Analog of Somatostatin Vapreotide Exhibits Biological Effects in vitro via Interaction with Neurokinin-1 Receptor

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Key Words

Substance P · Neurokinin-1 receptor · Somatostatin · Vapreotide · HIV · Somatostatin 2 receptor · Cyanamid-154806

Abstract

Objectives: Vapreotide, a synthetic analog of somatostatin, has analgesic activity most likely mediated through the blockade of neurokinin-1 receptor (NK1R), the substance P (SP)-preferring receptor. The ability of vapreotide to interfere with other biological effects of SP has yet to be investigated. **Methods:** We studied the ability of vapreotide to antagonize NK1R in three different cell types: immortalized U373MG human astrocytoma cells, human monocyte-derived macrophages (MDM) and a human embryonic kidney cell line, HEK293. Both U373MG and MDM express endogenous NK1R while HEK293 cells, which normally do not express NK1R, are stably transformed to express human NK1R (HEK293-NK1R). **Results:** Vapreotide attenuates SP-triggered intracellular calcium increases and nuclear factor- κ B activation in a dose-dependent manner. Vapreotide also inhibits SP-induced interleukin-8 and monocyte chemotactic protein-1 production in HEK293-NK1R and U373MG cell lines. Vapreotide inhibits HIV-1 infection of human MDM in

vitro, an effect that is reversible by SP pretreatment. **Conclusions:** Our findings indicate that vapreotide has NK1R antagonist activity and may have a potential application as a therapeutic intervention in HIV-1 infection.

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Introduction

Substance P (SP) is a major peptide regulator of the interaction between the immune and nervous systems [1, 2]. The biological response to SP is mediated by its preferred receptor, neurokinin-1 receptor (NK1R). NK1R is a member of the G-protein-coupled receptor superfamily (Gq/11) and has a typical structure of seven transmembrane domains, an extracellular N terminus and an intracellular C terminus [3]. Our previous investigations [4–11] have demonstrated the presence of NK1R at both mRNA and protein levels in cells of the human immune system and the nervous system, including human peripheral blood mononuclear cells [4], lymphocytes [5], microglia [6] and natural killer cells [11]. Subsequent to binding to NK1R, SP triggers the intracellular mobilization of calcium reserves [12–14], activation of nuclear factor- κ B (NF- κ B; a key transcriptional factor involved in

the control of cytokine expression) [15, 16], upregulation of tissue factor expression [17] and upregulation of interleukin (IL)-8 and monocyte chemotactic protein-1 (MCP-1) expression [15, 18–21]. SP also induces immediate early gene (EGR-1 and c-FOS) expression [22–25]. We have observed that SP and NK1R are actively involved in HIV infection of human immune cells and, in particular, monocyte-derived macrophages (MDM) [26, 27]. SP enhances HIV replication in human peripheral MDM [28]. Interruption of the interaction between SP and NK1R with NK1R antagonists (CP-96,345 or aprepitant) inhibits HIV infection of human MDM [27, 29].

Somatostatin is a peptide hormone which is distributed in many tissues including the immune system and the nervous system. Exposure to somatostatin causes inhibition of immunoglobulin G2a secretion by B lymphocytes [30] and interferon- γ secretion by T cells [31]. Vapreotide (RC-160) is a synthetic octapeptide analog of the naturally occurring somatostatin hormone. It has analgesic activity, which is most likely mediated by NK1R, the SP-preferring receptor [32]. Vapreotide displaces titrated SP ($IC_{50} = 3.3 \pm 1.8 \times 10^{-7}$ M) binding bronchial NK1R sites in a dose-dependent manner and it significantly and specifically reduces the SP-induced extravasations of the dye Evans Blue in the trachea and main bronchi via interaction with NK1R, suggesting that vapreotide also has an NK1R antagonist effect [32].

In order to further establish the NK1R antagonist effect of vapreotide in human cells, we investigated the effect of vapreotide on SP-induced elevation of cytosolic, free Ca^{2+} , activation of NF- κ B, and downstream effects such as activation of immediate early genes and cytokine and chemokine production. We also examined the effect of vapreotide on HIV-1 replication in human MDM in vitro.

Materials and Methods

Cells and Virus

HEK293, a human embryonic kidney cell line, and U373MG, a human astrocytoma cell line, were purchased from American Type Culture Collection (Rockville, Md., USA) and grown in DMEM supplemented with 10% fetal bovine serum, glutamine and antibiotics at 37°C in 5% CO₂. The culture medium was changed one day before SP treatment. U373MG cells express endogenous NK1R [33]. The HEK293 cell line, which stably expresses human NK1R (HEK293-NK1R), was established as previously described [18].

MDM were isolated from peripheral blood of normal adult donors (CFAR Immunology Core, University of Pennsylvania). The blood samples were identified as HIV-1 antibody negative by anonymous testing with the ELISA method (Coulter Immunology, Hialeah, Fla., USA). Monocytes were purified according to our

previously described techniques [34, 35] and cultured in 48-well plates at a density of 0.25×10^6 cells/0.5 ml/well for 7–10 days before HIV-1 infection.

The M-tropic HIV-1 R5 strain BaL was obtained from the NIH AIDS Reagent Program.

Reagents

pNF- κ B-luc multimer-driven luciferase construct [36] was generously provided by Dr. Randy Q. Cron. The Cell Line Nucleofector Kit V was purchased from Amaxa (Gaithersburg, Md., USA). The luciferase assay system was obtained from Promega (Madison, Wisc., USA). SP and vapreotide (RC-160) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). The NK1R antagonist, CP-96,345 [37], was generously provided by Pfizer Inc. (Groton, Conn., USA). Aprepitant (Emend®), manufactured by Merck & Co. (Whitehouse Station, N.J., USA), was purchased through the Children's Hospital of Philadelphia Pharmacy and extracted and purified by chromatography. Cyanamid-154806 (CYN), a somatostatin 2 receptor (SSTR2) antagonist, was obtained from Tocris Bioscience (Ellisville, Mo., USA). EIA kits for MCP-1 and IL-8 were purchased from Invitrogen Corporation (Carlsbad, Calif., USA). The sensitivity of the kits was <5 pg/ml.

SP-Induced Calcium Mobilization

Intracellular calcium measurements were performed in fura-2-loaded cells using the ratiometric method of Tsien and colleagues [38]. Cells were seeded in 96-well plates and allowed to adhere overnight at 37°C, 5% CO₂. Fura-2 loading was performed by incubating cells for 45 min at room temperature in medium containing 4 μ M fura 2-AM and 0.01% Pluronic F-127 (Molecular Probes, Eugene, Oreg., USA). Cells were washed in Hanks balanced salt solution containing 1 mM calcium chloride, and fluorescence was recorded in individual cells using an imaging system from Photon Technologies Inc. (Lawrenceville, N.J., USA). Recordings were performed using excitation wavelengths of 340 nm (Ca^{2+} bound Fura) and 380 nm (Ca^{2+} unbound Fura), and emitted light was measured at 510 nm over 240 s. The ratio of these measurements (340/380) in combination with a standardization kit (Molecular Probes) was used to calculate the intracellular calcium concentration. Peak intracellular calcium concentrations were determined after SP stimulation and were used to construct concentration-response curves. Logistic curves were fitted to data and used to derive EC₅₀ values for SP.

Luciferase Assay for NF- κ B Activation

pNF- κ B-luc plasmid (5 μ g) was transfected into HEK293-NK1R (2×10^6 cells per transfection) by Nucleofector II using the Cell Line Nucleofection Kit V following the manufacturer's protocol. After transfection, the cells were cultured in 24-well plates (0.25×10^6 cells/well) in DMEM containing 10% fetal bovine serum. Forty-eight hours after transfection, the cells were treated with SP (10^{-7} M) in the presence or absence of NK1R antagonists (aprepitant or CP-96,345 1 μ M) or vapreotide (10 μ M) for 6 h. Mock-treated cells were used as controls. Cell-free lysates were collected in 0.25 ml of 1 $\times$ Reporter Lysis Buffer (Promega) and centrifuged at 10,000 g for 1 min. The cell-free lysates were used to determine the NF- κ B-driven luciferase activity by the Luciferase assay system and a luminometer. The luciferase activity is expressed as relative light units (RLU). The results are presented as fold change in RLU compared to untreated controls, which are defined as 1.0.

Vapreotide Treatment

The HEK293-NK1R and U373MG cells were incubated with or without vapreotide for 10 min and then incubated with or without SP for 3 h. In some experiments, cells were first incubated with CYN for 10 min, and then vapreotide was added and incubated for an additional 10 min, followed by stimulation with SP for 3 h. Mock-treated cells were used as controls.

HIV Infection

MDM cultured in 48-well plates for 7–10 days (0.25×10^6 cells/well) were incubated with or without vapreotide for 2 h before infection with HIV-1. Cells were first incubated with vapreotide for 10 min and then SP was added to the MDM and the cells were incubated for an additional 2 h. Mock-treated MDM served as controls. After a 2-hour incubation with or without vapreotide and/or SP, the cells were infected overnight with cell-free HIV based on p24 antigen content ($16 \text{ ng}/10^6$ cells), and then extensively washed to remove unbound virus. Fresh medium supplemented with vapreotide and/or SP was added as described above. The culture medium and the reagents were replaced twice weekly. At day 7 after HIV infection, cellular RNA was extracted from the MDM for assessment of HIV gag mRNA expression using real-time RT-PCR assays.

RNA Extraction and Real-Time RT-PCR Assays

Total RNA was extracted from U373MG, HEK293-NK1R and MDM cells using RNeasy kit (Qiagen), and the potential DNA contamination was eliminated by on-column DNase digestion as instructed by the manufacturer (Qiagen). RNA concentration was determined by Qubit fluorometer using the Quant-iT RNA Assay kit (Invitrogen, Eugene, Oreg., USA).

Total RNA ($1 \mu\text{g}$) was reverse transcribed using the AffinityScript qPCR cDNA Synthesis kit (Stratagene, Cedar Creek, Tex., USA) with random primer, as instructed by the manufacturer. Reverse transcriptase-negative controls were used in order to control for genomic DNA contamination; $1 \mu\text{l}$ of the resulting cDNA was used as a template for real-time PCR amplification.

The sequences of the primers used in this study are listed in table 1. All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa., USA).

The mRNA levels of IL-8, MCP-1, EGR-1, c-FOS, HIV gag and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantitatively determined using the iQ iCycler system (Bio-Rad Laboratories, Inc., Hercules, Calif., USA). Thermal cycling conditions were designed as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. The specificity of the amplification was confirmed by SYBR dye dissociation curve and agarose gel electrophoresis. All amplification reactions were performed in duplicate. The averages of the threshold cycle (Ct) values of the duplicates were used to calculate the relative mRNA levels of these genes. In order to control the integrity of the RNA and normalize mRNA levels in these samples, the GAPDH gene was used as previously described [39]. The magnitude of change in IL-8, MCP-1, c-FOS and EGR-1 mRNA expression in response to SP and/or vapreotide or NK1R antagonists in the HEK293-NK1R or U373MG cells was calculated using the standard $2^{-(\Delta\Delta\text{Ct})}$ method [40]. For quantification of HIV gag cDNA in MDM, known amounts of HIV DNA isolated from ACH-2 cells were used to generate a standard curve. All standards and samples were run in duplicate.

Table 1. Primers used for real-time PCR assays

Gene		Sequence of primer (5'–3')
IL-8	Sense:	CTCTTGGCAGCCTTCCTGATTTCT
	Antisense:	TGTGGTCCACTCTCAATCACTCTC
MCP-1	Sense:	CTCGCTCAGCCAGATGCAATCAAT
	Antisense:	TGGAATCCTGAACCCACTTCTGCT
EGR-1	Sense:	GTGGCCTAGTGAGCATGACCAA
	Antisense:	AAATGGGACTGCTGTCGTTGGATG
c-Fos	Sense:	TGTCTGTGGCTTCCCTTGATCTGA
	Antisense:	TGGATGATGCTGGGAACAGGAAGT
HIV gag [56]	Sense:	CATGTTTTCAGCATTATCAGAAGGA
	Antisense:	TGCTTGATGTCCCCCACT
	Probe:	CACCCACAAGATTTAAACACCAT GCTAA
GAPDH	Sense:	GGTGGTCTCCTCTGACTTCAACA
	Antisense:	GTTGCTGTAGCCAAATTCGTTGT

Statistical Analyses

The results are expressed as the mean $\pm$ SD for replicate observations. Evaluation of the significance of differences between parameters was performed using Student's t test. In all cases, $p < 0.05$ was considered significant.

Results

Vapreotide Dose-Dependently Inhibits SP-Induced Intracellular Calcium Increases

SP induced an increase in calcium in U373MG cells in a dose-dependent manner with a maximum response at a concentration of $0.1 \mu\text{M}$, while the NK1R antagonist, aprepitant, completely antagonized this effect at $0.1 \mu\text{M}$ (fig. 1a). Vapreotide attenuated the effect of SP on calcium release in a concentration-dependent manner (fig. 1b). The concentration required for vapreotide to completely inhibit the effect of SP is about 100 times higher than that required for the NK1R antagonist aprepitant (fig. 1b).

Vapreotide Attenuates SP Activation of NF- κ B and Inhibits SP-Induced Gene Expression in HEK293-NK1R Cells

SP increased the NF- κ B-driven luciferase gene expression in HEK293-NK1R (fig. 2a), and this enhancement was antagonized by aprepitant or CP-96,345 and partially inhibited by vapreotide pretreatment.

Fig. 1. Aprepitant and vaporetide suppress SP-induced $[Ca^{2+}]_i$ increase in U373MG cells. **a** The effect of different concentrations of aprepitant (10, 30 and 100 nM) on SP-induced intracellular calcium increase was measured in U373MG cells stimulated with concentrations of SP ranging from 10^{-11} to 10^{-5} M. Each data point represents the average calcium concentrations measured in 8 indi-

vidual cells $\pm$ SD. **b** U373MG cells were pretreated with different concentrations of vaporetide as indicated, followed by stimulation with 100 nM or 10 nM of SP. Representative tracings show the average intracellular calcium concentration for 8 cells over 240 s of measurement.

SP treatment upregulates the mRNA expression of IL-8, EGR-1 and c-FOS genes in HEK293-NK1R cells. The NK1R antagonist, aprepitant, inhibited these effects (fig. 2b, c). Vapreotide pretreatment also reduced SP-mediated increases in IL-8, EGR-1 and c-FOS expression.

Vapreotide Inhibits SP-Induced Cytokine and Chemokine Expression in U373MG Cells

In order to further establish the NK1R antagonist effect of vaporetide, we examined its effect in U373MG cells that express endogenous NK1R. SP induced IL-8

Fig. 2. Vapreotide attenuates SP-induced NF-κB activation and IL-8, EGR-1 and c-FOS mRNA upregulation in HEK293-NK1R cells. **a** HEK293-NK1R cells that coexpress NF-κB-luc were preincubated with or without aprepitant (1 μM), CP-96,345 (1 μM) or vapreotide (10 μM) for 30 min followed by stimulation with SP (0.1 μM) for 6 h. Mock-treated cells were used as controls. The results are presented as fold change in RLU compared to the mock-treated control which is defined as 1.0. **b, c** HEK293-NK1R cells were preincubated with or without vapreotide (10 μM), aprepitant (1 μM)

or mock-treated as control for 30 min followed by 0.1 μM SP stimulation for 3 h. IL-8 (**b**), EGR-1 (**c**) and c-FOS (**c**) mRNA levels were determined by real-time PCR and are presented as fold change of mock-treated control, which is defined as 1.0. These results are representative of 3 independent experiments ± SD. NF-κB activation and IL-8, EGR-1 and c-FOS expression were significantly increased in SP-treated cultures (* p < 0.01) versus mock-treated control, aprepitant-treated, CP-96,345-treated or vapreotide-treated cultures by Student's t test.

expression at both mRNA and protein levels while both vapreotide and aprepitant inhibited this SP-induced IL-8 upregulation (fig. 3a, b). Similarly, SP enhanced MCP-1 secretion while both vapreotide and aprepitant antagonized this upregulation (fig. 3c, d). The effect of vapreotide on cell proliferation is mediated primarily by SSTR2 [41]. In order to further establish the NK1R antagonist effect of vapreotide, U373MG cells were pretreated with the SSTR2 selective antagonist CYN followed by incubation with vapreotide and SP stimulation. The data (fig. 3a) show that pretreatment with CYN did not reverse the inhibitory effect of vapreotide

on SP-stimulated IL-8 mRNA expression, suggesting that inhibition by vapreotide was not mediated by SSTR2.

Vapreotide Inhibits HIV-1 Infection of MDM in vitro

Vapreotide reduced HIV-1 replication in MDM as indicated by limited HIV gag mRNA expression compared to control MDM (fig. 4). In addition, SP treatment (10 μM) reversed vapreotide inhibition of HIV-1 replication in MDM (fig. 4). This observation indicates that the inhibition of HIV-1 replication by vapreotide is most likely due to its interaction with NK1R.

Fig. 3. Vapreotide inhibits SP-induced IL-8 and MCP-1 expression in U373MG cells. **a, b** U373MG cells were treated with SP alone, vapreotide and SP, with SSTR2 inhibitor CYN, vapreotide and SP, or with aprepitant and SP. U373MG cells were pretreated with vapreotide (10 μ M) or aprepitant (1 μ M) for 30 min followed by incubation in the presence or absence of SP (0.1 μ M) for 3 h. When cells were treated with both CYN and vapreotide, the cells were first incubated with CYN (10 μ M) for 30 min followed by vapreotide (10 μ M) for an additional 30 min and then stimulated with SP (0.1 μ M) for 3 h. IL-8 mRNA (**a**) levels were determined by real-time PCR and are presented as fold change of mock-treated control, which is defined as 1.0. Secreted IL-8 (**b**) in culture medium was measured by EIA, and the levels are presented as pg/ml.

ml. c, d U373MG cells were preincubated with or without vapreotide (10 μ M), aprepitant (1 μ M) or mock-treated as controls for 30 min followed by 0.1 μ M SP stimulation for 3 h when measuring MCP-1 mRNA (**c**) or for 18 h when measuring MCP-1 secretion in the culture supernatants (**d**). MCP-1 mRNA levels were determined by real-time PCR and are presented as fold of mock-treated control, which is defined as 1.0. Secreted MCP-1 in culture medium was measured by EIA, and data are presented as pg/ml. These results are representative of 3 independent experiments $\pm$ SD. SP-treated cultures had significantly higher levels of IL-8 and MCP-1 (* $p < 0.01$) compared to mock-treated control, aprepitant-treated and CYN and vapreotide-treated cultures by Student's *t* test.

Discussion

We studied the ability of vapreotide to antagonize NK1R in three different cell types: immortalized U373MG human astrocytoma cells, human MDM and a human embryonic kidney cell line, HEK293. Both U373MG and MDM express endogenous NK1R while HEK293 cells, which normally do not express NK1R, are stably transformed to express human NK1R (HEK293-NK1R). Previously, we showed a variety of biological effects in these

three cell types, which express NK1R and respond to SP treatment [33, 42–45]. In contrast, the HEK293 cell line lacking NK1R does not respond to SP treatment [42]. Vapreotide treatment antagonized SP-triggered intracellular calcium increases in a dose-dependent manner and reduced SP-induced NF- κ B activation of IL-8 expression in the HEK293-NK1R cell line. It also inhibited SP-induced IL-8 and MCP-1 production in U373MG astrocytoma cells, which express endogenous NK1R, indicating that vapreotide has NK1R antagonist activity. Further-

Fig. 4. SP reverses vapreotide inhibition of HIV-1 replication in human MDM. MDM cultured for 7–10 days were pretreated with SP and/or vapreotide at the concentrations indicated for 2 h followed by infection with HIV-1 for 7 days. HIV gag mRNA levels were quantified by real-time RT-PCR as copy numbers and normalized based on GAPDH levels. Representative data from 1 of 3 independent experiments $\pm$ SD are shown. Gag mRNA levels were significantly higher in SP-treated cultures (* $p < 0.01$) compared to mock-treated control, vapreotide-treated or both vapreotide and SP-treated cultures by Student's t test.

more, vapreotide attenuated HIV-1 replication in human MDM in vitro via interaction with NK1R.

Three downstream indicators of NK1R activation, SP-induced intracellular calcium increases, activation of NF- κ B and upregulation of cytokine expression, are antagonized by treatment with the NK1R antagonists CP-96,345 and aprepitant [14, 33, 43]. Vapreotide also reduced SP-induced calcium increases in a dose-dependent manner (fig. 2a, b). The concentration required for vapreotide to inhibit SP-induced calcium increase (10 μ M) is about 100 times higher than that for aprepitant (100 nM; fig. 1). In agreement with this observed difference, vapreotide displaced [3 H]SP with an IC_{50} of $3.3 \pm 1.8 \times 10^{-7}$ M on guinea pig bronchi [32], which is also about 100 times higher than that obtained with an NK1R antagonist, CP96,345 ($3.4 \pm 0.8 \times 10^{-9}$ M) [37]. Thus, this difference is most likely due to the weaker binding affinity of vapreotide to NK1R in comparison to the non-peptide NK1R antagonists aprepitant and CP-96,345.

SP activation of NK1R results in activation of NF- κ B in many cell systems, which, in turn, upregulates IL-8 expression [18, 19]. Thus, IL-8 expression is linked with NF- κ B activation. We previously demonstrated that both NK1R antagonists and NK1R siRNA inhibit SP-induced upregulation of IL-8 in the HEK293-NK1R cell line [18].

In the present study, our data show that vapreotide displayed an NK1R antagonist effect by reducing SP-induced NF- κ B activation and increased IL-8 and MCP-1 expression in both the HEK293-NK1R cell line and U373MG cells. Furthermore, the effect of vapreotide was not due to its binding to SSTR2, the dominant somatostatin receptor subtype expressed on inflammatory cells [46], because pretreatment of cells with a selective SSTR2 antagonist CYN [47], did not reverse vapreotide inhibition of SP-mediated upregulation of IL-8 expression (fig. 2).

SP-NK1R interaction has a modulatory effect on HIV infection [11, 27, 28, 48–51]. Plasma SP levels are elevated in HIV-infected men and women [52, 53]. We previously demonstrated that interruption of the interaction between SP and NK1R by NK1R antagonists (CP-96,345 or aprepitant) inhibits HIV-1 replication in MDM and decreases CCR5 expression [27, 29, 54]. Our data demonstrate that SP stimulates the expression of the proinflammatory chemokine MCP-1 in cells of astrocyte lineage, which may have a direct effect on HIV infection. MCP-1 contributes to HIV replication and disease progression including neuro-AIDS [for a review, see ref. 55]. Vapreotide reduced the proinflammatory response to SP, including MCP-1 expression, and inhibited HIV-1 replication in human MDM in vitro. We thus hypothesize that vapreotide, similar to other NK1R antagonists, may also interfere with the SP signaling via its interaction with NK1R, leading to the inhibition of HIV-1 infection.

Taken together, our data demonstrate that vapreotide has an NK1R antagonist effect and that it inhibits HIV-1 replication in human MDM in vitro via interaction with NK1R. This finding may have potential application for therapeutic intervention in HIV-1 infection.

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