This is a pre-print of an article published in *Molecular Biotechnology*. The final authenticated version is available online at: https://doi.org/10.1007/s12033-012-9642-4

Novel fusion protein derived from vasostatin 30 and vasoinhibin II-14.1 inhibits potently the coronary endothelial cells proliferation

Gabriela Vazquez Rodriguez[a], Carmen Gonzalez[b], Antonio De Leon Rodriguez[a*]

[a] Instituto Potosino de Investigación Científica y Tecnológica, División de Biología Molecular, Camino a la Presa San José Nº 2055 lomas 4ª sección CP 78216. San Luis Potosí; S.L.P., México.

[b] Universidad Autónoma de San Luis Potosí, Facultad de Ciencias Químicas. Av. Dr. Manuel Nava Nº 6 CP 78210, San Luis Potosí; S.L.P., México.

Submitted to: *Molecular Biotechnology*

*Corresponding author:

Tel: +52-444-8342000, Fax: +52-444-8342010

E-mail: aleonr@ipicyt.edu.mx

ABSTRACT

A novel fusion protein between the vasostatin 30 (Vs30) and the vasoinhibin of 14.1 kDa (Vi-II-

14.1) denominated as VS_VI was produced. The protein fusion genes were cloned into a T7 promoter-based vector, expressed in *Escherichia coli* BL21-SI and purified by affinity column chromatography. The recombinant VS_VI 10 nM inhibited the rat coronary endothelial cells proliferation at 65.5% whereas recombinant Vs30 and Vi-II-14.1 inhibited at 33 and 50.5% respectively at the same concentration. The results showed that VS_VI is more active than the Vs30 and Vi-II-14.1. In addition, a practical classification of the vasoinhibins based in the peptide origin and theorical molecular wheight is proposed. This is the first work that produces a new protein derived from Vs30 and Vi-II-14.1, both of them proposed as promising therapeutic agents.

Keywords: angiogenesis, cell proliferation, fusion protein, recombinant protein

1. Introduction

Angiogenesis is the physiological process which involves the growth of new blood vessel from pre-existing ones and it is considered as a key process for local invasion and the development of metastasis in tumor growth [1]. The discovery of endogenous inhibitors such as endostatin, angiostatin, tumstatin, vasostatin (Vs), vasoinhibins (Vi), and others [2,3], lead to the hypothesis that tumor growth can be blocked by inhibiting signaling pathways induced by mitogenic substances, in order to maintain a quiescent state and even reverse.

Vasoinhibins (Vi) are N-terminal peptides derived from human prolactin (hPRL), growth hormone (GH), or placental lactogen (PL) that inhibit endothelial cell proliferation and angiogenesis [4-6], the Vi derived from hPRL have been the most extensively studied. For instance, the vasoinhibin of 14.1 kDa (Vi-II-14.1), which is generated in the hypothalamic-neurohypophyseal system, derives from proteolytic cleavage of the hPRL by matrix metalloproteases (MMP) and cathepsin D [7,6]. The Vi-II-14.1 maintains the same antiproliferative activity as the vasoinhibin of 16 kDa (16-kDa fragment). The 16-kDa fragment is the most studied vasoinhibin, it suppresses angiogenesis *in vitro* and *in vivo* [8,9], induces apoptosis of endothelial cells in culture [10,11], interferes with cell cycle by inhibiting cyclins D1 and B1 [12], and blocks the mitogenic activity of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and vasoactive substances such as bradykinin (BK) and acetylcholine (ACh) [13,9,14,15].

On the other hand, the vasostatin (Vs) is an antiangiogenic peptide that derives from N-terminal end of human calreticulin (hCALR). The Vs inhibits angiogenesis *in vivo*, suppresses the neovascularization *in vivo* and prevents or reduces the experimental tumor growth [16,17]. Furthermore, Vs inhibits specifically the tumor blood vessel formation and does not affect the wound healing process [18]. The active domain of Vs is comprised in the 135-164 N-terminal amino acids of hCALR (Vs30). Vs30 is a potent antiproliferative and antiangiogenic agent that blocks the mitogenic activity of basic fibroblast growth factor (bFGF) in ECV304 cells and chick embryo chorioallantoic membranes [19].

The aim of this work is to develop a new fusion protein, derived from Vasostatin 30 and a vasoinhibin of 14.1 kDa (Vi-II-14.1), capable of inhibit potently the endothelial cell proliferation.

2. Materials and Methods

2.1 Expression vectors and strain

2.1.1 pET12a/Vi-II-14.1: The Vi-II-14.1 gen was amplified by polymerase chain reaction (PCR) using as template the pSG-hPRL plasmid (kindly provided by Dra. Carmen Clapp, UNAM), which contains the human prolactin's cDNA. To amplify Vi-II-14.1 gene we use the oligo forward 5'gtcgacgttgcccatctgtcccg 3' and the oligo reverse 5'ggatcc***tta***gtggtggtggtggtggtg*ctcaatctctacagctttggatagg 3' that contains a *Sal*I and *Bam*HI restriction enzyme site respectively (underlined), a termination codon (in bold) and a histidine tag (cursive).

2.1.2 pET12a/Vs30: The Vs30 gen was amplified from a synthetic gen of Vs. For Vs30 amplification we used the oligo forward 5'gtcgacggacatctgtggcc 3' and the oligo reverse 5'ggatcc***tta***gtggtggtggtggtggtg*cttgcaacggatgtcc 3' that contains a *Sal*I and *Bam*HI restriction enzyme site respectively (underlined), a termination codon (in bold) and a histidine tag (cursive).

2.1.3 pET12a/VS_VI fusion peptide: The fusion gen conformed by Vi-II-14.1 and Vs30 was generated by PCR overlapping. In a first round of PCR the Vs30 gen was amplified with the same oligo forward described in section 2.1.2 and the oligo reverse 5'cgggacagatgggcaacat**accacctggaccacc***cttgcaacggatgtcc* 3' with the Vs30 sequence

(underlined), a Gly-Gly-Pro-Gly-Gly bridge (in bold) and a Vi-II-14.1 homologue sequence (cursive). Then, in a second round of PCR the Vi-II-14.1 gen was amplified with the oligo forward 5'ggacatccgttgcaag**ggtggtccaggtggt***atgttgcccatctgtcccg* 3' with a Vs30 homologue sequence (underlined), a Gly-Gly-Pro-Gly-Gly bridge (in bold) and the Vi-II-14.1 sequence (cursive), and the oligo reverse previously described in section 2.1.1. Finally, in a third round of PCR we used as DNA template the PCR products from the first and second round where the homologue sequences functioned as primers.

All the PCR products were cloned into the pGEM T-EASY vector (Promega) and then subcloned into pET12a expression vector (Novagen) using the same restriction sites described in section 2.1. The resulting constructions designed as pET12a/Vi-II-14.1, pET12a/Vs30 and pET12a/VS_VI were confirmed by sequencing (MacLab, San Francisco CA). Since the Vi-II-14.1 gen was amplified from the native gen of hPRL, it contains rare codons for *E. coli*. Therefore, all vector constructions were transformed in an *E. coli* BL21-SI (Life technologies) previously transformed with the pLysSRARE plasmid (Novagen), which contains the tRNA´s reported as rare in *E. coli* [20].

2.2 Production and purification of the recombinant Vs30, Vi-II-14.1, and VS_VI fusion protein.

Escherichia coli BL21-SI/pLysSRARE transformed with each of the constructions previously described in section 2.1 was grown in BSG minimal medium (5 g glucose, 3.68 g $(NH_4)_2HPO_4$, 3.68 g KH_2PO_4, 1.0 g $MgSO_4$, 0.1 µg thiamine, a 5µM solution of trace element previously described in [21]) plus ampicillin 100 µg/mL and chloramphenicol 10 µg/mL. The production of the recombinant proteins was induced with 0.3 M NaCl at 20ºC. The recombinant Vi-II-14.1, Vs30 and VS_VI fusion protein (rVi-II-14.1, rVs30 and rVS_VI) produced in soluble phase of *E. coli* were purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity column with the ProBond

Purification System (Invitrogen) following manufacturer instructions and visualized by silver stain (BioRad). The purified recombinant proteins were identified by Western Blot with a polyclonal mouse anti-His tag primary antibody. A fraction of the purified recombinant proteins were lyophilized to prevent degradation.

2.3 Cell proliferation assay

Lyophilized recombinant proteins were reconstituted in an HGT buffer (25 nM HEPES, 1% glycerol (v/v) and 0.01% triton X-100). The anti-proliferative activity was assessed in coronary endothelial cells (CEC) isolated according to Sierra-Ramírez *et al*. [22]. CEC were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin/streptomycin (Sigma). Cells were cultured in 96-well culture plates (2000 cells/well) and treated with 0.1, 1, or 10 nM of the rVi-II-14.1, rVs30 or rVS_VI. All wells were treated with 10μg/mL polymyxin B (PMB, Sigma) in the presence or absence of 10 μM Bradykinin (BK, Sigma) and were incubated for 24 h. Cell proliferation was measured by the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) into formazan dye by a mitochondrial enzyme of living cells as previously described [23]. Briefly, 10 µL of 5 mg/ml MTT reagent was added to each well and incubated for 4 h at 37ºC, after which 100 µL of acidified isopropanol were added to stop the reaction. Culture plates were incubated overnight at room temperature under light protection. The formazan blue formed was measure in an iMark microplate reader (BioRad,) at a wavelenght of 570 nm. Controls with 10μg/mL polymixin B (PMB) were included to discard the background effect of the bacterial lipopolysaccharides.

2.4 Statistics

The statistical analysis of the treatments was determined by analysis of variance (ANOVA) and unpaired Student's T-test. Values are expressed as mean ± SEM. Treatments with $p<0.05$ were

statistically significant. The statistical analysis was performed using the KaleidaGraph software v.4.1. Treatments with PMB controls were done at least 4 times and treatments with each recombinant protein 3 times

3. Results

3.1 Classification of the vasoinhibins

There are a large number of peptides designated generically as vasoinhibins. Until now, they have been referred to by their electrophoretic molecular weight, but it is subjetive and does not indicate the origin of the peptide. Therefore, here we have proposed a classification based on the theoretical molecular weight and the parental protein origin. Vi that derive from placental lactogen (PL) are categorized as group I, and they have less range in molecular weight. Group II are those Vi that derive from the PRL, whereas those derived from growth hormone (GH) are group III, and they have the greatest range in molecular weight (Table 1). Thus, the vasoinhibin derived from PRL and molecular weight of 14.1 kDa, is designated as Vi-II-14.1. Vi derived from PRL occurs in tissues like the mammary gland, prostate, liver, kidney, spleen, hypothalamo-neurohypophyseal system, endothelial cells, and pituitary gland. Until now, there has been no evidence that Vi derived from PL occurs naturally *in vivo* [5]. Vi derived from PRL, have a higher antiangiogenic activity than those derived from GH and PL [24]. Therefore, we chose to produce a fusion protein based on the Vi-II-14.1 because it is smaller and conserves the same biological activity as the 16kDa fragment of PRL, which is the most extensively studied [7,25].

3.2 Production of rVs30, rVi-II-14.1, and rVS_VI fusion protein

The strains BL21-SI/pLysSRARE/pET12a-VS_VI, pET12a-Vi-II-14.1, and pET12a-Vs30 were induced at the culture mid-logarithm time. The expression of a ~18.5, ~15, and ~4.5 kDa proteins

corresponding to rVS_VI, rVi-II-14.1, and rVs30 were obtained, respectively. The recombinant proteins production was made at 20ºC as post-induction temperature to improve their solubility; this issue is supported by previously reports that recommend doing the expression of recombinant proteins at low temperatures [26,27]. The total production of the rVS_VI fusion protein was 32.7 mg/L, while the maximum concentration for rVs30 and rVi-II-14.1 was 9 and 8 mg/L, respectively. This suggest that the fused construction could be more stable than rVs30 and rVi-II-14.1 separately, that could be more susceptible to proteases.

To analyze the production of the recombinant proteins in soluble phase of *E. coli*, the total extract (TF) and the supernatant (SF) fractions were analyzed by Western blot. All the recombinant proteins rVs30, rVi-II-14.1, and rVS_VI were found in the SF fraction at a concentration of (mg/L): 6.8, 0.4, and 0.83, respectively (Figure 1).

Only the SF was processed to further purification using Ni-NTA columns where 2.98, 1.9, and 0.22 mg/L of final concentration were recovered for rVs30, rVi-II-14.1, and rVS_VI, respectively (Figure 2).

3.3 Biological activity of rVs30, rVi-II-14.1, and rVS_VI

Since the remaining bacterial components in the purified recombinant proteins could affect the CEC proliferation, controls were performed using *E. coli* BL21-SI/pLysSRARE/pET12a extract (ECOL). CEC cultures with the ECOL extract showed an overstimulated response in the proliferation cell, whereas the CEC cultures with PMB and ECOL plus PMB reverted to basal levels. In subsequent experiments using BK to overstimulate CEC proliferation, similar results were obtained (Figure 3). The CEC overproliferation by the ECOL extract can be explained by presence of lipopolysaccharides (LPS). LPS elevate the level of phosphatidylcholine-specific phospholipase C (PC-PLC) that induces monocyte chemotactic protein-1 (MCP-1) and IL-8 production [28]. IL-8 is known as a potent angiogenic agent [29]. Our results showed that PMB

was able to revert the effect of the bacterial LPS. Therefore, we used PMB in the subsequent experiments for the bioactivity assays.

The effect of the rVs30, rVi-II-14.1, and rVS_VI on the basal CEC proliferation is shown in Figure 4. The rVs30 inhibited 14.6 and 9.4% of CEC proliferation at 0.1 and 1 nM, respectively whereas 10 mM showed no effect (Figure 4A). Meanwhile, the rVi-II-14.1 reached a maximum inhibition of 59.7% at 10 nM (Figure 4B). The rVS_VI fusion protein stimulated 10.13% of CEC proliferation at 1 nM, and inhibited cell proliferation up to 48% at 10 nM (Figure 4C).

Figure 5 shows the effect of the recombinant proteins in CEC proliferation stimulated with BK. These cultures showed an overstimulated proliferation, up to 124% respect to basal proliferation. This value was normalized to 100% in order to be used as reference in the analysis of subsequent cultures with the recombinant proteins. Figure 5A shows that rVs30 inhibit 33% of CEC proliferation induced by BK at 0.1 nM. While, the three different concentrations evaluated for rVi-II-14.1 inhibited the cell proliferation up to 50.5% at 10 nM. Interestingly, the inhibitory effect was reverted in presence of the monoclonal antibody anti N-terminal of the human prolactin (anti-NhPRL) [30], which does not interfere with the BASAL CEC proliferación (Figure 3). On the other hand, the rVS_VI fusion protein inhibited cell proliferation up to 65.5% at 10 nM (Figure 5C). This suggests a possible potentiation due to the fusion of the antiangiogenic peptides rVs30 and rVi-II-14.1. Also, the antiproliferative effect of rVS_VI in presence of the antibody anti-NhPRL was reverted and the cell proliferation induced by BK was recovered almost in 100% at 0.1, and 1 nM of rVS_VI. However, at 10 nM the antiproliferative effect was reverted only 61% (Figure 5C). This fact can be explained by the antibody specificity, since it cannot recognize the Vs30 fragment conforming the rVS_VI, and it could maintains its antiproliferative activity that can be more evident at 10 nM.

Among the three recombinant proteins produced in this work, the rVS_VI fusion protein had the highest inhibitory effect in the proliferation assay with BK, whereas the rVs30 was the less active.

4. Discussion

The angiogenic process has become an interesting target for cancer treatment and other angiogenic-dependent disseases. The development of novel antiangiogenic peptides with high inhibitory capability that inhibit cancer cells, in a selective and efficient way without affecting normal cells and tissues is mandatory. These novel peptides can be evaluated as promising candidates in antiangiogenic therapy against wide types of cancer. An ideal agent would be one that besides blocking the angiogenic process also prevents tumor growth and/or metastasis process and greatly reduces considerably or totally the secondary effects.

The Vs30 has been produced as fusion protein to other antiangiogenic peptides resulting in novel proteins with better inhibitory activity in comparison with those peptides evaluated separately.

One-fusion protein between the Vs N-terminal end of hCALR (Vs30) and the C-terminal end fragment of platelet factor 4 (C13) inhibited ~80% the ECV304 cells proliferation at 8 μM, while the separated peptides Vs30 and C13 both inhibited ~50% at the same concentration [19]. Sun et. al. [31] produced a fusion protein (ALV) based on the aminoterminal fragment of urokinase (ATF) and Vs. The concentration of ALV and Vs required to inhibit 50% of HMVEC proliferation (ED_{50}) was about 30 and 60 nM, respectively, indicating that ALV fusion protein has higher antiproliferation activity than the Vs. VS_VI fusion protein produced in this work inhibited 65.5% the endothelial cell proliferation at low concentration (10 nM), this data suggests that our fusion protein could be more active than those PFV and ALV. This findings open new avenues and research lines related to

study the stability and biologic activity of VS_VI in reduction, inhibition or disappearance of certain type of cancer induced in animals models.

5. Acknowledgements

We thank Dr. Carmen Clapp and Dr. Gabriel Nava from the Instituto de Neurobiología, UNAM for the pSG-hPRL plasmid, and Héctor Rosas Hernández (UASLP) and Leandro G. Ordóñez Acevedo (IPICYT) for technical assistance. G. Vazquez thanks to CONACyT for her scholarship No. 211450.

6. Disclosure statement

This work was supported by CONACyT-BASICAS 82010 and PROMEP/103.5/072574.

7. Role of the funding source

The sponsors had no role in study design, in the collection, analysis and interpretation of data, in writing the report or in the decision to submit the paper for publication.

8. References

1. Persano L, Crescenzi M, Indraccolo S (2007) Anti-angiogenic gene therapy of cancer: current status and future prospects. Mol Aspects Med 28 (1):87-114

2. Ribatti D (2009) Endogenous inhibitors of angiogenesis: a historical review. Leuk Res 33 (5):638-644. doi:S0145-2126(08)00507-9 [pii]
10.1016/j.leukres.2008.11.019

3. Ma WW, Adjei AA (2009) Novel agents on the horizon for cancer therapy. CA Cancer J Clin 59 (2):111-137

4. Struman I, Bentzien F, Lee H, Mainfroid V, D'Angelo G, Goffin V, Weiner RI, Martial JA (1999) Opposing actions of intact and N-terminal fragments of the human prolactin/growth hormone family members on angiogenesis: an efficient mechanism for the regulation of angiogenesis. Proc Natl Acad Sci U S A 96 (4):1246-1251

5. Corbacho AM, Martinez De La Escalera G, Clapp C (2002) Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis. J Endocrinol 173 (2):219-238. doi:JOE04352 [pii]

6. Macotela Y, Aguilar MB, Guzman-Morales J, Rivera JC, Zermeno C, Lopez-Barrera F, Nava G, Lavalle C, Martinez de la Escalera G, Clapp C (2006) Matrix metalloproteases from chondrocytes generate an antiangiogenic 16 kDa prolactin. J Cell Sci 119 (Pt 9):1790-1800

7. Clapp C, Torner L, Gutierrez-Ospina G, Alcantara E, Lopez-Gomez FJ, Nagano M, Kelly PA, Mejia S, Morales MA, Martinez de la Escalera G (1994) The prolactin gene is expressed in the hypothalamic-neurohypophyseal system and the protein is processed into a 14-kDa fragment with activity like 16-kDa prolactin. Proc Natl Acad Sci U S A 91 (22):10384-10388

8. Clapp C, Martial JA, Guzman RC, Rentier-Delure F, Weiner RI (1993) The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. Endocrinology 133 (3):1292-1299

9. Gonzalez C, Corbacho AM, Eiserich JP, Garcia C, Lopez-Barrera F, Morales-Tlalpan V, Barajas-Espinosa A, Diaz-Munoz M, Rubio R, Lin SH, Martinez de la Escalera G, Clapp C (2004) 16K-prolactin inhibits activation of endothelial nitric oxide synthase, intracellular calcium mobilization, and endothelium-dependent vasorelaxation. Endocrinology 145 (12):5714-5722

10. Tabruyn SP, Sorlet CM, Rentier-Delrue F, Bours V, Weiner RI, Martial JA, Struman I (2003) The antiangiogenic factor 16K human prolactin induces caspase-dependent apoptosis by a mechanism that requires activation of nuclear factor-kappaB. Mol Endocrinol 17 (9):1815-1823

11. Martini JF, Piot C, Humeau LM, Struman I, Martial JA, Weiner RI (2000) The antiangiogenic factor 16K PRL induces programmed cell death in endothelial cells by caspase activation. Mol Endocrinol 14 (10):1536-1549

12. Tabruyn SP, Nguyen NQ, Cornet AM, Martial JA, Struman I (2005) The antiangiogenic factor, 16-kDa human prolactin, induces endothelial cell cycle arrest by acting at both the G0-G1 and the G2-M phases. Mol Endocrinol 19 (7):1932-1942. doi:me.2004-0515 [pii]
10.1210/me.2004-0515

13. D'Angelo G, Martini JF, Iiri T, Fantl WJ, Martial J, Weiner RI (1999) 16K human prolactin inhibits vascular endothelial growth factor-induced activation of Ras in capillary endothelial cells. Mol Endocrinol 13 (5):692-704

14. Clapp C, Weiner RI (1992) A specific, high affinity, saturable binding site for the 16-kilodalton fragment of prolactin on capillary endothelial cells. Endocrinology 130 (3):1380-1386

15. Stéphanie Thebault CG, Celina García, David Arredondo Zamarripa,, Gabriel Nava LV, Fernando López-Casillas, Gonzalo Martínez de la Escalera, Clapp. C (2011) Vasoinhibins Prevent Bradykinin-Stimulated Endothelial Cell Proliferation by Inactivating eNOS via Reduction of both Intracellular Ca2+ Levels and eNOS Phosphorylation at Ser1179. Pharmaceuticals 4:1052-1069. doi:10.3390/ph4071052

16. Pike SE, Yao L, Jones KD, Cherney B, Appella E, Sakaguchi K, Nakhasi H, Teruya-Feldstein J, Wirth P, Gupta G, Tosato G (1998) Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. J Exp Med 188 (12):2349-2356

17. Pike SE, Yao L, Setsuda J, Jones KD, Cherney B, Appella E, Sakaguchi K, Nakhasi H, Atreya CD, Teruya-Feldstein J, Wirth P, Gupta G, Tosato G (1999) Calreticulin and calreticulin fragments are endothelial cell inhibitors that suppress tumor growth. Blood 94 (7):2461-2468

18. Lange-Asschenfeldt B, Velasco P, Streit M, Hawighorst T, Pike SE, Tosato G, Detmar M (2001) The angiogenesis inhibitor vasostatin does not impair wound healing at tumor-inhibiting doses. J Invest Dermatol 117 (5):1036-1041

19. Li X, Jiang L, Wang Y, Xiao Y, Huang Y, Yao Q, Yang Y, Wu X (2007) Inhibition of angiogenesis by a novel small peptide consisting of the active fragments of platelet factor-4 and vasostatin. Cancer Lett 256 (1):29-32

20. Nakamura Y, Gojobori T, Ikemura T (2000) Codon usage tabulated from international DNA sequence databases: status for the year 2000. Nucleic Acids Res 28 (1):292. doi:gkd078 [pii]

21. Rosales-Colunga Luis Manuel R-FE, Ordoñez Leandro G., Alatriste-Mondragón Felipe, De León-Rodríguez Antonio (2010) Hydrogen production by Escherichia coli ΔhycA ΔlacI using cheese whey as substrate. International Journal of Hydrogen Energy 35 (2):491-499. doi:doi: 10.1016/j.ijhydene.2009.10.097

22. Sierra-Ramirez A, Morato T, Campos R, Rubio I, Calzada C, Mendez E, Ceballos G (2004) Acute effects of testosterone on intracellular Ca2+ kinetics in rat coronary endothelial cells are exerted via aromatization to estrogens. Am J Physiol Heart Circ Physiol 287 (1):H63-71

23. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65 (1-2):55-63. doi:0022-1759(83)90303-4 [pii]

24. Nguyen NQ, Tabruyn SP, Lins L, Lion M, Cornet AM, Lair F, Rentier-Delrue F, Brasseur R, Martial JA, Struman I (2006) Prolactin/growth hormone-derived antiangiogenic peptides highlight a potential role of tilted peptides in angiogenesis. Proc Natl Acad Sci U S A 103 (39):14319-14324. doi:0606638103 [pii]

10.1073/pnas.0606638103

25. Lopez-Gomez FJ, Torner L, Mejia S, de la Escalera GM, Clapp C (1995) Immunoreactive prolactins of the neurohypophyseal system display actions characteristic of prolactin and 16K prolactin. Endocrine 3 (8):573-578. doi:10.1007/BF02953021

26. Sun QM, Chen LL, Cao L, Fang L, Chen C, Hua ZC (2005) An improved strategy for high-level production of human vasostatin120-180. Biotechnol Prog 21 (4):1048-1052

27. Maldonado LM, Hernandez VE, Rivero EM, Barba de la Rosa AP, Flores JL, Acevedo LG, De Leon Rodriguez A (2007) Optimization of culture conditions for a synthetic gene expression in Escherichia coli using response surface methodology: the case of human interferon beta. Biomol Eng 24 (2):217-222

28. Zhang L, Li HY, Li H, Zhao J, Su L, Zhang Y, Zhang SL, Miao JY (2011) Lipopolysaccharide activated phosphatidylcholine-specific phospholipase C and induced IL-8 and MCP-1 production in vascular endothelial cells. J Cell Physiol 226 (6):1694-1701. doi:10.1002/jcp.22500

29. Li A, Varney ML, Valasek J, Godfrey M, Dave BJ, Singh RK (2005) Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. Angiogenesis 8 (1):63-71. doi:10.1007/s10456-005-5208-4

30. Piwnica D, Touraine P, Struman I, Tabruyn S, Bolbach G, Clapp C, Martial JA, Kelly PA, Goffin V (2004) Cathepsin D processes human prolactin into multiple 16K-like N-terminal fragments: study of their antiangiogenic properties and physiological relevance. Mol Endocrinol 18 (10):2522-2542

31. Sun Q, Xu Q, Dong X, Cao L, Huang X, Hu Q, Hua ZC (2008) A hybrid protein comprising ATF domain of pro-UK and VAS, an angiogenesis inhibitor, is a potent candidate for targeted cancer therapy. Int J Cancer 123 (4):942-950

Figure captions

Figure 1. Western blot analysis for the recombinant rVs30, rVi-II-14.1, and rVS_VI produced in *E. coli* BL21-SI/pLysSRARE cultures at 20ºC post-induction temperature. C: Control, TF: total extract of lysate cells and SF: supernatant or soluble fraction.

Figure 2. Recombinant rVs30, rVi-II-14.1, and rVS_VI purified by the Ni-affinity chromatography and visualized by silver stain. Lane 1: first eluated; lane 2: the recombinant protein. Frame indicates the recombinant protein.

Figure 3. Effect of bacterial cell extract and Polymyxin B (PMB) on CEC proliferation with or without bradykinin (BK). Subscript “a” means significant difference to BASAL cultures with $p<0.05$ and subscript “b” means significant difference to BK control with $p<0.05$. A control with the antibody anti-NhPRL, which does not have any effect in BASAL CEC proliferation, is included.

Figure 4. Effect of rVs30, rVi-II-14.1, and rVS_VI on CEC proliferation without BK. Both proliferative and antiproliferative activity for each protein was tested at 0.1, 1, and 10 nM concentrations. While rVi-II-14.1 (B) and rVS_VI (C) show a significant inhibition in CEC proliferation at 10 nM, rVs30 (A) shows no significant inhibitory effect. Subscript “a” means significant difference to BASAL cultures with $p<0.05$. Negative values mean proliferation.

Figure 5. Inhibition of CEC proliferation by rVs30, rVi-II-14.1, and rVS_VI on presence of BK. The separately rVs30 (A) and rVi-II-14.1 (B) show an inhibition of CEC proliferation lower than the rVS_VI fusion protein (C), and this effect is reverted in presence of an antibody anti-NhPRL. Subscript “a” means significant difference to BASAL culture induced by BK with $p<0.05$ and subscript “b” means significant difference to its countepart with antibody with $p<0.05$.