

The NK-1 Receptor Is Expressed in Human Primary Gastric and Colon Adenocarcinomas and Is Involved in the Antitumor Action of L-733,060 and the Mitogenic Action of Substance P on Human Gastrointestinal Cancer Cell Lines

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Key Words

NK-1 receptor antagonists · L-733,060 · NK-1 receptor isoforms · Apoptosis · Tachykinins · Substance P · Gastrointestinal carcinoma · Human primary gastrointestinal adenocarcinoma

Abstract

Background/Aims: It has been demonstrated that substance P (SP) and neurokinin-1 (NK-1) receptor antagonist L-733,060 induces cell proliferation and inhibition, respectively, in several human cancer cell lines. At present, it is unknown whether such actions are exerted on human gastric and colon adenocarcinomas. We carried out an in vitro study of the growth-inhibitory capacity of L-733,060 against human gastric and colon adenocarcinomas. **Methods:** A coulter counter was used to determine viable cell numbers followed by application of the tetrazolium compound MTS. Immunoblot analysis was used to determine the NK-1 receptors and the DAPI method was applied to demonstrate apoptosis. Immunohistochemistry was used to demonstrate NK-1 receptors in primary human gastric and colon adenocarcinomas. **Re-**

sults: We observed the presence of several NK-1 receptor isoforms in human gastric and colon adenocarcinomas. Nanomolar concentrations of SP increased the growth of both cell lines and micromolar concentrations of L-733,060 inhibited the growth of such cell lines, with and without previous administration of SP. L-733,060 inhibited the growth of the 23132/87 and SW-403 cell lines in a dose-dependent manner. After administration of L-733,060, apoptosis was observed in both cell lines. In both human primary gastric and colon adenocarcinomas, a high density of NK-1 receptors was observed. Immunoreactivity, showing a diffuse cytoplasmic staining, was observed in the epithelial cells of normal and tumor glands and in numerous stromal elements. **Conclusions:** We demonstrated that NK-1 receptors were expressed in 23132/37 and SW-403 cell lines and in human primary gastric and colon adenocarcinomas, that SP is a mitogen and that the antitumor action of L-733,060 on both human cell lines occurs through the NK-1 receptor. Data also indicate that the cell death observed is produced by apoptosis. These data suggest that the NK-1 receptor is a new and promising target in the treatment of human gastrointestinal adenocarcinomas.

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Introduction

Gastric cancer accounts for approximately 12% of all cancer deaths. It remains the world's second leading cause of cancer mortality [1] and is predicted to be the eighth leading cause of all deaths worldwide by 2010 [2]. Surgery is the only potentially curative treatment for localized gastric cancer, but most cases present at an advanced stage. The prognosis for this disease is extremely poor, with overall 5-year survival rates ranging from 10 to 15% in the United States and most developed countries [3]. Colorectal cancer remains one of the leading causes of cancer death in the Western world and was estimated to have affected 148,000 people in 2002 in the United States alone [4]. Despite advances in multimodality therapies, 5-year survival in colorectal cancer is approximately 55% and has remained essentially unchanged over the last 40 years. Because metastatic disease is the major cause of treatment failure, new therapeutic strategies are essential [5].

Substance P (SP) is an undecapeptide that belongs to the tachykinin family of peptides. It is known that SP, neurokinin (NK) A, neuropeptide K and neuropeptide γ (the 2 latter elongated forms of NKA) are derived from the preprotachykinin A gene (PPT-1), whereas NKB is derived from the preprotachykinin B gene (PPT-2). The biological actions of SP, NKA and NKB are mediated by 3 receptors, named NK-1, NK-2 and NK-3, the NK-1 receptor showing preferential affinity for SP. After binding to the NK-1 receptor, SP regulates many biological functions [6], and this neuropeptide has also been implicated in neurogenic inflammation, pain and depression [7]. Moreover, SP is known to have a widespread distribution in both the central and peripheral nervous systems, and it is also known that the undecapeptide is released from primary sensory nerve fibers. Moreover, activation of the NK-1 receptor induces mitogenesis in several tumor cells [8–12].

L-733,060 is a selective, potent and long-acting central nonpeptide tachykinin NK-1 receptor antagonist showing high affinity for the human NK-1 receptor *in vitro* [13]. The administration of L-733,060 produces analgesia [14] and antidepressive effects [15]. Moreover, the compound has been used in the treatment of a broad range of anxiety and mood disorders [16] as well as in inflammatory liver disease; its action is most likely due to an inhibition of the effects of SP [17]. In addition, *in vitro* and *in vivo* studies have demonstrated that SP antagonists inhibit the growth of both small cell lung cancer and glioma [18, 19]. Recently, we have also demonstrated that L-

733,060 shows antitumor activity against human neuroblastoma, glioma, melanoma, retinoblastoma and pancreas carcinoma cell lines [10–12, 20]. However, to our knowledge no study has been carried out on the antitumor effect of the potent and long-acting NK-1 receptor antagonist L-733,060 against human gastric 23132/87 and colon adenocarcinoma SW-403 cell lines. It is also unknown whether SP exerts a mitogenic action or not on both tumor cell lines, and the presence of NK-1 receptors in those cell lines is also unknown. Thus, the aims of this study were: (1) to demonstrate the presence of isoforms of the NK-1 receptor in the human gastric adenocarcinoma 23132/87 and the human colon adenocarcinoma SW-403 cell lines; (2) to study the role of SP and the NK-1 receptor in the induction of the proliferation of human gastric adenocarcinoma 23132/87 and the human colon adenocarcinoma SW-403 cell lines; (3) to demonstrate, using an MTS colorimetric method to evaluate cell viability, the antitumor action of the NK-1 receptor antagonist L-733,060 on both cancer cell lines and to show that this antitumor action occurs through the NK-1 receptor; (4) to know whether the antagonist produces, or not, apoptosis in both gastrointestinal tumor cell lines; (5) to demonstrate, in several human primary human gastric and colon adenocarcinomas, the presence and distribution of the NK-1 receptor.

Materials and Methods

Cell Cultures

We used the human gastric adenocarcinoma cell line 23132/87 (DSMZ, Braunschweig, Germany) and the human colon adenocarcinoma cell line SW-403 (DSMZ). These cell lines were maintained in DMEM or RPMI 1640 (Gibco, Barcelona, Spain) supplemented with 10% heat-inactivated fetal bovine serum according to the culture conditions suggested by the ATCC, the ICLC and the DSMZ. Cell lines were seeded in 75-cm² tissue culture flasks (Falcon, Heidelberg, Germany). The medium was renewed every 2 days and the cells were harvested by treatment with trypsin (0.05 and 0.02% EDTA without Ca²⁺ and Mg²⁺; Sigma-Aldrich, Madrid, Spain) on the sixth day after seeding. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂.

Drug Treatments

The NK-1 receptor antagonist (2S, 3S) 3-([3,5-Bis(trifluoromethyl)phenyl]methoxy)-2-phenylpiperidine, MW 438.9, (L-733,060; Sigma-Aldrich) was dissolved in distilled water containing 0.2% dimethylsulphoxide (DMSO) before sample treatment. In order to determine the 50% inhibition concentration (IC₅₀), different concentrations (5, 10, 15, 20, 25 and 30 μ M) of L-733,060 were evaluated. SP, acetate salt (Sigma-Aldrich), was dissolved in distilled water. In order to determine SP-induced cell proliferation, different concentrations of SP (5, 10, 50 and 100 nM) were

evaluated. The most effective SP concentration for each cell line was incubated 1 h before the addition of L-733,060.

Proliferation Assays

Cell proliferation was evaluated using the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), according to the manufacturer's instructions (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega Corp., Madison, Wisc., USA). At the time of the assay, cells cultured for 4–5 days were harvested by trypsinization and cell viability was evaluated by Trypan blue exclusion. Cell numbers were quantified using a Coulter counter. Cells were cultured in 96-well plates: each well contained 10^4 cells in a total volume of 100 μ l. Each assay included 1 plate. The plate included blank wells (0 cells/0.1 ml), control wells (10^4 cells /0.1 ml), control wells with DMSO, control wells treated with L-733,060, control wells treated with exogenous SP at different concentrations and control wells treated with the most effective SP concentration and L-733,060. The plates were inoculated with L-733,060 (5, 10, 20 and 30 μ M for 23132/87 or 10, 15, 20 and 25 μ M for SW-403) and were incubated for a period of 50 or 48 h, respectively. The plates were also inoculated with exogenous SP (5, 10 and 100 nM for 23132/87 or 5, 50 and 100 nM for SW-403) with (10 μ M for 23132/87 or 15 μ M for SW-403) and without L-733,060 for their first doubling times (50 or 48 h). For the proliferation assay, 20 μ l of the MTS reagent was added to each well 90 min before reading the samples on a multiscanner microplate reader (Spectra Classic; TECAN, Barcelona, Spain) at 492 nm. The quantity of the product, as measured by optical density, is directly proportional to the number of living cells. Each experimental condition (blank wells, control wells and control wells treated with different concentrations of L-733,060 or SP) was assayed in duplicate and all experiments were performed at least 3 times. The IC_{50} of L-733,060 was calculated using the regression straight-line function based on the least-squares technique.

Statistical Analyses

Data are expressed as means \pm SD. Statistical analysis was performed with SPSS statistical software for Microsoft Windows, release 13.0 (SPSS Inc., Chicago, Ill., USA). The homogeneity of the variance was tested using the Levene test. If the variances were homogeneous, the data were analyzed by using the one-way ANOVA test with Bonferroni's correction for multiple comparisons. For data sets with nonhomogeneous variances, the ANOVA test with T3 Dunnett post hoc analysis was applied. The criterion for significance was $p < 0.05$ for all comparisons.

Western Blot Analyses

We followed a previously reported methodology [10]. Thus, total protein was prepared from subconfluent human gastric adenocarcinoma cell line and human colon adenocarcinoma cell cultures in 125-cm² culture flasks. As a control, we included a protein extract from rat pheochromocytoma cell line (PC12 cells). Briefly, cells were harvested by trypsin treatment, washed with phosphate-buffered saline (PBS), pH 7.4, and resuspended in HEN buffer (5 mM EDTA, 250 mM NaCl, 50 mM HEPES, pH 7.3) containing 5 mM DTT, 1 mM Na₃VO₄, 0.2% IGEPAL CA-630 (Sigma) and 1% protease inhibitor cocktail (Sigma). Once resuspended, cells were vortexed, incubated on ice for 5 min, and centrifuged for 15 min at high speed in a microcentrifuge, after which

the protein-containing supernatant was collected. Protein concentrations were determined with the protein assay kit from Bio-Rad according to the manufacturer's instructions.

Fifty micrograms of protein, from each sample, was separated by electrophoresis on 10% SDS-polyacrylamide gels and electroblotted onto PVDF membranes. Blots were incubated in blocking solution (5% nonfat milk in PBS, 0.1% Tween-20), followed by overnight incubation with a polyclonal antibody (S8305; Sigma) developed in rabbit against the KTMTESSSFYSNMLA conserved domain, corresponding to the C terminus of the NK-1 receptor (Sigma), and diluted 1:1,000. The membranes were then washed with PBS/Tween-20 and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 2 h at room temperature (1:10,000 dilution). Antibody detection was performed with an enhanced chemiluminescence reaction (ECL Western blotting detection; Amersham Life Science, Piscataway, N.J., USA). As previously reported [10], we included a protein extract from a glioma cell line as a control (data not shown). Several isoforms of the NK-1 receptor shown by this cell line are known [10]. In addition, no bands were detected when incubation was carried out with the secondary antibody alone.

DAPI Staining

In order to determine whether apoptosis was induced by the NK-1 receptor antagonist used here, DAPI staining was performed. Briefly, cells were cultured on 4-chamber slides. After treatment with L-733,060 for their first doubling times (48–50 h), the cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde for 30 min. Following a second wash in PBS, cells were incubated in DAPI solution (Sigma-Aldrich) at a dilution of 1/1,000 (1 μ g/ml) for 30 min in the dark and the supporting slides were mounted with a mixture of PBS and glycerol at 70%. The cells were then observed through a fluorescence microscope (Zeiss, Oberkochen, Germany). Apoptotic cells were defined by the condensation and fragmentation of nuclear chromatin. We counted the number of apoptotic cells observed in the following cases: human gastric adenocarcinoma 23132/87 and human colon adenocarcinoma SW-403 cells not treated with the NK-1 receptor antagonists; 23132/87 cells treated with the NK-1 receptor antagonist L-733,060 (30 μ M) and SW-403 cells treated with the NK-1 receptor antagonist L-733,060 (25 μ M). In each case, the count was repeated in 3 different slides. Finally, in each slide, we counted the number of apoptotic cells located in 5 different sequential fields (we considered a field as the image observed with the 40 \times lens). Images of such fields were captured by means of a microscope equipped with a digital camera.

Immunohistochemistry for NK-1 Receptors in Human Primary Adenocarcinomas

After surgical intervention, 3 human primary gastric adenocarcinomas and 4 human primary colon adenocarcinomas were obtained from patients at the Department of Pathology, Hospital 'Virgen del Rocío', Sevilla, Spain. The experimental design, protocols and procedures were performed under the ethical guidelines and legal recommendations of Spanish and European law, as well as in accordance with the Declaration of Helsinki.

Formalin-fixed and paraffin-embedded tumors (gastric and colon adenocarcinomas) were cut at 5 μ m and dried overnight at 37°C. The sections were deparaffinized with xylene, hydrated through a series of solutions containing decreasing concentra-

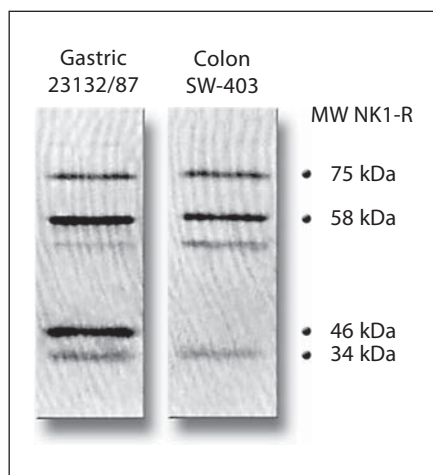


Fig. 1. Western blot analysis of NK-1 receptors in human gastric adenocarcinoma 23132/87 and colon adenocarcinoma SW-403 cell lines showing the presence of different NK-1 receptor complex isoforms. Dots indicate bands with molecular weights similar to those previously reported.

tions of ethanol, and immersed in water. After pressure cooker antigen retrieval in 10× citrate buffer, pH 6.0, slides were cooled at room temperature for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min at room temperature. After washing with 0.05 M Tris, the sections were incubated with 10% nonimmune pig serum for 30 min at room temperature. Subsequently, they were incubated overnight at 4°C with 1:500 diluted anti-NK-1 receptor primary antibody (Sigma-Aldrich). The sections were then washed in 0.05 M Tris at room temperature. The next step was the addition of Envision System-HRP (Dako) reagents during 30 min at room temperature. The slides were rinsed with 0.05 M Tris, and immunoreactivity was visualized for light microscopy with 3,3'-diaminobenzidine chromogen solution (DAB+; Dako). Cell nuclei were lightly counterstained with hematoxylin. Finally, as previously published [21], in order to determine the specificity of the immunostaining, primary retinoblastoma was used as positive control. As negative control, the primary antibody was omitted, being replaced by nonimmune serum. In both cases, the results obtained confirmed the specificity of the NK-1 receptor antibody used here.

All slides were evaluated by 2 independent investigators. In each slide, 10 representative microscopic high-power fields were evaluated using a 40× lens. The presence or absence of staining and the intensity of immunoreactivity were noted, as well as the number of cells showing a brown staining and whether or not the staining was localized on the cytoplasm and/or in the plasma membrane. The number of immunoreactive cells was scored as follows: when less than 10% of the total tumor cells were stained, the number of immunoreactive cells was considered low, it was considered moderate when 10–40% were stained and high when more than 40% were stained. Tumor cells were recorded as positive when they showed a moderate or strong labeling.

Results

NK-1 Receptors

We performed Western blot analyses in order to test the presence of NK-1 receptor in both human gastric adenocarcinoma 23132/87 and colon adenocarcinoma SW-403 cell lines. Total cell protein extracts were loaded onto polyacrylamide gels, resolved, and transferred to membranes as described in Material and Methods. Incubation with an antibody against an epitope whose sequence is conserved in several species (see Materials and Methods) revealed the presence of different isoforms of the NK-1 receptor in both the 23132/87 and SW-403 cell lines (fig. 1). The relative amount of each protein form was similar between both cell lines. Four similar bands (isoforms of about 75, 58, 46 and 34 kDa) were observed in the human gastric adenocarcinoma 23132/87 cell line (fig. 1), whereas the colon adenocarcinoma SW-403 cell line expressed isoforms of 75, 58 and 34 kDa. An additional band was observed in both cell lines. In addition, no bands were detected when incubation was performed with the secondary antibody alone.

Using an immunohistochemistry technique, a high density of NK-1 receptors was localized in all human primary adenocarcinomas studied (fig. 2b, c, e, f). In both gastric and colon adenocarcinomas, a high number of tumor cells (>75%) expressing the NK-1 receptor was observed. In all adenocarcinomas studied, the immunoreactivity was observed in the epithelial cells of normal and tumor glands and in numerous stromal elements. This immunoreactivity was homogeneously expressed and showed a diffuse cytoplasmic staining. Moreover, in human colon adenocarcinomas, a uniform granular cytoplasmic staining was visualized in the apical areas of the epithelial cells of normal glands, whereas the most intense granular cytoplasmic staining was observed in the apical areas and other areas of the cytoplasm of the epithelial cells of the tumor glands.

Antitumor Action of L-733,060

Growth inhibition of the 23132/87 and SW-403 cell lines by L-733,060 was observed after the addition of increasing concentrations of L-733,060 (fig. 3a, 4a). Moreover, treatment of both cell lines with L-733,060 resulted in a concentration-dependent cytotoxicity (fig. 3a, 4a). These figures also show the IC_{50} at the first doubling times. Thus, the concentrations required for a 50% reduction in optical density (IC_{50}) observed in the controls treated with L-733,060 were 14.29 μ M for 50 h for 23132/87 and 14.45 μ M for 48 h for SW-403 (fig. 3a, 4a). Maximum inhibition

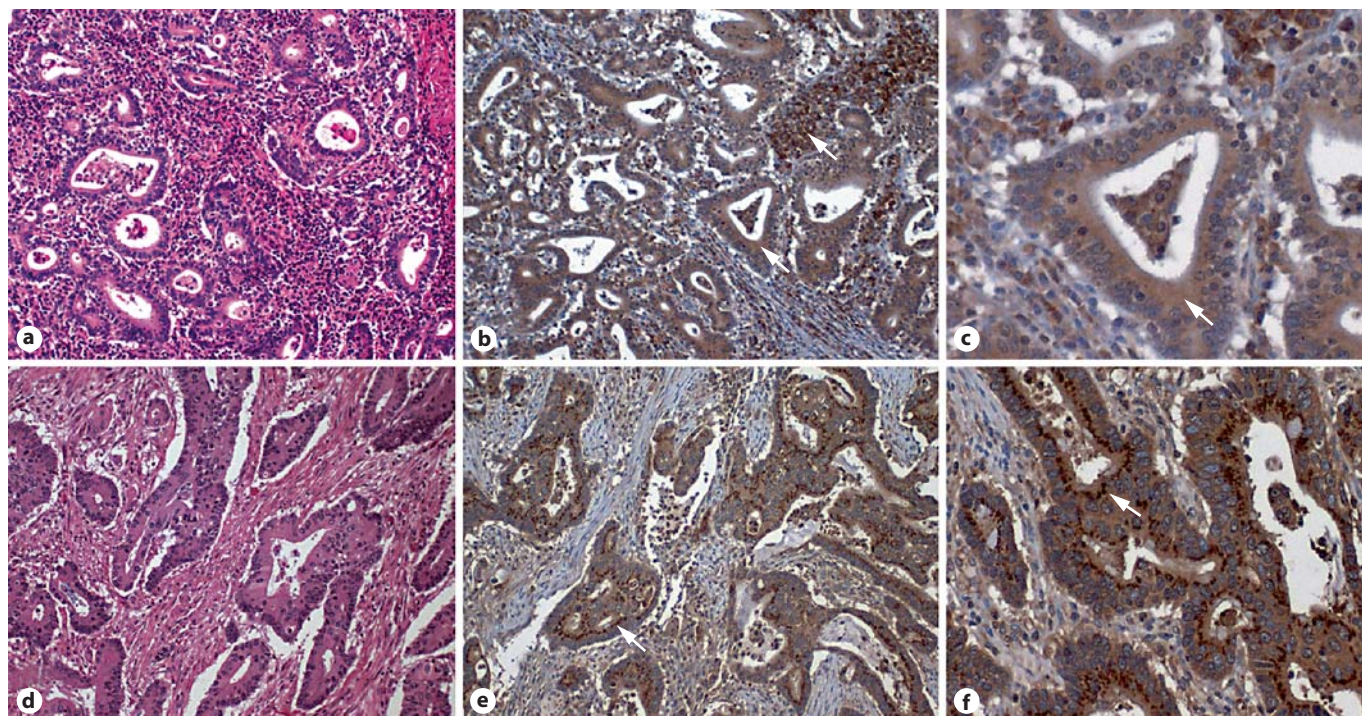


Fig. 2. Hematoxylin-eosin-stained sections of human primary gastric (a) and colon (d) adenocarcinomas. $\times 25$. The presence of NK-1 receptors (arrows) in human primary gastric adenocarcinoma (b, c) and in human primary colon adenocarcinoma (e, f) is shown. b, e NK-1 receptors (arrows) can be observed on the cytoplasm of the epithelial cells tumor glands and in numerous stromal elements. $\times 25$. c Magnification of the right-middle region observed in b. $\times 50$. e, f NK-1 receptors (arrows) showing an intense granular cytoplasmic staining in the tumor glands of human primary colon adenocarcinoma. $\times 25$ (e) and $\times 50$ (f).

was observed when the drug was present at a concentration of $30 \mu\text{M}$ (23132/87) and $25 \mu\text{M}$ (SW-403) during the culture periods. At the first doubling time, a strong decrease in the number of the 2 cell lines studied was found at intermediate concentrations and no remaining living cells were observed at the maximal concentration. A lower inhibition of growth of the 2 lines was observed in the presence of low doses of L-733,060. The figures also show that the SD values for the 2 cell lines studied were small, pointing to total agreement among the values obtained at the 3 times the experiments were carried out.

The NK-1 Receptor Antagonist L-733,060 Blocks SP-Induced Mitogen Stimulation

Growth of the 23132/87 and SW-403 cell lines was noted after the addition of SP and we observed that certain nanomolar concentrations of SP induced cell proliferation compared to the controls (fig. 3b, 4b). SP stimulation was evident at 5 nM and the maximum level was reached at 10 nM for 23132/87 and 50 nM for SW-403 (fig. 3b, 4b).

This indicates that the activation of SP receptors leads to mitogenesis in the 23132/87 and SW-403 human cancer cell lines. Thus, the percentage of cell proliferation of both cell lines increased from 10 to 20% in 23132/87 and from 10 to 25% in SW-403, depending on the dose of SP administered (fig. 3b, 4b).

Treatment with L-733,060, at $10 \mu\text{M}$ for 23132/87 and $15 \mu\text{M}$ for SW-403, partially inhibited the growth of both cell lines (fig. 3b, 4b). In order to examine whether the NK-1 receptor antagonist L-733,060 inhibited cell proliferation via an interaction with its receptor, we used the specific NK-1 receptor agonist SP in competition experiments. Thus, the cellular concentration at $10 \mu\text{M}$ (23132/87) and $15 \mu\text{M}$ (SW-403) of L-733,060 as well as 10 nM (23132/87) and 50 nM (SW-403) of SP was higher than that observed with L-733,060 alone for 23132/87 (fig. 3b) and SW-403 (fig. 4b). These results indicate that L-733,060 blocks SP mitogen stimulation, since L-733,060-induced growth inhibition was partially reversed by the administration of a nanomolar dose of exogenous SP.

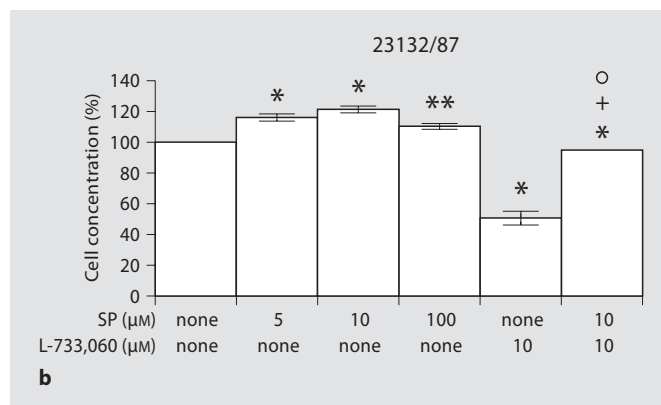
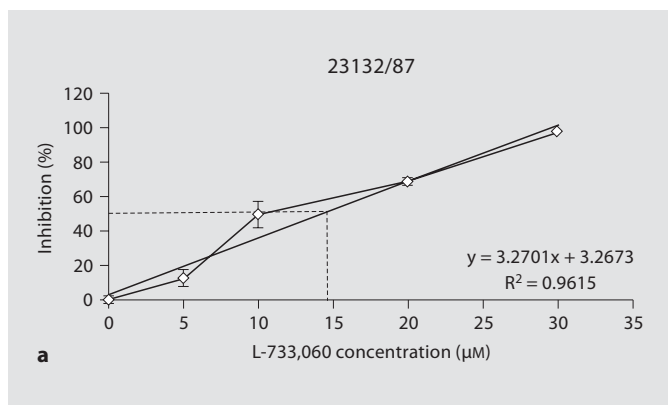


Fig. 3. a Percentage of growth inhibition of human gastric adenocarcinoma 23132/87 cells at 50 h in vitro cultures following the addition of increasing concentrations (5, 10, 20 and 30 μM) of L-733,060. The percentage of inhibition for the first doubling time of incubation is plotted on a linear graph. * $p \leq 0.05$. Values are means \pm SD (bars). The regression line is indicated, as well as the equation to obtain the IC_{50} . **b** Induction of cell proliferation of human gastric adenocarcinoma 23132/87 cells by SP at several

nanomolar concentrations (5, 10 and 100 nM). The NK-1 receptor antagonist L-733,060 was added (10 μM) in the presence (10 nM) or absence (none) of SP for 50 h. In both cases, L-733,060 inhibited 23132/87 cell proliferation. Using the ANOVA test, a significant difference between each group and the control group (none-none) was found. * $p \leq 0.01$; ** $p \leq 0.05$; + $p < 0.05$, 10-none vs. 10-10; ^o $p < 0.05$, 10-10 vs. none-10. Vertical bars indicate SD.

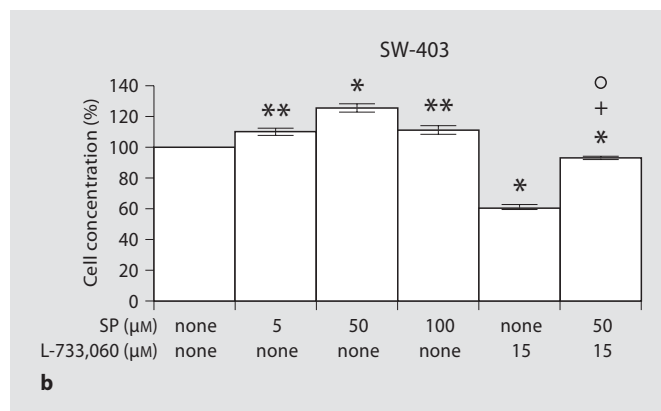
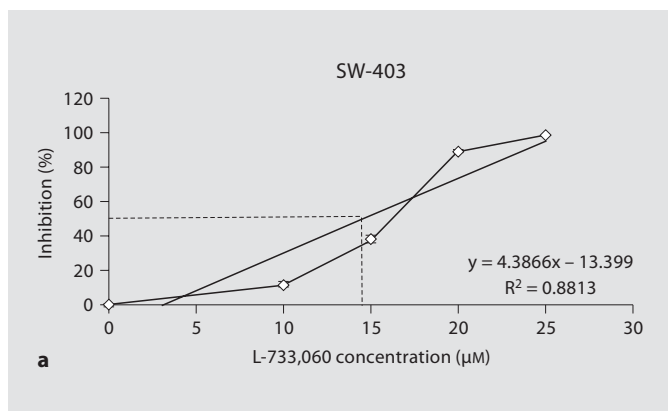


Fig. 4. a Percentage of growth inhibition of human colon adenocarcinoma SW-403 cells at 48 h in vitro cultures following the addition of increasing concentrations (10, 15, 20 and 25 μM) of L-733,060. The percentage of inhibition for the first doubling time of incubation is plotted on a linear graph. * $p \leq 0.05$. Values are means \pm SD (bars). The regression line is indicated, as well as the equation to obtain the IC_{50} . **b** Induction of cell proliferation of human colon adenocarcinoma SW-403 cells by SP at several

nanomolar concentrations (5, 50 and 100 nM). The NK-1 receptor antagonist L-733,060 was added (15 μM) in the presence (50 nM) or absence (none) of SP for 48 h. In both cases, L-733,060 inhibited SW-403 cell proliferation. Using the ANOVA test, a significant difference between each group and the control group (none-none) was found. * $p \leq 0.01$; ** $p \leq 0.05$; + $p \leq 0.05$, 50-none vs. 50-15; ^o $p \leq 0.05$, 50-15 vs. none-15. Vertical bars indicate SD.

This indicates the specificity of tachykinin NK-1 receptor activation in the growth of the human gastric adenocarcinoma (fig. 3b) and human colon adenocarcinoma cell lines (fig. 4b), since an increase in the cellular concentration (44.8 and 32.45%) was observed in the 23132/87

and SW-403 cell lines, respectively, (fig. 3b, 4b) with respect to the values found when the antagonist was administered alone. There were no significant differences between the control and the control-DMSO groups (data not shown).

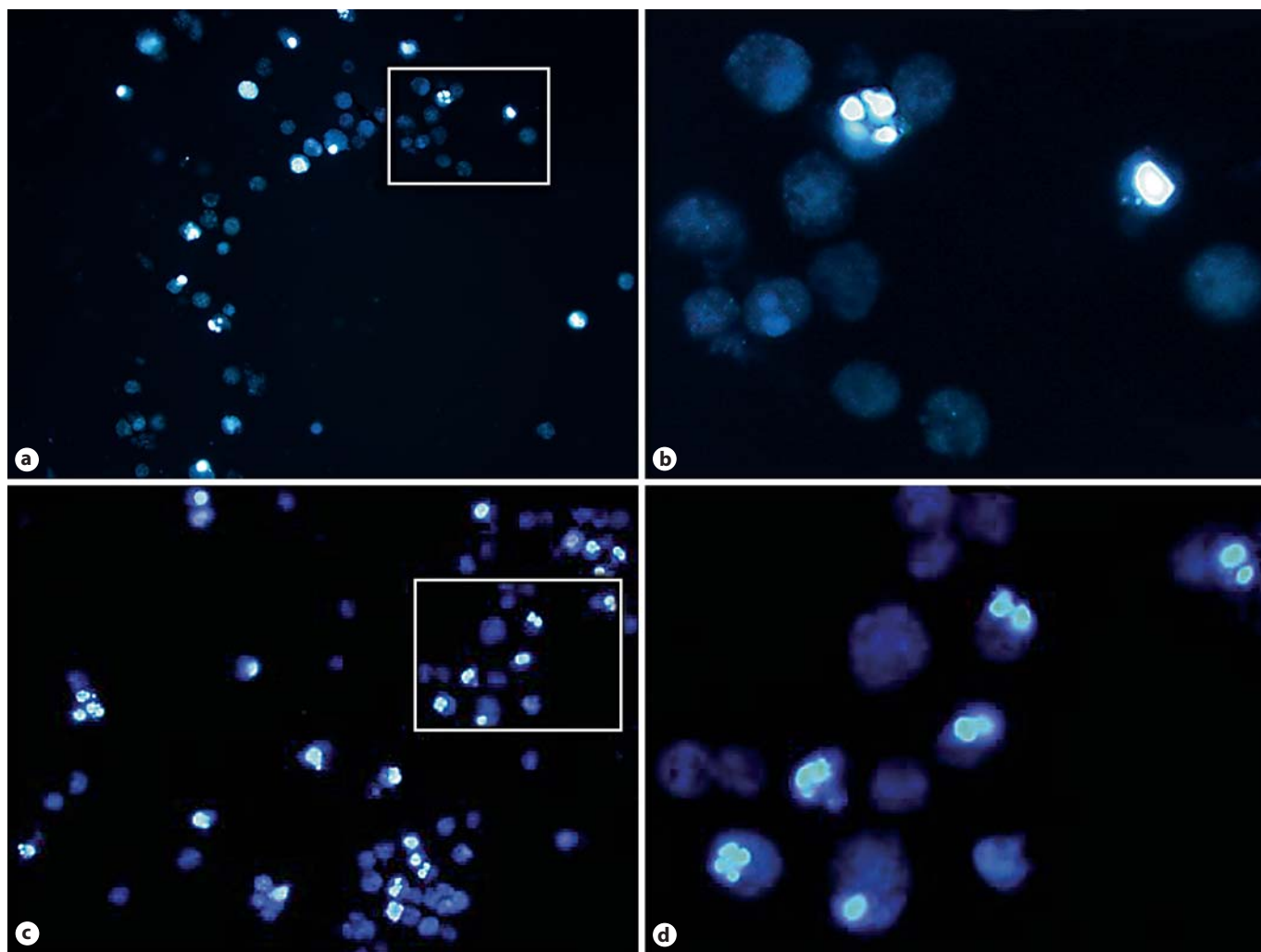


Fig. 5. Apoptosis features induced by L-733,060 in human gastric adenocarcinoma 23132/87 (**a, b**) and colon adenocarcinoma SW-403 (**c, d**) cell lines. **b, d** Higher-power magnification of the regions delimited in the rectangles shown in **a** and **c**, respectively. $\times 40$ (**a, c**) and $\times 100$ (**b, d**). Nuclei of treated cells show chromatin condensation and nuclear fragmentation.

Apoptosis

After administration of the NK-1 receptor antagonist L-733,060, a great number of apoptotic cells were found in both 23132/87 and SW-403 cell lines (fig. 5). In fact, we observed in DAPI-stained cultures a 50.39 and 45.83% increase in apoptotic cells in 23132/87 and SW-403 cell lines, respectively, after administration of L-733,060; SD values were 6.45 and 8.33, respectively. These apoptotic cells were not found in gastrointestinal carcinoma cell cultures not treated with NK-1 receptor antagonists.

Discussion

We have demonstrated the presence of isoforms of NK-1 receptors in gastrointestinal carcinoma cell lines. Several previous reports have shown that different isoforms of the NK-1 receptor can be found in both human and rat tissues [22]. For instance, 4 different proteins with molecular weights of 33, 58, 78 and 116 kDa can be specifically affinity labeled using [125 I]SP in human lymphocytes [23], the main SP-binding protein present on human lymphocyte cell membranes being a 58-kDa hydrophobic glycoprotein [24]. In rat tissues, several NK-1 receptor forms have been detected, with sizes ranging

from 46 to 54 kDa [25]. Recently, we have demonstrated the presence of isoforms of the NK-1 receptor in both SKN-BE(2) neuroblastoma and GAMG glioma cell lines [10]. Thus, in the SKN-BE(2) cell line, a major 54-kDa band was observed, whereas in GAMG cells, 2 additional and more abundant isoforms of about 33 and 38 kDa were detected. In addition, in human pancreas carcinoma CAPAN-1 and PA-TU 8902 cell lines, appearance of 75-, 58-, 46- and 34-kDa bands was reported [12, 26]. Thus, the present data are in agreement with those of previous studies, since we have demonstrated for the first time the presence of several NK-1 receptor isoforms in human gastric adenocarcinoma 23132/87 (34, 46, 58 and 75 kDa) and human colon adenocarcinoma SW-403 (34, 58 and 75 kDa) cell lines, although the functional roles of these NK-1 receptor isoforms in such cell lines are currently unknown.

It is known that NK-1 receptors are overexpressed in primary glioblastomas, breast and pancreatic carcinomas as well as retinoblastoma. However, up to now, the presence of NK-1 receptors in human primary gastric adenocarcinomas was unknown. Here, we have demonstrated for the first time the presence of NK-1 receptors in human primary gastric adenocarcinomas. In addition, such receptors were observed in the 3 gastric adenocarcinomas studied. Hennig et al. [27] only observed NK-1 receptors in 1 of 21 human primary colon adenocarcinomas studied, whereas Schultz et al. [28] did not observe such receptors in human primary colon adenocarcinomas (0/5). However, here, we have observed NK-1 receptors in all colon adenocarcinomas studied (4/4). Thus, NK-1 receptor visualization, after using immunohistochemical methods, will facilitate the identification of tumors with a sufficient NK-1 receptor overexpression for diagnostic or therapeutic intervention (administering NK-1 receptor antagonists).

Moreover, we have also demonstrated for the first time *in vitro* a potent growth inhibition on human gastric and colon adenocarcinoma cell lines through use of the non-peptide NK-1 receptor antagonist L-733,060. This is in agreement with previous studies, in which the use of SP peptide antagonists other than L-733,060 inhibited, both *in vitro* and *in vivo*, the growth of small cell lung cancer [18], and it is also known that the nonpeptide antagonist L-732,138 exerts antitumor action on a glioma cell line [29]. Finally, our observations are in agreement with recent findings showing that L-733,060 has antitumor activity against other human cancer cell lines such as neuroblastoma, glioma, melanoma, retinoblastoma and pancreas adenocarcinoma [9, 11, 12, 20]. It should be noted

that in cell lines as different as those mentioned above, as well as in gastric and colon adenocarcinoma, the same NK-1 receptor antagonist (L-733,060) elicits growth inhibition. This observation suggests the possibility of a common mechanism for cancer cell proliferation mediated by SP and NK-1 receptors. Were this the case, it would mean that NK-1 receptor antagonists (for example, L-733,060) could inhibit a large number of tumor cell types in which both SP and NK-1 receptors are expressed [10, 12, 26, 30] and may be candidates for a broad-spectrum antineoplastic drug.

We have demonstrated that treatment of the gastric and colon adenocarcinoma cell lines with L-733,060 produces growth inhibition and cell death. We have also demonstrated the cell death observed here was due to a specific toxic effect of L-733,060 and not to a nonspecific action of this substance. In our competition experiments, exogenous SP cell proliferation was partially reversed by the administration of L-733,060, suggesting the specificity of tachykinin NK-1 receptor blocking on human gastric and colon adenocarcinoma cell lines by L-733,060, which supports the specific effect. Moreover, the specific binding of L-733,060 to the NK-1 receptor in human breast carcinoma has been demonstrated [31]. Thus, the action on these cell lines by the NK-1 receptor antagonist L-733,060 is probably related to the ability of this antagonist to block the NK-1 receptor in such tumors. In addition, the findings of the present study demonstrate that treatment of both adenocarcinoma cell lines with this NK-1 receptor antagonist results in cell death and that such death occurs by apoptosis. This is in agreement with previous *in vitro* studies carried out in lung cancer [32] and in rhabdomyosarcoma cell lines [33]. In this sense, it is known that an SP antagonist (other than L-733,060) induces apoptosis in lung cancer and causes a concentration-dependent loss of cell viability [32], as we observed using L-733,060, since the antitumor activity of L-733,060 reported here was dose dependent. The blockade of NK-1 receptors in both gastric and colon adenocarcinoma cell lines by L-733,060 could inhibit both DNA synthesis and cell proliferation through the mitogen-activated protein kinase pathway [8]. Moreover, L-733,060 has been successfully used in preclinical assays at 10- to 20-mg/kg doses (30–60 μ M), for example, in order to decrease inflammatory liver damage [17].

All data mentioned above suggest that treatment with antagonists of the NK-1 receptor in cancer cell lines expressing the NK-1 receptor could improve cancer treatment. Further studies in this field are necessary in order to shed further light on the mechanisms that produce the

growth inhibition of cancer cell lines by antagonists of the tachykinin receptors. In this sense, it would be very interesting to know whether the treatment of cancer cell lines with the NK-1 receptor inhibitors that have been studied in humans [34] might produce the same growth inhibitory action as we found with L-733,060. At present, more than 30 compounds are known to act as NK-1 receptor antagonists.

We have also demonstrated for the first time that SP increases the growth of both the gastric and colon adenocarcinoma cell lines through binding the NK-1 receptor. This is in agreement with previous studies, since it has been reported that the activation of NK-1 receptors by SP induces mitogenesis in several cancer cell types [8–12] and that the NK-1 receptor is overexpressed in breast cancer and metastasis [30] as well as in human pancreatic cancer [26]. Furthermore, SP induces the migration of tumor cells to specific organs through binding to the NK-1 receptor on human breast carcinoma [31]. All above data suggest that SP and NK-1 receptors could play an important role in the development of human gastric and colon adenocarcinoma and metastasis, and that the NK-1 receptor antagonists (for example, L-733,060) could be useful in the treatment of tumors in which both SP and NK-1 receptors are expressed.

In summary, we here describe for the first time the presence of several isoforms of the NK-1 receptor in human adenocarcinoma gastric 23132/87 and colon SW-403 cell lines. Moreover, we have identified NK-1 receptors in all primary human gastric and colon adenocarcinoma cell lines studied. We also demonstrated the antitumor activity of the NK-1 receptor antagonist L-733,060 against both adenocarcinoma human cell lines. We also reported that this antagonist induces apoptosis in such tumor cell lines and that SP is a mitogen of both cancer cell lines. All these observations suggest that the NK-1 receptor could be a new and promising target in the treatment of human gastrointestinal adenocarcinomas and that the NK-1 receptor antagonists could improve gastrointestinal cancer treatment.

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