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LETTERS

The in vitro and in silico assessment of the proliferative and angiogenic potential of Substance K: Interaction of Substance K between the Neurokinin-1, Neurokinin-2, and Tie-2 receptor proteins

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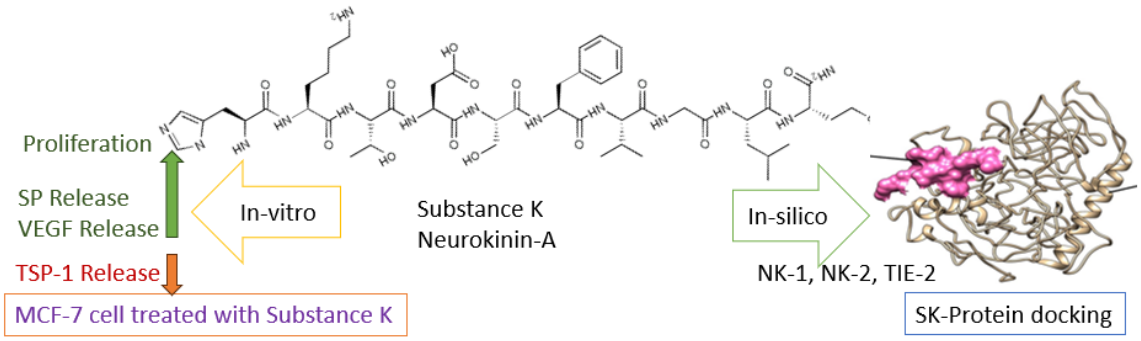
Article

ABSTRACT



Substance K is a neurotransmitter peptide, with neurological activity. SK shows similar effects with Substance P on cell proliferation. This study aims to determine whether SK has the potential to act on cell proliferation and angiogenesis via in vitro and in silico methods. MCF-7 cells were treated with different doses of SK and the cytotoxic effects on the proliferation of cells were determined via a WST-1 kit. VEGF, SP, and Thrombospondin-1 levels in media were evaluated via ELISA kits. In silico assessment was done to determine the possible interaction of SK with NK-1, NK-2, NK3, Scara5/SRCR domain, VEGFR-1, VEGFR2, CD36, NRP1, Tie-2, and PDGFR receptor proteins. Molecular docking studies were performed via HPEPDOCK 2.0, MDockPeP, and CABS-dock. Substance K exhibits both proliferative and angiogenic properties on MCF-7 human breast cancer cells, as predicted. According to our molecular docking results, SK effectively binds NK-1, NK-2, and Tie-2 receptors and probably this could be one of the possible reasons for its angiogenic properties. This is the first report showing that the SK could mimic SP and act as an angiogenic factor based on its interactions with NK-1, NK-2, and Tie2 important angiogenic receptor proteins.

Keywords: Substance K, Neurokinin-A, Angiogenesis, Substance P, NK-1, NK-2, Tie-2

INTRODUCTION

Substance K (SK), also known as Neurokinin-A, is an alternative splicing product of the preprotakikinin gene. It is neurotransmitter that generally acts on both the central and peripheral nervous systems. However, it also plays a role in the inflammatory response and pain. SK is a short peptide sequence consisting of 9 amino acids (N-Her; Lys; Asp; Ser; Phe; Val; Gly; Leu; Met-C).[1] It acts through the SK Neurokinin-1 receptor and triggers NF kappa B/ERK1/2 and PI3-kinase/Akt signaling pathways in macrophage cells.[2]

Like SP, excitatory neurons, and secretory cells in the hypothalamic-pituitary express SK. In addition, SK is also involved in the neurosensory system and plays a role in a wide range of inflammatory and tissue regeneration processes.[3] Inflammation, tissue healing, and cell proliferation are associated with the release of SP and SK from the damaged tissue.[4-6] In pathological depression studies related to stress-induced activation of the noradrenergic prefrontal, lobe system show that internally secreted corticotropin-releasing hormones may be under the control of the SP and SK. According to these studies, it was also suggested that some forms of depression may be directly related to the release of SK and SP.[7]

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Inflammatory responses in the central nervous system usually develop after traumatic injury or exposure to an infectious agent. SK plays an important role in the immune response against infection through Interleukin-1 and interleukin-6. Tachykinins act by selectively binding to neurokinin receptors 1,2 and 3, activating the G protein bound to these receptors. Although each receptor shows specific binding to SP and SK, SP and SK act as agonists of each other and have binding affinities for all three receptors.[8]

In this context, the first angiogenic factor examined in this study was SP. SP is an 11 aa neuropeptide released because of the proteolytic fragmentation of Tachykinins, a family of peptide hormones that have been preserved throughout the evolutionary process in vertebrates and invertebrates. Tachykinins are secreted from nerve cells as preprotachykinin and are broken down into substance P, Neurokinin-A, and Neurokinin-B. Discovered for the first time in 1931, SP has been evaluated as a neurotransmitter since the 1950s.[9]

SP and its receptor are found both in the central and peripheral nervous system and in peripheral tissues.[10] It is accepted that SP plays a role in the regulation of many biological functions such as the expansion of arteries, neuronal life, and degeneration, regulation of the cardiovascular system, sensory perception, movement control, regulation of respiratory mechanisms, and gastric motility. In addition, SP has been shown to play a role in inflammation, pain, and depression.[11] Especially in breast cancer, preprotachicinin expression increases when compared to normal breast epithelium cells, however, the SP was detected in both cytoplasm and the cores of tumor cells such as keratocystic odontogenic tumors, oral squamous cell carcinoma, and larynx carcinoma tissue.[12]

The SP's receptor NK-1R expression has been shown to increase in many cancerous cells as compared to normal cells, and it is accepted that the increase in receptor quantity is correlated with malignancy. It has also been determined that NK-1 receptors are present in intra and fairy tumoral blood vessels. In recent years, four or more NK-1 receptor isoforms have been identified in human tumor cell lines, but their functional roles are unknown. In studies with the SP and NK-1 receptor, activation of the receptor with the SP is also shown because of phospholipase D activation, Inositol phosphate formation, and interleukin-6, interleukin-8, taurine, and glutamate release.[13]

The data obtained from the studies showed that in many cancers the same NK-1 receptor isoforms were exterminated. The SP and its receptor are taken into the cell with the clathrin-dependent endocytosis and immediately after it is taken, the NK-1 receptor returns to the cell membrane as the SP is fragmented. The SP's stimulation of the proliferation of tumor cells can suggest a new mechanism for regulating regional tumoral activity through sensory neurons containing SP. The SP can then modulate the growth of tumor cells by establishing a direct relationship between the nervous system and tumor cells.[14] Given all these characteristics, the response to the question is whether SK affects the amount of SP released from breast cancer cells is sought within the scope of the study.

Another angiogenic factor that determines the amount of release is the Vascular Endothelial Growth Factor (VEGF). VEGF is the most important pro-angiogenic factor released from cancer cells. VEGF is a potential and selective mythology for cells, which, as its name suggests, induces the formation of a fast and complete angiogenic response. VEGF is produced and released by several normal cell types, and it is shown that VEGF exposure is significantly increased in tumor cells, including various breast cancer cells. VEGF is also exited by reactive stromal cells of the breast tumor. However, unlike many other signals secreted by cells that form the breast tumor, VEGF often interacts with endothelial cells. Because VEGFR-2, the receptor where VEGF is connected to high affinity, is only available on endothelial cell surfaces.[15] After connecting to VEGF, VEGFR-2, the endothelial activates an in-cell signal transmission path that causes a change in the activity of genes that encourage cell migration and proliferation. The angiogenesis triggered by the breast tumor is associated with the increase in the exposure of VEGF, synthesized by both tumor cells and cells in tumor stroke.[16]

The last parameter we evaluated in our study is Thrombospondin-1 (TSP-1). TSP-1 is the first discovered endogen angiogenesis inhibitor. The TSP-1 was defined as a thrombin-sensitive protein by Baenziger and his friends in early 1970. In response to the thrombin activation of the TSP-1, it was then understood that platelets were subunits of a large protein secreted from α-granules. TSPs are multifunctional glycoproteins, and their affinity to matrix proteins, plasma proteins, ions, and different cell surfaces is very high. Because the TSP-1 structure has many bonding zones, different types of cell membrane proteins play a role as receptors for the TSP-1.[17]

TSPs can connect to heparin, fibronectin, fibrinogen, type IV collagen, and calcium. From its discovery to date, it has been associated with cell growth and replication, cell mobility, cell skeletal organization, inflammatory response, differentiation to different types of cells, and regulation of angiogenesis that plays a role in wound healing and tumor formation. As compared to normal or benign cell lines, the TSP-1 is exhibited at much lower levels in cancer cell lines. In cell lines with low metastasis capability, the TSP-1 is exterminated at a higher volume than in metastatic cell lines. This is later shown in vivo experiments. The result of the TSP-1 transfection has shown that the formation of the primary tumor has been initiated, and tumor vasculature and metastasis have decreased. However, many studies show that the TSP-1 has been able to inhibit the proliferation, migrations, and tube generation of endothelial cell migration experiments and corneal neovascularization testing.[18]

This study aims to determine whether SK affected the amounts of three angiogenic factors released from breast cancer cells. To be able to talk about the effect of substance K on angiogenesis, the effect of substance K on vascular endothelial cells in vitro should also be investigated. However, the most important limitation of our study is our project budget. Therefore, to clarify the role of substance K in angiogenesis, we determined its effect on receptor proteins on the endothelial cell surface that are directly related to angiogenesis by molecular docking method.

In this context, we evaluated the interaction of substance K both with its receptors (NK-1, NK-2, and NK-3) and with VEGFR-1, VEGFR-2, CD36, NRP1, Tie-2, and PDGFR proteins, which are among the angiogenic receptors on the endothelial cell surface, in silico by peptide-protein molecular docking method using HPEPDOCK 2.0., CABS-dock and MDockPeP web servers.

The computational method is a widely utilized approach in the field of drug design and discovery. It facilitates the drug discovery and design process by accelerating the procedure while also reducing the likelihood of human errors. Computational methods, predominantly the molecular docking method, have been extensively utilized in the field of anticancer drug discovery. Docking is a virtual screening method that can be performed on a large database of compounds. Molecular docking helps in identifying the predominant binding modes of a ligand with a protein whose three-dimensional structure is known. The docking process can predict the method of inhibition of the target molecule by the ligand molecule. Utilities of molecular docking include structure-activity relationship studies, lead identification by virtual screening, optimization of the identified lead, combinatorial library design, and more.[19]

Angiogenesis is the formation of new blood vessels from vasculature due to various stimuli. It is a complex and well-coordinated process that involves extensive signaling networks between and within endothelial cells (ECs), mural cells (VSMCs and pericytes), and other cell types such as immune cells. To date, there is no study investigating the effect of SK on angiogenic and antiangiogenic factors released from cancer cells, and this is the first report showing that SK orchestrates the angiogenic factor release from cancer cells and strongly interacts with certain angiogenic receptors.

Experimental Protocols

Cell Culture Studies

The MCF-7 breast cancer cells (ATCC Cat No: HTB-22) which express the Neurokinin-1 receptor were chosen and used in the present study. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L glutamine, 1 mM sodium pyruvate and 0.02 mM non-essential amino acids at 37˚C with 5% CO2 in a humidified atmosphere. Confluent cells (90%) were used in all experiments. All cell lines used in the present study were assessed and demonstrated to be free of mycoplasma contamination.

Preparation of Substance K

Substance K was purchased from (Merck N4267, Darmstadt, Germany) and was dissolved in sterile distilled water, and a 1mg/ml stock solution was prepared.

Cell Proliferation

Cell proliferation was determined using WST-1 assay (Promega Corporation, Madison, WI, USA), which is based on the cleavage of 3-(4,5 dimethylthiazol-2-yl)- 5-(3-carboxy methoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) into a formazan product which is soluble in tissue culture medium. Briefly, cells were seeded at 5×10^3 cells per well in 200 µl complete medium onto 96-well plates and allowed to attach for 24 h. After the cells reached 80–90% confluence, the media was removed, and cells were washed with phosphate-buffered saline (PBS). Subsequently, cells were treated with different concentrations of SK (100 nM; 10 nM; 1 nM, and 0.1 nM) and were prepared in 1% FBS containing a complete medium. Each treatment was performed in eight-well replicates. The cells were grown at 37°C for 24 h, 48 h, and 72 h. The medium was gently aspirated to terminate the experiment, 180 µl serum-free complete medium and 20 µl of WST-1 were added to each well, and cells were incubated for 4 h. The absorbances at 440 nm were measured in a microplate reader (Allsheng AMR-100 Elisa Reader, Hangzhou Allsheng Instruments Co., Ltd. Zhejiang, China) using wells without cells as background. Sample readings were calculated by subtracting the average of background absorbances. All experiments were performed at least four times.

Estimation of VEGF, SP, and TSP-1

To determine the possible effects of SK on angiogenic factor levels in MCF-7 cells, AFG Bioscience Human ELISA kits were used following the manufacturer's protocols (Cat. No: EK711520; EK710535; EK711455, AFG Bioscience, Northbrook, IL USA, respectively). Briefly, the cells were divided into 6 well-plates as 50,000 cells/well, and cells were treated with different doses of SK (100 nM, 10 nM, 1 nM, 0.1 nM) and incubated for 24,48- and 72-hour incubation periods. The experiments were terminated, and the media were collected and studied by the kit protocols. Human angiogenic factor-specific polyclonal antibody-coated wells were used. Serial dilutions of purified human VEGF, SP, and TSP-1 (as standards) and cell media (200 µl) were added into each well. The procedure was started with the incubation of a primary antibody and a biotinylated secondary antibody for three factors. Then, streptavidin-peroxidase enzyme and the relative substrate solution were added and the absorbances were determined at 450 nm in an ELISA plate reader. All measurements were performed three times and average results were compared to non-treated cells. Average results from three independent experiments were used to compare non-treated and treated cells.

In Silico Studies

Protein Structure Preparation

X-ray crystal structures were obtained from the Protein Data Bank (PDB). The peptide sequence (H-DL-His-DL-Lys-DL-xiThr-DL-Asp-DL-Ser-DL-Phe-DL-Val-Gly-DL-Leu-DL-Met-NH2; letter form: HKXDSFVGLM) was obtained PubChem and PDB file names are as follows: human SRCR domain of Scara5 receptor, PDB ID: 7C00; human NEUROKININ-1 receptor, PDB ID: 6E59; human NEUROKININ-2 receptor, PDB ID: 7XWO; human NEUROKININ-3 receptor, PDB ID:1f9p; Human vascular endothelial growth factor receptor-1, PDB ID: 1VR2; Human vascular endothelial growth factor receptor-2, PDB ID: 1YWN; Human CD36 receptor, PDB ID: 5LGD; Human NRP1 receptor, PDB ID:1KEX; Human Tie 2 receptor PDB ID: 1FVR and Human PDGFR PDB ID: 3MJG. BIOVIA Discovery Studio was used for Protein Preparation.

Molecular Docking Studies

HPEPDOCK 2.0, MDockPeP, and CABS-dock web servers were used for peptide-protein docking studies. The SK peptide was docked to the binding site cavity by the automized system in the servers. Docking calculations were performed with an exhaustiveness option of 10 (average accuracy). The servers give docking results as binding energy scores for ten positions and create a list for the top ten positions and name them 1 to 10 (1 is the best, 10 is the worst). For each receptor-docking study, model 1 was chosen for evaluation and visualization.

Statistical analysis

Data are expressed as the mean ± SEM. Results were analyzed using one-way ANOVA followed by Dunnett's Multiple Comparisons Test for comparison of group means to control or by the Student's t-test. (Graph Pad InStat., USA). A P value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

This study focused on the possible proliferative and angiogenic effects of SK on MCF-7 breast cancer cells. The proliferative effects of different concentrations of SK (100 nM, 10 nM, 1 nM, 0.1 nM) were evaluated via the WST-1 assay. Cell viability was determined 24, 48, and 72 h after SK treatments. As shown in Figures 1, 2, and, SK triggers the proliferation of cancer cells in a time-dependent manner ($P > 0.05$).

Determination of VEGF, SP, and TSP-1 levels via the ELISA method

The protein levels of angiogenic factors were examined at the end of 24, 48, and 72 h incubation periods following different concentrations of SK administration. To characterize the levels of the factors in the MCF-7 cell line were investigated by enzyme- linked immunosorbent assay. According to the results, At the end of the 24 hours, especially in cells treated with 0.1 nM concentration of SK, the amount of SP release increased as compared to the control group. This situation was not observed at other concentrations. At the end of the 48 hours, 10 nM and 0.1 nM SK treatments could cause a significant increase in SP levels as compared to the control group. Similarly, for a 72-hour incubation period, 100 nM and 0.1 nM SK treatment increased SP levels. VEGF release was notably increased especially for the 48-hour incubation period. On the other hand, for all incubation periods and all doses, SK treatment could cause a significant decrease in TSP-1 release except 100 nM SK-treated group. SK alone did not increase SP release, however, it has been determined that SK can affect VEGF and TSP 1 release from MCF-7 cells. SK might have an SP-like, angiogenic effect at least for breast cancer cells. Results are representative of three independent experiments and are given in Table 1, Table 2, and Table 3.

In silico results

In order to determine the possible interaction of SK with angiogenic receptor proteins molecular peptide-protein docking method was performed via HPEPDOCK 2.0 and MDockPeP servers. The energy scores of each peptide/receptor protein docking are given in Table 4.

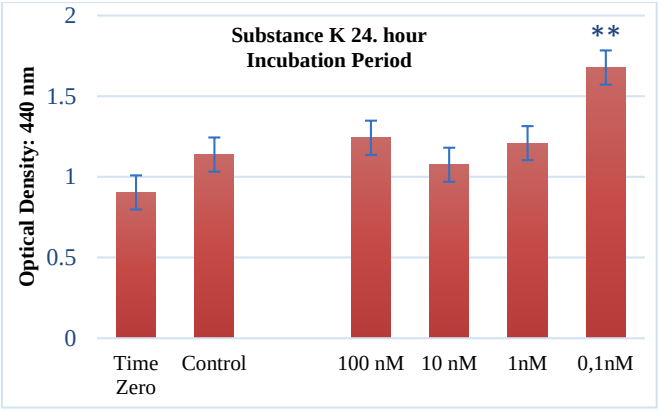


Figure 1. In vitro proliferation of MCF-7 cells for 24 hours. Cell growth was determined after 24 h using a WST-1 solution. Time 0 represents the cell number at the beginning of the experiments. Control represents the untreated control group. Results are representative of four independent experiments with eight replicates and are reported as means ± SE.

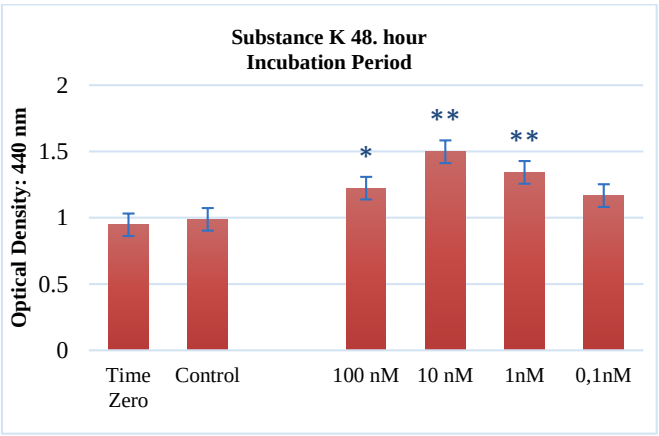


Figure 2. In vitro proliferation of MCF-7 cells for 48 hours. Cell growth was determined after 48 h using a WST-1 solution. Time 0 represents the cell number at the beginning of the experiments. Control represents the untreated control group. Results are representative of four independent experiments with eight replicates and are reported as means ± SE.

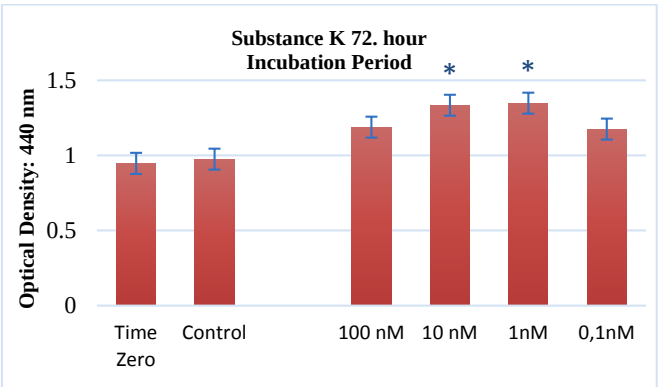


Figure 3. In vitro proliferation of MCF-7 cells for 72 hours. Cell growth was determined after 72 h using a WST-1 solution. Time 0 represents the cell number at the beginning of the experiments. Control represents the untreated control group. Results are representative of four independent experiments with eight replicates and are reported as means ± SE.

Table 1 SP release from MCF-7 cells. C: Control; SK doses (100,10,1,0.1 nM) SP release expressed as ng per L.

Groups	24 h incubation period	48 h incubation period	72 h incubation period
C	83 ng/L	84 ng/L	85 ng/L
SK (100 nM)	74 ng/L	49 ng/L	105 ng/L
10 nM	79 ng/L	81 ng/L	81 ng/L
1 nM	80 ng/L	77 ng/L	83 ng/L
0,1 nM	96 ng/L	88 ng/L	92 ng/L

Table 2 VEGF release from MCF-7 cells. C: Control; SK doses (100,10,1,0.1 nM) VEGF release expressed as ng per L

Groups	24 h incubation period	48 h incubation period	72 h incubation period
C	57 ng/L	56 ng/L	68 ng/L
SK (100 nM)	51 ng/L	64 ng/L	70 ng/L
10 nM	53 ng/L	66 ng/L	62 ng/L
1 nM	58 ng/L	71 ng/L	76 ng/L
0,1 nM	55 ng/L	62 ng/L	67 ng/L

Table 3 TSP-1 release from MCF-7 cells. C: Control; SK doses (100,10,1,0.1 nM) TSP-1 release expressed as ng per L.

Groups	24 h incubation period	48 h incubation period	72 h incubation period
C	274 ng/L	261 ng/L	224 ng/L
SK (100 nM)	225 ng/L	260 ng/L	252 ng/L
10 nM	226 ng/L	247 ng/L	106 ng/L
1 nM	262 ng/L	243 ng/L	150 ng/L
0,1 nM	249 ng/L	221 ng/L	137 ng/L

According to our peptide-protein docking results, SK strongly binds to NK-1, NK-2, and Tie2 receptor proteins with a binding energy score of -256,454; -226,565, and -187,506 respectively. The binding position schemes, pairs of peptide/receptor residues, and contact map of interactions obtained from the HPEPDOCK 2.0 and CABS-dock are given in Figures 4,5 and 6 below.

Due to the high binding potential of SK to NK-1, we wanted to know if this interaction is stronger than SP-NK-1 interaction or not. To test this, we further examined the interaction between SP to NK-1, NK-2, and Tie-2 receptor proteins via molecular peptide-receptor docking via the HPEPDOCK 2.0 and CABS-dock servers. According to our results, SK more strongly binds to the NK-1 receptor than SP does with a binding energy of -242,567. The binding position schemes of SP/NK-1, SP/NK-2, and SP/Tie2 interactions are given in Figure 7.

This study aims to investigate the possible angiogenic roles of SK on human breast cancer cell line MCF-7 in vitro and its interactions with angiogenic receptors in silico. For this purpose, firstly the proliferative effects of SK on MCF-7 cells were examined via WST-1 assay. Secondly, we tried to determine the changes in angiogenic factor levels that are released from the cell to the media. The possible interaction of SK with certain angiogenic receptors by peptide-protein molecular docking method via HPEPDOCK 2.0, CABSdock, and MDockPeP servers. SK is a 9 amino acid (N- His; Lys; Asp; Ser; Phe; Val; Gly; Leu; Met -C) peptide belonging to the Tachykinin gene family like Substance P.[20]

Table 4 SK refers to Substance K. The amino acid sequence is HKXDSFVGLM. Full Names of Receptor proteins: The Scavenger receptor cysteine-rich domain of scavenger receptor class A member 5 (SRCR domain of Scara5); Neurokinin (NK); Vascular endothelial growth factor receptor (VEGFR); Cluster of differentiation 36 (CD36); Neuropilin-1 (NRP1); Angiopoietin-1 receptor (Tie-2); Platelet-derived growth factor receptor (PDGFR). * refers to the top three best scores

Peptide/Protein Docking	Binding Energy scores
SK/ SRCR domain of Scara5 receptor	-182,093
SK/ NK-1 receptor	**-256,454***
SK/ NK-2 receptor	**-226,565****
SK/ NK-3 receptor	-124,515
SK/ VEGFR-1 receptor	-179,249
SK/ VEGFR-2 receptor	-177,326
SK/ CD36 receptor	No score was calculated
SK/ NRP1 receptor	-168,651
SK/ Tie2 receptor	**-187,506*****
SK/ PDGFR receptor	-170,822

It has been known that both peptides have agonist effects on their receptors. Tachykinin receptors are expressed in many cancer cells, and they affect the proliferation, apoptosis, and metastasis pathways of cancer cells via autocrine/paracrine/neuroendocrine signals.[21]

In studies conducted on Substance K, it is accepted that it is an indicator of poor prognosis with increased levels of it in circulation (SK≥ 50 ng/L). The decrease in the amount in circulation indicates that there is a significant improvement in the prognosis.[22]

According to our proliferation results, 0.1 nM SK treatment could lead to an increase in proliferation ((** $p< 0.001$) for the 24-hour incubation period. 48-hour treatment of SK in three doses (100nM -** $p< 0.005$-, 10nM - ** $p< 0.001$- and 1 nM - ** $p< 0.001$) also enhanced the proliferation of MCF-7 cells. 10nM (* $p< 0.005$) and 1 nM (* $p< 0.005$) SK treatments also triggered the cell proliferation. Accordingly, SK assumed a proliferative role in the viability of MCF-7 cells, as we predicted.

According to our test results, the amounts of SP released from MCF-7 cells to the medium differ depending on the 24-, 48- and 72-hour periods. Although this difference is not significant, it is not correlated with the concentration of SK. According to our results, the amount of VEGF released from MCF-7 cells to the medium increased significantly at the 48th hour. In this context, it can be accepted that Substance K is also an angiogenic peptide like SP and may play an important role in cancer angiogenesis.

The biological functions of tachykinins are carried out by three receptors. These receptors are neurokinin-1 (NK-1), neurokinin-2 (NK-2), and neurokinin-3 (NK-3).[23] The receptor of SP is NK-1. SP is a natural ligand with the highest affinity binding to the

NK-1 receptor. Stimulation of NK-1s generates several secondary messengers that can trigger many of the effector mechanisms necessary for the stimulation and function of cells. The binding of SP to its specific receptor activates the G protein and because of this activation, the formation of a complex including SP, NK-1, β-arrestin, SRC, and ERK1/2 proteins is activated. ERK1/2 in this activated complex moves toward the nucleus increases cell proliferation and protects the cell from apoptosis by activating MAP kinases.[24]

On the other hand, the amount of TSP 1 released from MCF-7 cells to the medium decreased due to the presence of Substance K in the medium. Thus, Substance K has the potential to reduce the release of factors that cause inhibition of angiogenesis.

Protein-peptide interactions are fundamental to various cellular functions such as transcription regulation, signal transduction, and immune response however, especially for cancer these interactions could be a promising target for novel therapeutic strategies[25]. Understanding the structure of protein-peptide complexes is crucial for grasping the mechanisms of protein-peptide interactions and advancing peptide-based therapeutics. However, only a fraction of the entire protein-peptide interaction universe has available resolved complex structures on the Protein Data Bank (PDB), due to the technical and financial challenges involved in determining complex structures through experimental techniques like X-ray crystallography.

Several in silico methods have recently been developed to predict the structures of protein-peptide complexes, addressing this challenge.[26] Thus, in addition to our in vitro results, due to the limitation in the project budget, the interaction potentials of SK with 10 important receptors involved in the angiogenesis process were investigated in silico via peptide-protein molecular docking method by HPEPDOC 2.0 and MDockPeP servers.[26,27]

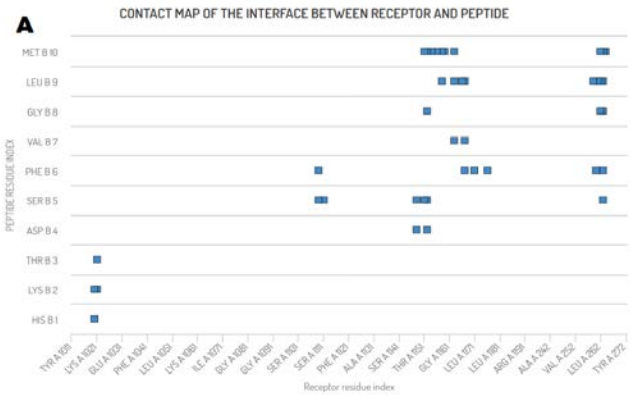
Despite their well-known neuronal and neuroendocrine functions, neuropeptides play a significant role in regulating cancer progression and metastasis. They are critical factors that either stimulate or suppress tumor cell growth through autocrine and paracrine signaling pathways[28]. Especially for breast cancer, Neuropeptide Y (NPY) functions as a breast cancer suppressor, with the effects being mediated by neuropeptide Y receptors 1 and 5 (Y1R and Y5R). Y1R is mainly expressed in breast cancer. Moreover, the NPY/Y1R system was found to inhibit forskolin-stimulated cAMP production and mobilize intracellular Ca2+ in MCF-7 cells, leading to the suppression of estrogen-induced cancer cell proliferation.

Y5R expression was detected in MCF-7, T47D, MDA-MB-231, MDA-MB-468, HS578T, and BT-549 cell lines. Upon activation of Y5R, cAMP production is inhibited in BT-549 cells, leading to an increase in mitogen-activated protein kinase (MAPK) activity and a concomitant rise in extracellular signal-regulated kinase (ERK1/2) phosphorylation. This subsequently promotes cell proliferation.[10]

The role of the neuropeptide/GPCR family in multiple types of cancer as tumor regulators is widely recognized. Research on the involvement of the neuropeptide/Receptor system in cancer development highlights the intricacies of the neuroendocrine/receptor pathway. Multiple neuropeptides are acknowledged for their influence on cancer cell signaling. For instance, VIP is reported to operate through cAMP/PKA in non-tumor cells, and via cAMP/ERK/PI3K in prostate tumor cells.[28]

However, there is no study related to the angiogenic effects of SK on breast cancer cells. Although we investigated the effects on the amounts of proangiogenic and anti-angiogenic factors released from cancer cells in vitro, we would also need to study the effect on vascular endothelial cells in order to talk about a full angiogenic effect. For this reason, we wanted to evaluate this point, which we could not achieve in vitro due to a limited budget, at least in silico, in terms of whether it would interact with the ten important receptors of angiogenesis.

Interestingly, according to our docking results, SK binds to the NK receptors NK-1 and NK-2 with very high affinity.



B:

Pairs of peptide /receptor residues closer than 4.5A in the selected complex					
Receptor residue	Peptide residue	Receptor residue	Peptide residue	Receptor residue	Peptide residue
PRO A 263	MET B 10	PHE A 264	MET B 10	PRO A 263	LEU B9
PRO A 263	PHE B 6	PRO A 263	GLY B 8	PRO A 263	SER B 5
LEU A 262	LEU B9	LEU A 262	MET B 10	LEU A 262	GLY B 8
CYS A 260	PHE B 6	TRP A 261	LEU B9	ILE A 259	LEU B 9
LEU A 1171	PHE B 6	LEU A 1176	PHE B 6	ALA A 1167	LEU B 9
ALA A 1167	PHE B 6	ALA A 1167	VAL B 7	LEU A 1166	LEU B 9
PRO A 1163	LEU B 9	PRO A 1163	MET B 10	PRO A 1163	VAL B 7
VAL A 1158	MET B 10	LYS A 1159	MET B 10	VAL A 1158	LEU B9
THRA 1154	MET B 10	ILE A 1156	MET B 10	ASN A 1152	MET B 10
ASN A 1152	SER B 5	ASN A 1152	GLY B 8	ASN A 1152	ASP B 4
THRA 1151	SER B 5	THR A 1151	MET B 10	ASPA 1148	SER B 5
SER A 1111	SER B 5	ASP A 1148	ASP B4	TYRA 1109	PHEB 6
LYS A 1021	THR B 3	TYR A 1109	SER B 5	LYS A 1021	LYS B 2
LYS A 1020	HIS B 1	LYS A 1020	LYS B 2		

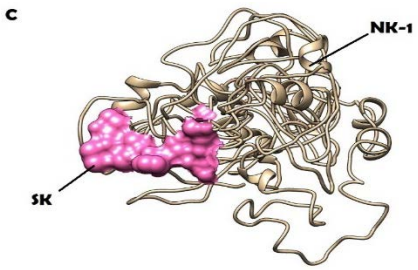
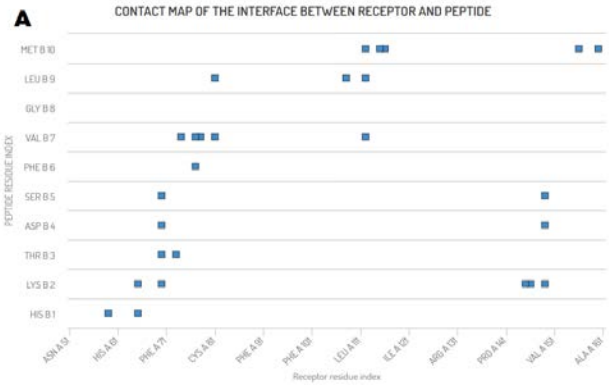


Figure 4 SK refers to Substance K. NK-1 refers to Neurokinin-1; A: Contact map of the interface between NK-1 and SK. B: There are 41 Pairs of peptide/receptor residues closer than 4.5 Å in the interaction site, C: Schematic view of the docking



B: Pairs of peptide /receptor residues closer than 4.5A in the selected complex					
Receptor residue	Peptide residue	Receptor residue	Peptide residue	Receptor residue	Peptide residue
LYS A 149	SER B5	TRPA 156	MET B 10	LEU A 160	MET B 10
PRO A146	LYS B2	LYS A 149	LYS B2	LYSA 149	ASPB4
THRA 115	MET B 10	ALAA 116	MET B 10	ALAA 145	LYS B2
PHE A 112	VALB7	PHE A 112	LEU B9	PHEA 112	MET B 10
CYSA 81	VAL B7	CYSA81	LEU 89	PHE A 108	LEU B9
LEU A 77	PHE B 6	LEU A 77	VAL B7	ALAA 78	VALB7
TYRA 70	SERB5	VALA 73	THRB 3	ASNA 74	VAL B7
TYRA 70	LYS B2	TYRA 70	THRB 3	TYRA 70	ASP B 4
LEUA 59	HIS B1	ARGA 65	HIS B1	ARGA 65	LYS B2

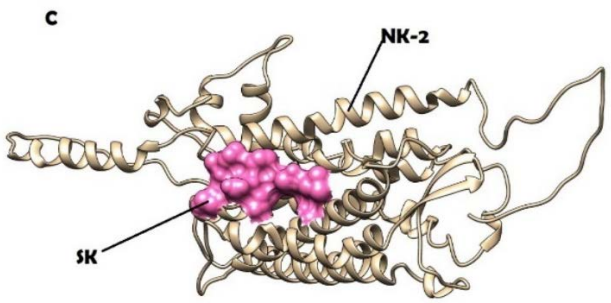
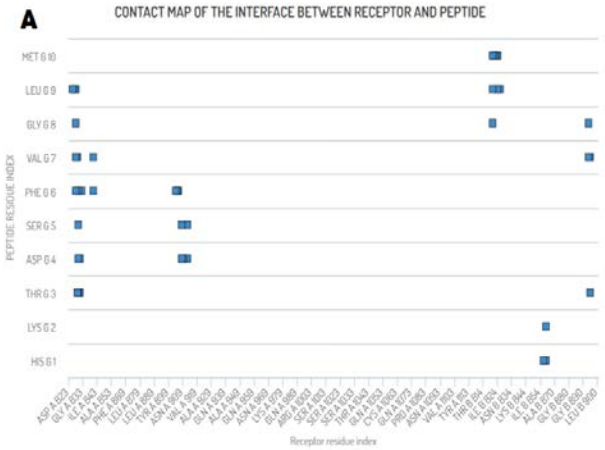


Figure 5. SK refers to Substance K. NK-2 refers to Neurokinin-2; A: Contact map of the interface between NK-2 and S, B: There are 27 Pairs of peptide/receptor residues closer than 4.5 Å in the interaction site, C: Schematic view of the docking.



B: Pairs of peptide /receptor residues closer than 4.5A in the selected complex					
Receptor residue	Peptide residue	Receptor residue.1	Peptide residue.1	Receptor residue.2	Peptide residue.2
ARG B 895	VAL G 7	HIS B 894	GLY G8	ARG B 895	THR G 3
HIS B 894	VAL G 7	LYS B 858	HIS G 1	LYS B 858	LYS G2
ARG B 856	HIS G1	ILE B 824	MET G 10	PHE B 826	LEU G 9
ILE B 824	LEU G 9	ASN B 822	MET G 10	ASP B 823	MET G 10
TRP B 821	MET G 10	TRP B 821	GLY G 8	TRP B 821	LEU G 9
ASP A 912	SER G 5	ASN A 909	ASP G 4	ASP A 912	ASP G4
GLY A 908	SER G5	PRO A 906	PHE G 6	GLY A 908	ASP G4
ALAA 905	PHE G 6	LYS A 840	VAL G 7	TYR A 904	PHE G 6
LYS A 840	PHE G	GLY A 831	ASP G4	GLU A 832	PHE G 6
GLY A 831	THR G3	ILE A 830	SER G5	ILE A 830	PHE G 6
ILE A 830	ASP G4	VAL A 829	VAL G 7	ILE A 830	THR G 3
VAL A 829	THR G 3	ASP A 828	GLY G 8	ASP A 828	LEU G 9
ASP A 828	VAL G7	GLN A 827	LEU G 9	ASP A 828	PHE G 6
PHE A 826	LEU G 9				

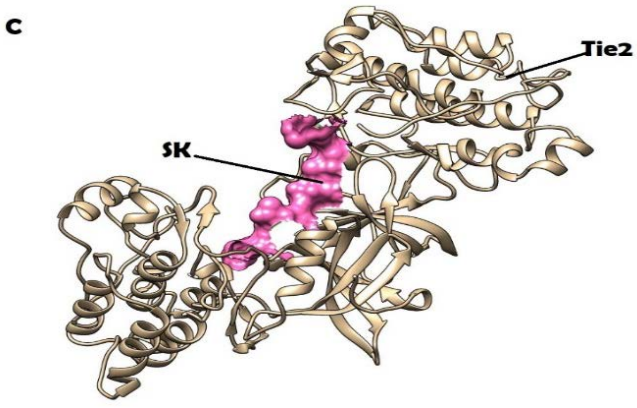


Figure 6. SK refers to Substance K. Tie2 refers to the Angiopoietin-1 receptor; A: Contact map of the interface between SK and Tie2, B: There are 40 Pairs of peptide/receptor residues closer than 4.5 Å in the interaction site, C: Schematic view of the docking.

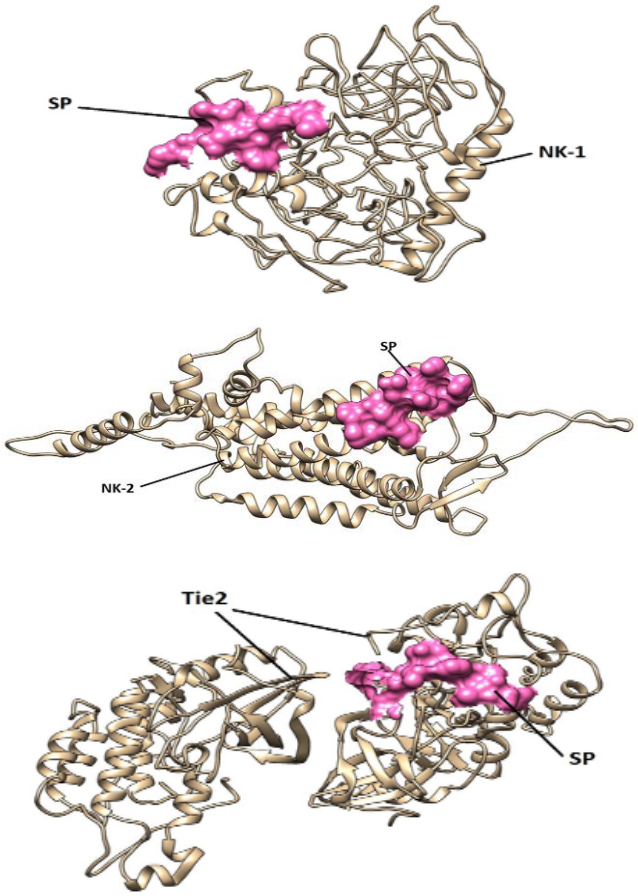


Figure 7. Schematic view of the Docking. A: SP/NK-1, B: SP/NK-2, C: SP/Tie2.

NK-1 is the receptor of SP, and NK-2 is the actual receptor of SK, however, SK binds to NK-1 with higher affinity than its own receptor. Although the main angiogenic receptors are VEGFR1 and VEGFR2, SK may show its angiogenic effect through the Tie-2 receptor. According to our docking binding energy results, the receptor that SK binds with the highest affinity, except for its own receptor, is the Tie2 receptor. In this context, new therapeutic compounds that will block interactions between both the SP receptor NK-1 and the Tie2 receptor of SK may be promising for cancer treatment.

Conclusion

In conclusion, this is the first report showing that Substance K might be a novel angiogenic peptide, and blocking its receptor could lead to a novel anti-cancer strategy. Understanding the molecular mechanisms of Substance K on a cellular level and its interactions with angiogenic receptors may display important outputs for cancer therapy. In vitro, in vivo, and silico studies must be needed.

Abbreviations

Ala: Alanine
Arg: Arginine
Asn: Asparagine
Asp: Aspartic acid
BT-549: Human Breast Cancer Cell Line
cAMP: Cyclic adenosine monophosphate
CD36: Platelet Glycoprotein 4
Cys: Cysteine
DMEM: Dulbecco's modified Eagle's medium
ECs: Endothelial Cells
ERK1/2: extracellular signal-regulated kinase
FBS: FETAL BOVİNE SERUM
Gln: Glutamine
Glu: Glutamic acid
Gly: Glycine
His: Histidine
HS578T: Human Breast Cancer Cell Line
Ile: Isoleucine
Leu: Leucine
Lys: Lysine
MAPK: Mitogen-activated protein kinase
MCF-7: Human Breast Cancer Cell Line
MDA-MB-231: Human Breast Cancer Cell Line
MDA-MB-468: Human Breast Cancer Cell Line
Met: Methionine
NK-1: Neurokinin-1
NK-1R: Neurokinin-1 Receptor
NK-2: Neurokinin-2
NK-3: Neurokinin-3
NPY: Neuropeptide Y
NRP1: Neuropilin-1
PBS: Phosphate-Buffered Saline
PDB: Protein Darta Bank
PDGFR: Platelet-Derived Growth Factor Receptor
Phe: Phenylalanine
PI3K: Phosphatidylinositol 3-kinase
Pro: Proline
Ser: Serine
SK: Substance K
SP: Subtance P
T47D: Human Breast Cancer Cell Line
Thr: Threonine
Tie-2: Angiopoietin-1 Receptor
Trp: Tryptophan
TSP-1: Thrombospondin-1
Tyr: Tyrosine
Val: Valine
VEGF: Vascular Endothelial Growth Factor
VEGFR-1: Vascular Endothelial Growth Factor Receptor 1
VEGFR-2: Vascular Endothelial Growth Factor Receptor 2
WST-1: (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt)

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Conflict of Interest Statement

Authors declare that there is no conflict of interest.

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