0.05 were considered statistically significant.

Results

Effect of the A β Derived Decapeptide on Tumor Cell Proliferation and Survival In vitro

Different culture conditions were tested to determine the possible cytotoxic and antiproliferative activities of the A β derivative decapeptide on human MCF-7 breast cancer cells. Proliferation was evaluated by quantifying the amount of live cells using the Quick Cell proliferation assay whereas toxicity was determined by monitoring the release of lactate dehydrogenase in the culture medium surrounding the cells following 24 h of treatment with different doses of peptide. We first evaluated the effect of a dose range of the decapeptide (1–20 μ M) using regular culture conditions employing 10% fetal calf serum. No toxic or antiproliferative effects of the peptide were observed following 24 and 48 h of treatment for any of these doses (data not shown). We next investigated the effect of the decapeptide using a culture medium containing a low serum concentration (1%) and supplemented with 17- β -Estradiol. Following 24 h of treatment, the in vitro assays showed that the A β derived decapeptide did not induce toxicity nor did it significantly inhibit the proliferation of human MCF-7 breast cancer cells (Fig. 1). Similarly, in the absence of 17- β -Estradiol in the culture medium no significant toxicity or antiproliferative activity was observed following the treatment of MCF-7 cells with the A β derived decapeptide for 24 h (Fig. 1). 4-hydroxy tamoxifen was used as a positive control in the assays and as expected significantly induced cellular toxicity and reduced the amount of live cells following 24 h of treatment. Altogether, these data suggest that the A β derived decapeptide does not exert direct toxic or antiproliferative effects on MCF-7 tumor cells in vitro contrary to tamoxifen.

Effect of the A β Derived Decapeptide on the Growth and Vascularization of Human Breast Carcinoma MCF-7 Tumor Xenografts

Human MCF-7 breast carcinoma cells were injected subcutaneously in the right and left flank of nude mice, previously implanted with a pellet of 17 β -Estradiol since the growth of MCF-7 tumors is estrogen dependent (Lehnes et al. 2007). MCF-7 tumor volumes were first measurable 42 days after the implantation of MCF-7 cells. Treatments were initiated 59 days after the implantation of MCF-7 cells when the tumors reached an approximate volume of 150 mm³. In this tumor xenograft model both tamoxifen and the A β derivative decapeptide inhibited the growth of MCF-7 tumors in nude mice: 74.3 $\pm$ 29.6 mm³ in decapeptide-treated (50 mg/kg/day) and 159.5 $\pm$ 64.3 in tamoxifen-treated (4.6 mg/kg/day), versus 328.7 $\pm$ 62.9 in control animals at day 74 (Fig. 2). There were no weight differences between the animal groups suggesting that none of the treatments were eliciting overt toxicity (data not shown). Interestingly, at 50 mg/kg the A β derived decapeptide appears more potent than tamoxifen in this tumor xenograft model, and leads to the regression of tumors.

The tumor xenografts were further analyzed for the extent of angiogenesis by evaluating the vascularization of the tumors following PECAM-1 immunostaining. Both tamoxifen and the A β derivative decapeptide appeared to significantly reduce PECAM-1 stained positive microvessels (Fig. 3). In addition reduction of Ki67 immunopositive proliferative tumor cells following tamoxifen and the A β derivative decapeptide treatments were observed (Fig. 4). Similarly, mitosis counts were reduced in tumor sections from tamoxifen and decapeptide treated animals compared to animals treated with the placebo. Since a reduced vascularization was observed in the MCF-7 tumor xenografts following tamoxifen treatment, we investigated the effect of dose range of tamoxifen on angiogenesis using an in vitro capillary morphogenesis assay. Only at the highest dose of tamoxifen tested (10 μ M) which is approximately 20 times higher than the plasma level detected in patients treated with tamoxifen (Daniel et al. 1979), a slight inhibition of capillary formation was observed and was associated with cellular toxicity (Fig. 5).

Discussion

The studies described in this article were conducted to determine whether a small derivative decapeptide of the Alzheimer A β peptide, that we previously identified as an antiangiogenic peptide (Patel et al. 2008), may also have antiangiogenic and antitumoral properties in a human breast tumor xenograft model. We first determined whether the A β derivative decapeptide was able to affect the proliferation or survival of human MCF-7 breast cancer cells in vitro. Even high doses of the decapeptide (20 μ M) were unable to significantly impact the proliferation or viability of MCF-7 cells both in the presence or absence of estrogen whereas the antiestrogen tamoxifen was cytotoxic to MCF-7 tumor cells. These data suggest that the decapeptide does not display direct toxic or antiproliferative activities on MCF-7 cells. In vivo, the decapeptide appeared to potently suppress the growth of MCF-7 tumor xenografts. Decreased tumor vascularization, as evidenced by reduced PECAM-1 immunostaining, was observed following treatment with the decapeptide whereas no direct cytotoxic or antiproliferative effects of the decapeptide were observed in vitro on MCF-7 cells suggesting that the peptide is halting tumor growth by inhibiting angiogenesis. By contrast, tamoxifen appears to directly impact the proliferation and viability of MCF-7 cells in vitro. A reduction of MCF-7 tumor xenografts vascularization was also observed following tamoxifen treatment but no significant effect of tamoxifen on in vitro angiogenesis was detected with physiologically relevant concentrations suggesting that the reduced vascularization observed in MCF-7 tumor xenografts is related to the impact of tamoxifen on tumor cell growth and viability.

Measurement of proliferation in breast tumors provides a strong prognostic factor as proliferation plays an important role in the clinical behavior of invasive breast cancer and increased proliferation correlates strongly with a poor prognosis (van Diest et al. 2004). Tumor expression of the proliferation antigen Ki67 is widely used to assess prognosis of cancer patients. Change in Ki67 expression is also used as a pharmacodynamic marker of efficacy and decreased Ki67 expression has been observed following endocrine therapy of breast tumor with tamoxifen (Dowsett et al. 2007). We observed that both tamoxifen and the decapeptide treatment lead to a significant reduction of Ki67 immunostaining in MCF-7 tumor xenografts. The fact that the decapeptide does not present any cytotoxic or antiproliferative activity towards MCF-7 tumor cells in vitro suggests that the decreased proliferation observed in the A β decapeptide treated tumor xenografts is secondary to an inhibition of angiogenesis induced by the peptide.

We have previously shown that the full length A β peptide (42 amino-acids) which accumulates in the brain and cerebrovessels of AD patients displays some antiangiogenic properties and is potent enough to inhibit the growth and vascularization of human lung adenocarcinoma and glioblastoma tumor xenografts (Paris et al. 2004a, b, 2005). Although the physiological

function of A β still remains unknown, our data clearly suggest that high levels of soluble A β are antiangiogenic. A β levels appear elevated in the blood circulation of patients before the development of AD (Abdullah et al. 2007, 2009; Blasko et al. 2008; Ringman et al. 2008) and interestingly, the risk of developing cancer in AD patients is lower than in non-demented patients and reciprocally, the risk of developing AD is lower in patients with a history of cancer (Roe et al. 2005). Epidemiological studies have also shown that cancer-related deaths are significantly underrepresented in patients with AD (Ganguli et al. 2005). In addition, previous cross-sectional and case-control studies have suggested that the prevalence of cancer is lower in people with a diagnosis of AD (DeSouky 1992; Yamada et al. 1999; Tirumalasetti et al. 1991; Yashin et al. 2009) which could suggest that a common biological mechanism impacting many tumor types underlies this intriguing relationship. We suggest that the anti-angiogenic role of A β may be one such mechanism.

In the present study, we extended our previous work by investigating the potential antitumoral and antiangiogenic activity of a decapeptide fragment of A β on the growth and vascularization of MCF-7 tumor xenografts. This and related peptides may represent novel therapeutic molecules but clearly further work is required to determine the pharmacokinetic characteristic of the peptide and whether peptide modifications can improve its stability and efficacy in different tumor models.

Acknowledgments

This work was supported by NIH/NIA grant #R01A619250. We would like to thank Diane and Robert Roskamp for their generosity in helping to make this work possible.

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Fig. 1.

a Effect of the short A β derivative decapeptide EVHHQKL VFF on the proliferation of MCF-7 breast carcinoma cells. ANOVA revealed significant main effect of tamoxifen ($P < 0.001$) but not for the A β derivative decapeptide ($P = 0.752$) on MCF-7 cell proliferation. Post hoc analysis showed significant differences between the control conditions, tamoxifen at 5 μ M ($P < 0.05$) and tamoxifen at 10 μ M ($P < 0.001$) and between estradiol and estradiol + tamoxifen at 10 μ M ($P < 0.001$) showing that in absence of estradiol, tamoxifen inhibits the proliferation of MCF-7 at 5 and 10 μ M whereas in the presence of estradiol, 10 μ M of tamoxifen are required to significantly inhibits the proliferation of MCF-7 cells. **b** Effect of the short A β derivative decapeptide EVHHQKL VFF on the viability of MCF-7 breast carcinoma cells. ANOVA

revealed significant main effect of tamoxifen ($P < 0.001$) on cellular viability (measured by the release of lactico dehydrogenase (LDH)) but no significant main effect of the decapeptide ($P > 0.05$). Post hoc comparisons showed significant differences between the control conditions and tamoxifen at 10 μM ($P < 0.001$) and between the estradiol treatment and estradiol + tamoxifen at 10 μM ($P < 0.001$) showing that tamoxifen is cytotoxic at 10 μM

Fig. 2.

Effect of the short A β derivative peptide EVHHQKLVFF on the growth and vascularization of human MCF-7 breast carcinoma tumor xenografts in immunodeficient mice. **a** Representative pictures showing the tumor masses on the right and left flank of the animals following treatment (from *left to right*) with 50 mg/kg of body weight/Day of a scrambled control peptide (placebo), treatment with the antiestrogen tamoxifen and treatment with 50 mg/kg/Day of EVHHQKLVFF peptide. **b** Tumor volumes were evaluated every 3 days with an electronic caliper when the tumors became palpable (42 days after the implantation of MCF-7 cells) until the completion of the study (day 74 following the implantation of MCF-7 cells). Treatments were initiated on day 59 (after MCF-7 cells implantation) when the tumors reached

an approximate volume of 150 mm³ and animals were treated daily as indicated above for a period of 15 days. ANOVA revealed statistically significant main effect of treatments ($P < 0.001$) and post hoc analyses revealed significant differences between the tumor volumes in the scrambled peptide treatment group and in the tamoxifen treatment group ($P < 0.03$), between the scrambled peptide treatment and the EVHHQKL VFF peptide treatment ($P < 0.001$) and between the tamoxifen treatment and the EVHHQKL VFF peptide treatment ($P < 0.001$) showing that the EVHHQKL VFF potentially suppresses the growth of MCF-7 tumor xenografts

Fig. 3.

a Representative pictures of PECAM-1 immunostained tumor sections ($\times 40$ magnification) for the different treatment groups (from left to right: scrambled peptide treatment (placebo), tamoxifen treatment and EVHHQKLVEF peptide treatment), **b** The histogram represents the vascularization (PECAM-1 burden expressed as a % of the area examined) of the tumors for the different treatment groups. ANOVA revealed statistically significant effect of treatments ($P < 0.01$) and post hoc analysis showed significant differences between the scrambled peptide treatment and the EVHHQKLVEF peptide treatment ($P < 0.001$), between the scrambled peptide and tamoxifen ($P < 0.003$) but no significant differences between the EVHHQKLVEF peptide and tamoxifen ($P = 0.072$)

Fig. 4.

a Representative pictures of hematoxylin-eosin stained tumor sections ($\times 100$ magnification) revealing the presence of mitosis and of Ki-67 immunostained tumor sections ($\times 20$ magnification) for the different treatment groups. **b** The histogram represents the average number of mitosis and number of Ki-67 immunostained cells observed per microscopic field ($\times 60$ magnification) for the tumor sections of the different treatment groups. ANOVA revealed statistically significant effect of treatments ($P < 0.001$) and post hoc analysis showed significant differences between the scrambled peptide treatment and the EVHHQKLVFF peptide treatment ($P < 0.001$), between the scrambled peptide (placebo) and tamoxifen ($P < 0.05$) but no significant difference between the EVHHQKLVFF peptide and tamoxifen ($P = 0.089$) for

Ki67 immunostaining. Similarly, statistical significant main effect of the treatments ($P < 0.001$) and statistically significant differences were observed following post hoc analysis for the mitosis count between the scrambled peptide and the EVHHQKLVFF treatment ($P < 0.001$), between the scrambled peptide and tamoxifen treatment ($P < 0.001$) but no significant difference between tamoxifen and EVHHQKLVFF treatment ($P = 0.179$)

Fig. 5.

Effect of 4-hydroxy tamoxifen on angiogenesis in vitro. **a** Representative pictures depicting the formation of capillary networks in response to a dose range of 4-hydroxy tamoxifen following the plating of human brain microvascular endothelial cells onto a layer of Matrigel. **b** The graph represents the quantification of capillary network length by image analysis and the amount of LDH activity detected in the culture medium surrounding the capillary like structures following 24 h of treatment with a dose range of 4-hydroxy tamoxifen. ANOVA revealed a significant main effect of 4-hydroxy tamoxifen on LDH released ($P < 0.03$) but no significant main effect on capillary formation ($P = 0.743$) and post hoc analysis showed

significant differences for LDH released between control conditions and 10 μ M of 4-hydroxy tamoxifen ($P < 0.009$)