ARTICLE
Journal of Cellular Biochemistry 110:1142–1146 (2010)

Journal of Cellular Biochemistry

The Effects of Peptides With Estrogen-Like Activity on Cell Proliferation and Energy Metabolism in Human Derived Vascular Smooth Muscle Cells

D. Somjen,[1*] E. Knoll,[1] B. Gayer,[2] R. Kasher,[3] F. Kohen,[2] and N. Stern[1]

[1]*Institute of Endocrinology, Tel-Aviv Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv 64239, Israel*

[2]*Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel*

[3]*Department of Desalination and Water Treatment, Zuckerberg Institute for Water Research, The Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sde-Boqer Campus 84990, Beersheba, Israel*

ABSTRACT

Hormone replacement therapy (HRT) for post-menopausal symptoms in diabetes is associated with increased risk of coronary heart disease and stroke. Therefore, there is a need for new HRT with no adverse effects on diabetic post-menopausal women. We developed peptides as potential estrogen mimetic compounds and now we evaluated the effects of the most efficacious peptide; hexapeptide estrogen-mimetic peptide 1 (EMP-1) (VSWFFE) in comparison to estrogen (E_2) and peptides with weak activity A44 (KAWFFE) and A45 (KRAFFE) on modulation of cell proliferation of vascular smooth muscle cells (VSMC) growing in normal (ng) or high glucose (hg) concentrations. In ng EMP-1-like E_2 inhibited cell proliferation at high concentration, and stimulated at low concentration. EMP-1 did not affect E_2 stimulation of DNA, but inhibited E_2 inhibition of cell proliferation at high concentration. All effects by the combination of EMP-1 and E_2 were abolished at hg. A44-stimulated cell proliferation at all concentrations and A45 had no effect. When A44 was co-incubated with E_2 at both concentrations, DNA synthesis was stimulated, but abolished at hg. A45 abolished E_2 stimulation and inhibition of cell proliferation at both glucose concentrations. All peptides tested except A45-stimulated CK-specific activity at both glucose concentrations. In hg A44 stimulation of DNA was unaffected as well as its inhibition by EMP-1. EMP-1 and A44 similar to E_2-stimulated MAPK activity in ng or hg, suggesting similar mechanism of action. The results presented here suggest that EMP-1 provided it acts similarly in vivo can replace E_2 for treatment of post-menopausal women in hyperglycemia due to diabetes. J. Cell. Biochem. 110: 1142–1146, 2010.

KEY WORDS: ESTROGEN-MIMETIC PEPTIDES; VASCULAR SMOOTH MUSCLE CELLS; CELL PROLIFERATION; CK ACTIVITY; MAPK ACTIVITY

INTRODUCTION

Estrogen is a key regulator of vascular biology. In previous reports we have observed that estradiol-17β (E_2) exerts a biphasic effect on human vascular smooth muscle cells (VSMC) growth, such that low concentrations (in the range observed in pre-menopausal women) stimulate whereas higher concentrations (supraphysiological range) inhibit VSMC proliferation [Somjen et al., 1998]. In a more recent study we have reported that effects of E_2 on cell growth on VSMC are not operative in the presence of a high glucose concentration [Somjen et al., 2004]. Cardiovascular protection in healthy pre-menauposal women is probably due to dual effects of E_2 on VSMC in addition to its other known effects on other cells in the arterial wall and through other factors such as plasma lipoprotein. By contrast, several studies have indicated that a pre-menopausal status does not confer cardiovascular protection in diabetic women [Manning et al., 2003; Scott et al., 2004; Gooding et al., 2005; Bowden et al., 2006; Masding et al., 2006]. Estradiol-17β depletion through menopause or ovariectomy (OVX) leads to altered activity that can be improved by hormone replacement therapy (HRT) which has been reported to modulate vascular function and cardiovascular risk in healthy post-menopausal women. However, treatment of postmenopausal symptoms in diabetes with conventional HRT containing conjugated equine estrogens and medroxyprogesterone acetate causes concerns about increased risk of coronary heart disease and stroke [Scott et al., 2004]. Accordingly, there is a need to develop estrogen-like compounds exhibiting dual effects on VSMC for HRT of diabetic post-menopausal women.

*Correspondence to: D. Somjen, Institute of Endocrinology, Metabolism and Hypertension, Tel Aviv-Sourasky Medical Center, Weizman Street, Tel-Aviv 64239, Israel. E-mail: dalias@tasmc.health.gov.il

Received 12 January 2010; Accepted 23 March 2010 • DOI 10.1002/jcb.22627 • © 2010 Wiley-Liss, Inc.
Published online 19 May 2010 in Wiley InterScience (www.interscience.wiley.com).

We have recently developed synthetic peptides with estrogen-like activity [Venkatesh et al., 2002]. Using a strategy that combines (i) an anti-E_2 monoclonal antibody (mAb-E_2) as a bait for screening a phage-display peptide library [Venkatesh et al., 2002]; (ii) systematic-residue replacement, a new method for peptide affinity optimization, we identified a linear hexapeptide, VSWFFE that we named estrogen-mimetic peptide 1 (EMP-1) [Kasher et al., 2004]. EMP-1 binds monoclonal antibody to E_2 with high affinity ($IC_{50} = 0.006$ nM). It inhibits the binding of ^{3}[H] E_2 to both estrogen receptor α (ERα) and estrogen receptor β (ERβ) ($IC_{50} = 100$ nM) and possesses estrogen-like activity in vitro and in vivo. Moreover, in the OVX mouse model EMP-1 did not prevent bone loss, but enhanced the age-related diminution in growth plate thickness and inhibited longitudinal bone growth, suggesting that EMP-1 can be a lead compound for treatment of acromegaly [Kasher et al., 2009].

As a continuation of our work on estrogenic mimetic peptides, in the present study we explored the effects of EMP-1 and two estrogenic mimetic peptides A44 and A45 on cell proliferation of VSMC in normal and hyperglycemic conditions. Unlike E_2, EMP-1 exhibited dual effects of cell proliferation of VSMC growing under normal or high glucose concentrations and can be considered as a lead compound for HRT in hyperglycemia due to diabetes.

MATERIALS AND METHODS

REAGENTS

All reagents used were of analytical grade: chemicals, steroids, anti-MAP-kinase antibody (clone # ERK-PT115), and goat anti-rabbit peroxidase were purchased from Sigma (St. Louis, MI). General anti-MAP-kinase rabbit antibody [ERK-2 (c-14), sc-154] was purchased from Santa-Cruz Biotechnology (Santa-Cruz, CA). Enhancement solution was purchased from Wallac (Turku, Finland). The europium-chelating agent was a generous gift from I. Hemmilla (Wallac).

PEPTIDE SYNTHESIS AND PURIFICATION

The peptides EMP-1 (VSWFFE), A44 (KAWFFE), and A45 (KRAFFE) were synthesized and subsequently purified by HPLC as described [Kasher et al., 2004].

CELL CULTURES

Human umbilical vascular smooth muscle cells (VSMC). Human umbilical artery VSMC were prepared as previously described with minor modifications [Somjen et al., 1998]. Cells were used only at passages 1–3 when expression of smooth muscle α-actin was clearly demonstrable. To obtain "high glucose" conditions, the medium including the FCS, was supplemented with glucose up to a final concentration of 22 mM (4.5 g/L) whereas in the regular medium was 5.5 mM (1.0 g/L). Cells were grown to sub-confluence (4–7 days) at different glucose concentrations as described [Somjen et al., 2004].

ASSESSMENT OF DNA SYNTHESIS

Sub-confluent cells were treated with various hormones or agents as indicated. Twenty-two hours later, [^{3}H] thymidine was added for 2 h and [^{3}H] thymidine incorporation into DNA was determined as described before [Somjen et al., 2004].

CREATINE KINASE EXTRACTION AND ASSAY

Cells were treated for 4 h with the various hormones and agents as specified, and creatine kinase brain type-specific activity (CK), an established genomic response marker of E_2 was determined as described by a coupled spectrophotometric assay described previously [Somjen et al., 2004]. Protein was determined by Coomasie blue dye binding using BSA as standard.

EUROPIUM-LABELED REAGENTS

Activated anti-MAP-kinase antibody (0.2 mg IgG in 0.9 ml PBS) was dialyzed against 50 mM carbonate/bicarbonate buffer for 2 h. Europium-labeling reagent (300 nmol in 100 μl of water) was then added. The reaction mixture was incubated overnight at 4°C and the labeled protein was purified by gel filtration on Sephadex G-25M. The europium-labeled antibody was eluted with 50 mM Tris–HCl buffer (pH 7.5) and stored at 4°C until use.

PREPARATION OF CELL EXTRACTS FOR MAP KINASE

Each treatment was performed in quadruplet. Cells were grown until sub-confluence and then tested with the different hormones as indicated. Effects on MAP-kinase were tested in cells, 5 days after the medium has been changed, at a time in which replacement with fresh medium would have taken place. Following 15 min exposure to the various hormones, cells were washed twice with calcium- and magnesium-free cold phosphate-buffered saline (PBS). Subsequently, 0.3 ml of lysis buffer was added to each plate. Lysis buffer consisted of 20 mM Hepes, pH 7.5, containing 150 mM NaCl, 1% Triton-X 100, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM β-glycerol phosphate, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors (1 mM benzamidine, 2 nM sodium vanadate, leupeptin 10 μg/ml, Aprotinin 10 μg/ml, and pepstatin 10 μg/ml). The plates were gently agitated at 4°C for 10 min. The cells were then scraped from each plate, and transferred to Eppendorf tubes. After centrifugation of the tubes at 4°C for 10 min at 14,000*g*, the supernatants (lysates) containing total cell extracts were removed. The cell lysate corresponding to each treatment was combined and divided into two aliquots. One aliquot was used for protein determination with Coomassie blue using BSA as standard. Another aliquot of the cell lysate was used for a two-site MAP-kinase assay.

TWO-SITE MAP-KINASE ASSAY

Microtiter strips (Labsysterms, Oy, Helsinki, Finland) were coated over 70 h at 4°C with the general anti-MAP-kinase antibody (2.5 μg/ml PBS, pH 7.4, 200 μl/well). The antibody solution from each well was then decanted, and the microtiter strips were blocked with 200 μl/well blocking buffer (PBS containing 2% BSA) for 2 h at room temperature. The microtiter strips were then washed twice with buffer after which the cell lysates were then transferred (100 μl/well) in triplicate to the microtiter wells. Assay buffer (100 μl) was then added to each well and the strips were incubated overnight at 4°C and washed three times. Subsequently, Europium-labeled, activated anti-MAP-kinase antibody (192 ng/well in 200 μl assay buffer) was

added, and the strips were incubated under shaking conditions for 2 h at room temperature. The strips were then washed four times and processed for time-resolved fluorescence as described previously [Somjen et al., 2004]. This assay actually determines the net change due to the combined effects on both kinase and phosphatase activities.

STATISTICAL ANALYSIS

The significance of differences between the mean values obtained from experimental and controls were evaluated by two ways analysis of variance (ANOVA).

RESULTS

THE EFFECT OF A44, A45, AND EMP-1 ESTROGENIC-LIKE PEPTIDES WITH AND WITHOUT E_2 ON DNA SYNTHESIS AND CK-SPECIFIC ACTIVITY IN VSMC CELLS IN NORMAL AND HIGH GLUCOSE IN THE GROWTH MEDIUM

VSMC cells were treated with the different peptides (at 50 nM), and E_2 (0.3 nM (l) and 30 nM (h)) or the peptides together with 0.3 or 30 nM E_2 for 24 h. At the end of the incubation DNA synthesis and CK were determined. DNA synthesis was stimulated by 0.3 nM (E_2l) and inhibited by both EMP-1 at 50 nM and E_2 at high concentrations as was shown previously [Somjen et al., 2004] while A44-stimulated cell proliferation at 50 nM. By contrast A45 had no effect on DNA synthesis in VSMC (Fig. 1a). A44 with E_2l- or E_2h-stimulated DNA synthesis whereas the lack of effect by A45 was not affected by addition of E_2l or E_2h. EMP-1 with E_2l-stimulated cell proliferation whereas EMP-1 with E_2h had no effect (Fig. 1a, upper panel). Growing the cells in high glucose concentration in the medium resulted in no effect on the stimulation by E_2l and A44 and on the inhibition by EMP-1 at 50 nM. All other treatments were the same as in normal glucose (Fig. 1b, lower panel). CK was stimulated by all peptides tested, except A45 (Fig. 1b). In high glucose in the growth medium CK stimulation was not affected as shown with E_2 (Fig. 1b).

DOSE-DEPENDENT STIMULATION BY A44 AND EMP-1 ESTROGENIC-LIKE PEPTIDES ON DNA SYNTHESIS IN VSMC CELLS IN NORMAL AND HIGH GLUCOSE IN THE GROWTH MEDIUM

VSMC cells were treated with different doses of the peptides, EMP-1 at low concentrations stimulated DNA synthesis, but at high concentration it was inhibiting in VSMC (Fig. 2a). DNA synthesis was stimulated dose-dependently by A44 (Fig. 2b); these patterns of response by these peptides were not affected by growing the cells in high glucose in the growth medium (Fig. 2a,b). Similar behavior to EMP-1 was with E_2 except that the beneficial inhibitory effect which was apparent in hg with EMP-1 was lost with E_2 (Fig. 2c), and A44 behaved like some phytoestrogens and stimulated it dose-dependently (unpublished data).

THE EFFECT OF A44, A55 AND EMP-1 ESTROGENIC-LIKE PEPTIDES ON MAPK ACTIVITY IN VSMC CELLS

VSMC cells were treated with 30 nM E_2, and 50 nM A44, A45 and EMP-1, and MAPK activity was stimulated by all compounds tested except A45 (Fig. 3) in normal or high glucose in the growth media.

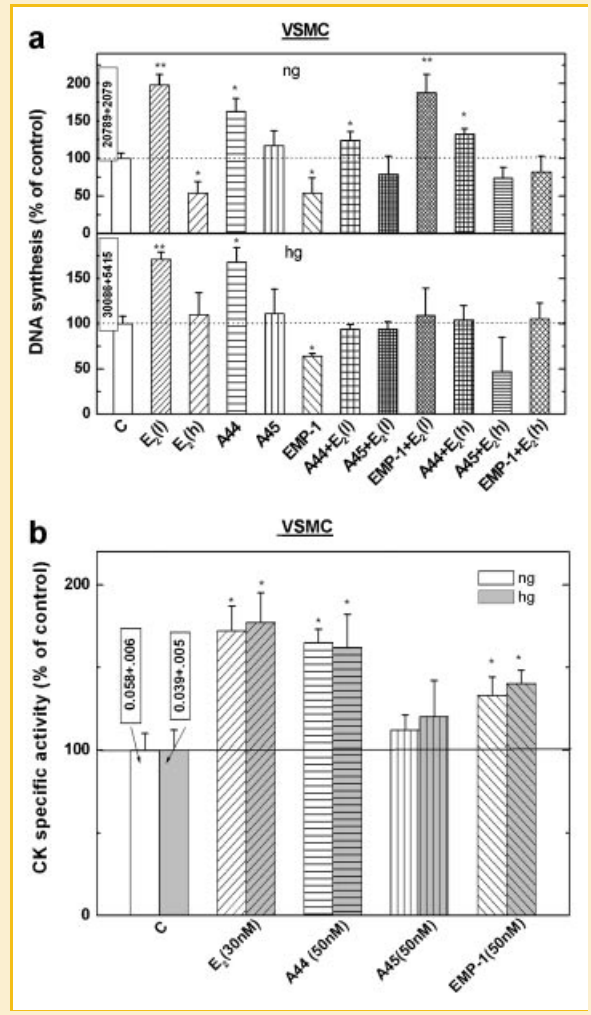

Fig. 1. VSMC cells were treated for 24 h with 0.3 or 30 nM E_2 and the different peptides A44, A45, and EMP-1 with and without E_2 at 50 nM in normal glucose (ng, upper panel) or at high glucose (hg, lower panel) in the growth medium. a: DNA synthesis and (b) CK were prepared and assayed as described in the Materials and Methods Section. Results are expressed as % of control for n = 6–9. In the figure is added in the control column the basal levels of ^{3}[H] thymidine incorporation in dpm/well ± SEM.

DISCUSSION

We have recently reported that chronic exposure to high glucose concentration substantially modifies the effects of estradiol-17β (E_2) on human vascular cell growth [Somjen et al., 2004]. Thus, the inhibitory effect exerted by high E_2 concentrations (such as found in pre-menopausal women or under HRT), on DNA synthesis in VSMC in a normal glucose concentrations was not discernible under high glucose conditions. Apparently, high glucose blocks potentially cell growth-related effects by which estrogens may confer one of its cardiovascular protection in pre-menopausal women. The high glucose effects does not modify the stimulation of CK activity which is a well-known nuclear estrogenic activity marker [Somjen et al., 2004], suggesting membranal interaction modified by high glucose, rather then nuclear one. This might imply that EMP-1 might activate

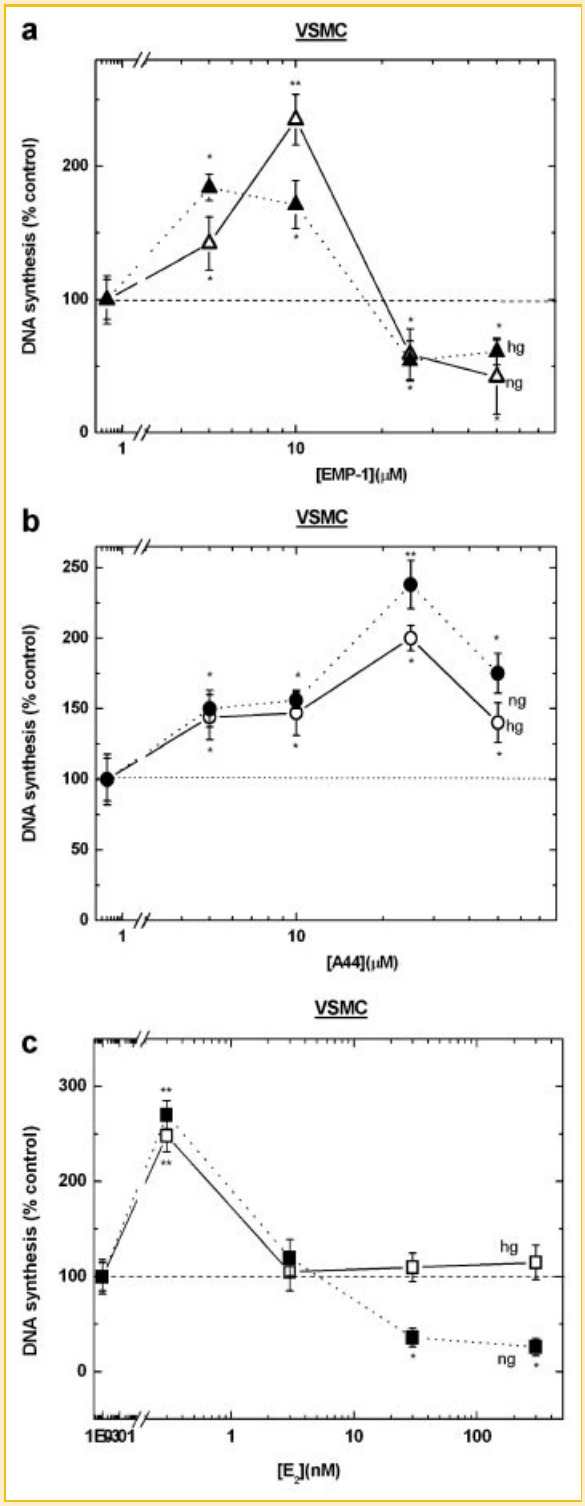

Fig. 2. VSMC cells were treated for 24 h with different concentrations of different peptides. a: EMP-1, (b) A44, and (c) E_2 in normal glucose (ng) or at high glucose (hg) in the growth medium. DNA synthesis was assayed as described in the Materials and Methods Section. Results are expressed as % of control for n = 6–9. In the figure is added in the control column the basal levels of CK specific activity in nmol/min/mg protein.

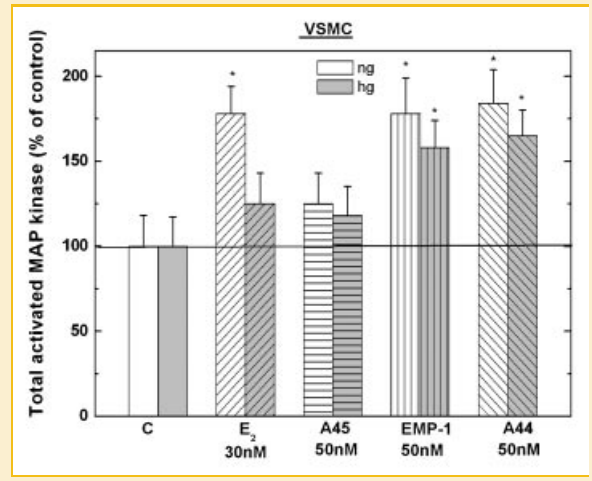

Fig. 3. The effects of E_2 at 30 nM and the peptides A44, A45, and EMP-1 at 50 nM for 24 h on MAPK activity in VSMC cells. Enzyme was prepared and assayed as described in the Materials and Methods Section. Results are expressed as % of control for n = 3–6.

more efficiently membranal putative binding sites and to less extent although effectively nuclear binding sites [Somjen et al., 2004]. By contrast, only EMP-1 and not A44 and A45 were capable of activating VSMC in normal as well as in hyperglycemic concentration. Thus, EMP-1 at a low concentration stimulated DNA synthesis and at high concentration inhibited DNA synthesis in cells growing in either normal or high glucose, conditions where E_2 is not active any more. The different behavior of the other peptides might be due to its interaction with the different estrogenic binding sites, which is the subject of future studies.

EMP-1 and A44 similar to E_2 and not to A45-stimulated CK-specific activity and MAPK activity in VSMC in low or high glucose concentration, suggesting that these peptides behave like E_2 in these cells in the specific parameters we used.

The results shown in this study implicate that EMP-1 is a biologically active estrogen mimetic peptide, and due to its dual effects in VSMC in both normal and hyperglycemic conditions, may be used in HRT if it acts similarly in vivo in diabetic patients where the estrogenic compounds fail to be effective.

REFERENCES

Bowden DW, Lohman K, Hsu FC, Langefeld CD, Carr JJ, Lenchik L, Wagenknecht LE, Freedman BI, Herrington DM. 2006. Hormone replacement therapy is associated with increased C-reactive protein in women with type 2 diabetes in the Diabetes Heart Study. Diabetes Med 23:763–767.

Gooding KM, MacLeod KM, Spyer G, Ewings P, Tooke JE, Shore AC. 2005. Impact of hormone replacement therapy on microvascular function in healthy and type 2 diabetic postmenopausal women. Diabetes Med 22: 536–542.

Kasher R, Gayer B, Kulik T, Somjen D, Venkatesh N, Fridkin M, Katchalski-Katzir E, Kohen F. 2004. Design, synthesis, and evaluation of peptides with estrogen-like activity. Biopolymers 76:404–420.

Kasher R, Bajayo A, Gabet Y, Nevo N, Fridkin M, Katchalski-Katzir E, Kohen F, Bab I. 2009. Restrain of bone growth by estrogen-mimetic

peptide-1 (EMP-1): A micro-computed tomographic study. Peptides 30: 1181–1186.

Manning PJ, Sutherland WH, Allum AR, de Jong SA, Jones SD. 2003. HRT does not improve urinary albumin excretion in postmenopausal diabetic women. Diabetes Res Clin Pract 60:33–39.

Masding MG, Stears AJ, Burdge GC, Wootton SA, Sandeman DD. 2006. The benefits of oestrogens on postprandial lipid metabolism are lost in post-menopausal women with type 2 diabetes. Diabetes Med 23:768–774.

Scott AR, Dhindsa P, Forsyth J, Mansell P. 2004. Effect of hormone replacement therapy on cardiovascular risk factors in postmenopausal women with diabetes. Diabetes Obes Metab 6:16–22.

Somjen D, Kohen F, Jaffe A, Amir-Zaltsman Y, Knoll E, Stern N. 1998. Effects of gonadal steroids and their antagonists on DNA synthesis in human vascular cells. Hypertension 32:39–45.

Somjen D, Paller CJ, Gayer B, Kohen F, Knoll E, Stern N. 2004. High glucose blocks the effects of estradiol on human vascular cell growth: Differential interaction with estradiol and raloxifene. J Steroid Biochem Mol Biol 88: 101–110.

Venkatesh N, Zaltsman Y, Somjen D, Gayer B, Boopathi E, Kasher R, Kulik T, Katchalski-Katzir E, Kohen F. 2002. A synthetic peptide with estrogen-like activity derived from a phage-display peptide library. Peptides 23:573–580.