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PRECLINICAL STUDIES

The NK-1 receptor is expressed in human leukemia and is involved in the antitumor action of aprepitant and other NK-1 receptor antagonists on acute lymphoblastic leukemia cell lines

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Summary Substance P and neurokinin-1 (NK-1) receptor antagonists respectively induce cell proliferation and cell inhibition in human cancer cell lines. In acute lymphoblastic leukemia (ALL), substance P is expressed in human blast cells. However, the possible presence of NK-1 receptors in human ALL and the issue of whether the antitumor action of NK-1 receptor antagonists is exerted or not on human ALL (T-ALL BE-13 and B-ALL SD-1 cell lines) remain unknown. An immunoblot analysis was performed and an in vitro study of the cytotoxicity of three NK-1 receptor antagonists (L-733,060, L-732,138, aprepitant) was carried out on both cell lines. NK-1 receptors were found in those cell lines, and both expressed mRNA for this receptor. Using a knockdown method, we demonstrate that NK-1 receptors are involved in the viability of tumor cells. *TAC1R* cDNA was detected in the ALL cell lines by real-time quantitative RT-PCR. We also observed that the three NK-1 receptor antagonists elicited the inhibition of ALL cell growth; that the specific antitumor action of the NK-1 receptor antagonists occurs through the NK-1 receptor, and that ALL cell death is due to apoptosis. These findings suggest that NK-1 receptor antagonists could be considered as new antitumor drugs for the treatment of human ALL.

Keywords L-733,060 · L-732,138 · Substance P · Apoptosis · T-ALL BE-13 cell line · B-ALL SD-1 cell line

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children. In the United States, leukemias are the most common forms of childhood cancer, representing approximately 31% of all cancer cases occurring in children under the age of 15 [1]. ALL represents approximately 75% of all leukemias. Despite the dramatic advances in the treatment of the disease achieved over the past 40 years, the 5-year event-free survival rate is nearly 80% for children with ALL and approximately 40% for adults [2].

Substance P (SP) is an undecapeptide that belongs to the tachykinin family of peptides. The biological actions of SP, NKA and NKB are mediated by three receptors, named neurokinin (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. This neuropeptide has been implicated in neurogenic inflammation, pain and depression, as well as in tumor cell proliferation, neoangiogenesis and metastasis [3]. SP is a major mediator in the growth of capillary vessels in vivo and in the proliferation of cultured endothelial cells in vitro, and it has also been demonstrated that NK-1 receptor agonists induce neoangiogenesis [4]. Additionally, the activation of NK-1 receptors by SP induces mitogenesis in several tumor cell lines [5–12] and modulates the functions of the immune and hematopoietic systems [13].

Also, the active migration of tumor cells, a crucial requirement for invasion or infiltration, is regulated by SP signals [14].

NK-1 receptors have been localized and characterized in several human neoplasms and in human cell lines such as glioma, neuroblastoma, melanoma, retinoblastoma and pancreatic, laryngeal, gastric and colon carcinomas [6–8, 11, 15–20]. Moreover, human blood T-lymphocytes and human IM-9 B-lymphoblasts cell express 7,000–10,000 and 25,000–30,000 NK-1 receptors per cell, respectively [21]. In addition, SP is expressed in human blast cells in ALL [22].

The NK-1 receptor antagonist L-733,060 (a piperidine derivative) shows high affinity for the human NK-1 receptor in vitro [23], and L-732,138 (an L-tryptophan derivative) shows a competitive and selective antagonism for this receptor. It has also been demonstrated that L-733,060 exerts antitumor activity against human melanoma, neuroblastoma, glioma, retinoblastoma, pancreas, larynx, gastric and colon carcinoma cell lines [6–8, 10, 11, 24, 25] and that L-732,138 has antitumor activity against glioma, neuroblastoma, retinoblastoma, larynx carcinoma and melanoma [9, 10, 12, 26, 27]. The drug aprepitant is a selective, high-affinity antagonist of the human NK-1 receptor. To date it has been used as an antidepressant, anxyolitic and antiemetic [28, 29]. Additionally, it has been reported that aprepitant is a broad-spectrum antitumor drug [30], eliciting, for example, antitumor action against melanoma cells [20]. However, to our knowledge the presence of NK-1 receptors in ALL or the issue of whether the antitumor action of the NK-1 receptor antagonists L-733,060, L-732,138 and aprepitant is exerted on human ALL cells remain unknown. Thus, the aims of this study were: 1) To demonstrate in vitro the presence of NK-1 receptors in human T-ALL BE-13 and B-ALL SD-1 cell lines; 2) To demonstrate that ALL cell lines express mRNA for the NK-1 receptor; 3) To evaluate the involvement of the NK-1 receptor in the viability of ALL cells; 4) To demonstrate, using a WST-8 colorimetric method to evaluate cell viability, the antitumor action of the NK-1 receptor antagonists L-733,060, L-732,138 and aprepitant against human ALL cell lines and to show that this antitumor action occurs through the NK-1 receptor, and 5) To determine whether these NK-1 receptor antagonists produce, or not, apoptosis in the ALL cell lines studied.

Materials and methods

Cell cultures and Western blot analyses

We used the human peripheral blood T-lymphoblastoid BE-13 (from an 11-year-old girl with T-acute ALL) and B-lymphoblastoid SD-1 (from a patient with B-acute ALL) cell lines (DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen). These cell lines were maintained in RPMI 1640 (GIBCO, BRL) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL), 2 mML-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES buffer (Bio Whittaker Europe), the morphology of these cells growing in suspension being round and ovoid-to-round respectively. The cell lines were cultured in 75 cm^2 tissue culture flasks (Falcon, Heidelberg; Germany) and maintained at $0.3–1.0\times10^6$ cells/ml. Cells were fed twice-weekly. They were incubated at 37°C in a humidified atmosphere (95% air/5% CO_2).

As previously reported [6], total protein was prepared from subconfluent BE-13 and SD-1 human leukemia cell cultures. Protein concentrations were determined using the protein assay kit from BIO-RAD, according to the manufacturer's instructions. From each sample, 50 μg of protein was separated by electrophoresis on 10% SDS-polyacrylamide gels and electroblotted onto PVDF membranes. Blots were incubated in blocking solution (5% non-fat milk in PBS, 0.1% Tween-20 [PBS-T]), followed by overnight incubation with an Ig G polyclonal antibody against the KTMTESSSFYSNMLA conserved domain, corresponding to the C-terminus of the NK-1 receptor (Product Number S8305, Sigma-Aldrich, Madrid, Spain) and diluted 1:4,000. Membranes were then washed with PBS-T 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, United Kingdom).

Drug treatments

Three different NK-1 receptor antagonists were used in this study: (2S, 3S) 3-([3, 5-Bis (trifluoromethyl)phenyl]methoxy)-2-phenylpiperidine, MW 438.9 (L-733,060) (Sigma-Aldrich, Madrid, Spain); 3, 5-bis (trifluoromethyl) benzyl ester, MW 472.39 (L-732,138) (Sigma-Aldrich, Madrid, Spain); and 5-[[(2*R*, 3*S*)-2-[(1*R*)-1-[3,5-bis(trifluoromethyl) phenyl] ethoxy]-3-(4-fluorophenyl) -4-morpholinyl]methyl]-1,2-dihydro-3*H*-1,2,4-triazol-3-one, MW 534.43 (aprepitant) (the drug was kindly supplied by Merck Research Laboratories, Madrid, Spain). L-733,060 and L-732,138 were dissolved in distilled water containing 0.2% dimethyl-sulphoxide (DMSO) and aprepitant was dissolved in distilled water containing acetonitrile before sample treatment. In order to determine the IC_{50}, different concentrations (2.5–50 μM) of L-733,060; (10–100 μM), of L-732,138, and (10–60 μM) of aprepitant were evaluated for both tumor cell lines. Substance P (SP),

acetate salt (Sigma-Aldrich, Madrid, Spain), was dissolved in distilled water and different concentrations (5, 10, 50, 100 and 500 nM) were used. The most mitogenic nanomolar SP concentration for each cell line was incubated for 1 h before the addition of each NK-1 receptor antagonist.

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, USA). Cell numbers were quantified using a Coulter counter. The plate included blank wells (0 cells/0.1 ml), control wells (10^4 cells/0.1 ml), control wells with acetonitrile, control wells treated with SP, control wells treated with the NK-1 receptor antagonist, and control wells treated with the most mitogenic exogenous SP nM concentration and the NK-1 receptor antagonist (50% μM inhibition concentration (IC_{50}) of antagonist for their first doubling times). For the proliferation assays, 20 μl of the MTS reagent was added to each well 90 min before reading the samples on a multiscanner microplate reader (TECAN Spectra classic, Barcelona, Spain) at 492 nm. Each experimental condition (blank wells, control wells, and control wells treated with the different concentrations of each antagonist and/or SP) was assayed in duplicate and all experiments were performed at least three times.

The IC_{50} of the NK-1 receptor antagonists was calculated using the regression straight line function based on the least squares technique.

Statistical analyses

Data were expressed as means ± SD. Statistical analysis was performed with SPPS statistical software for Microsoft Windows, release 14.0 (Professional Statistic, Chicago). The homogeneity of variance was tested using the Levene test. If the variances were homogeneous, the data were analyzed using the one-way ANOVA test with Bonferroni's correction for multiple comparisons. For data sets with non-homogeneous variances, the ANOVA test with T3 Dunnett post-hoc analysis was applied. The criterion for significance was $p<0.05$ for all comparisons.

DAPI staining

In order to determine whether apoptosis was induced by the NK-1 receptor antagonists in both tumor cell lines studied here, DAPI staining was performed. Briefly, after treatment with each antagonist (L-733,060, L-732,138 or aprepitant) for approximately their first doubling times the cells were fixed in 4% paraformaldehyde. Following a second wash in PBS, the cells were incubated in DAPI solution (Sigma-Aldrich) at a dilution of 1/1,000 (=1 μg/ml) for 15 min in the dark. The cells were then observed under a fluorescence microscope (Zeiss, Oberkochen, Germany). Apoptotic cells were defined by chromatin condensation and nuclear fragmentation. We counted the number of apoptotic cells, repeating the counts on three different slides. Finally, on each slide we counted the number of apoptotic cells located in five different sequential fields.

Western blot analyses

PCR (Polymerase Chain Reaction)

a) RNA extraction and reverse transcription

From cultured cells (BE-13 or SD-1), total RNA isolation was achieved with the NucleoSpin RNA II Kit (Macherey-Nagel), allowing the purification of approximately 5×10^6 cultured cells. The final RNA was dissolved in RNase-free water. The purity and quality of the purified RNA were also checked. Reverse transcription with the elimination of genomic DNA was performed according to the manufacturer's instructions (QuantiTect Reverse transcription Handbook, QIAGEN). All reactions were carried out on ice in order to minimize the risk of RNA degradation. The cDNA obtained was kept at −80°C.

b) Amplification

From the cDNA preparation, 4 μl was used in PCR with specific primers according to the modified method of Bigioni et al. [31] based on the common sequence of the *TAC1* human isoforms (NM 001058, NM 015727), *TAC1R-Forward* (CTG CTG GTG ATT GGC TAT GC) and *TAC1R-Reverse* (AGG AGG AAG AAG ATG TGG AAG G), which yielded a 186 bp fragment. Amplification of the specimens was performed in a final reaction volume of 20 μl, and they were incubated at 95°C for 7 min, subjected to 40 cycles of 95°C for 30 s, 62°C for 40 s and 72°C 30 s, followed by a final extension cycle at 72°C for 7 min. The amplification products were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide.

Real time quantitative RT-PCR

Real-time quantitative RT-PCR was performed as described previously [32, 33]. Reverse transcription with elimination of genomic DNA was performed according to the manufacturer's instructions (QuantiTect Reverse transcription Handbook, QIAGEN). Real-time quantitative RT-PCR

analysis was performed using a de Roche Light Cycler with a fluorogenic detection system (SYBR green). The beta-actin gene was chosen as the housekeeping gene for normalization. The sequences of primers for the human NK-1 receptor were based on the common sequence of the *TAC1R* human isoforms (NM 001058, NM 015727), *TAC1R-Forward* (CTG CTG GTG ATT GGC TAT GC) and *TACR1-Reverse* (AGG AGG AAG AAG ATG TGG AAG G), which yielded a 186 bp fragment and for beta-actin gene were used forward primer CGGCATCGTCACC AACTG, and reverse primer CACGCAGCTCATTGTAG AAGGT, which yielded a 70 bp fragment. All amplification reactions were performed in a final volume of 20 μl containing 2 μl of the Master Mix PCR and 2 μl (1 μg) of cDNA. The primer concentrations were optimized as follows: 500 nM for the NK-1 receptor forward primer and reverse primer; 300 nM for the beta-actin forward primer and reverse primer. PCR for the NK-1 receptor included 40 cycles. In each cycle, the temperature was set at 92°C for 10 s; 62°C for 15 s, and 72°C for 10 s, while the conditions for PCR for beta-actin were 95°C for 5 s; 58°C for 15 s, and 72°C for 10 s. Data were analyzed using the relative standard-curve method, as reported previously [32]. Experiments were performed in duplicate for each data point. Each PCR run included five standard samples, two negative controls, and the experimental samples. Standard curves for both NK-1 receptor and beta-actin were generated using cDNAs from the BE-13, SD-1 and HEK 293 cell lines. For each experimental sample, the relative amounts (copy-number) of NK-1 receptor mRNA and beta-actin mRNA were respectively determined from the standard curve. The normalized amount of NK-1 receptor was determined by dividing the amount of NK-1 receptor mRNA by the amount of beta-actin mRNA for each sample. For BE-13, SD-1 cancer cell lines and the HEK 293 normal cell line, NK-1 receptor mRNA and beta-actin mRNA analysis was repeated four times in duplicate.

Small interfering RNA (siRNA) gene-silencing method

We carried out this method according to the manufacturer's instructions (Invitrogen, Madrid, Spain) and according to a previous study [34]. One day before transfection, 2×10^4 cells per well from the BE-13 and SD-1 human leukemia cell lines were seeded in 6-wells plates containing 2 ml of normal growth medium. Total cell number was determined the day before the transfection. Cells were incubated at 37° C in a CO_2 incubator for 17–20 h for normalization. Then, the normal growth medium was removed and an antibiotic-free growth medium (Opti-MEM; GIBCO) was added and incubated for 1 h. The latter medium was removed and the transfection mixture was added, which contained the siRNA *TAC1R* (Tachykinin 1 gene) (Invitrogen) and the diluted transfection reagent medium (Hiperfect, Qiagen); the latter two were previously incubated for 30 min. Following this, the final volume obtained was 200 μl. This method was applied in the same way to siRNA-negative control solution. For each transfection, 200 μl siRNA transfection reagent mixture was added to each well containing 800 μl of the antibiotic-free growth medium. The final siRNA concentration used for each transfection was 20 nM. It was incubated for 4–5 h at 37°C in a CO_2 incubator. Finally, 2 ml of normal growth medium was added for additional 72-h of incubation.

Results

NK-1 receptors

We performed Western blot analyses in order to test the presence of the NK-1 receptor in the human T-ALL BE-13 and B-ALL SD-1 cell lines. Total cell protein extracts were loaded onto polyacrylamide gels, resolved, and transferred to membranes. Incubation with an antibody against an epitope whose sequence is conserved in several species revealed the presence of different isoforms of the NK-1 receptor in both the BE-13 and the B-ALL SD-1 cell lines (Fig. 1). The relative amount of each protein form was similar in both cell lines. Two bands (isoforms of about 33 and 58 kDa) were observed in the human T-ALL BE-13 cell line (Fig. 1), whereas the B-ALL SD-1 cells expressed isoforms of 33, 58 and 75 kDa. In addition, no bands were detected when incubation was performed with the secondary antibody alone.

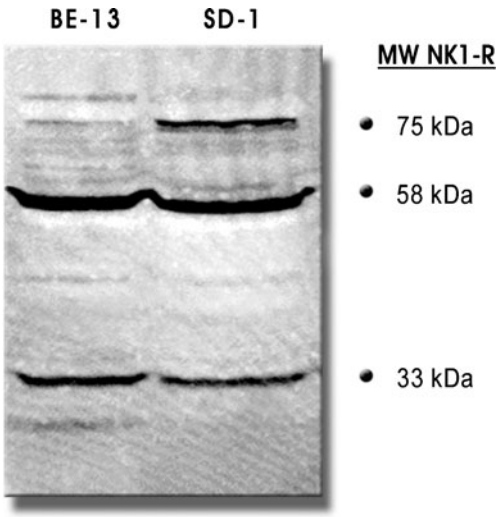

Fig. 1 Western blot analysis of NK-1 receptors in human T-ALL BE-13 and B-ALL SD-1 cell lines showing the presence of different NK-1 receptor complex isoforms. Dots indicate bands with molecular weights similar to those reported previously

Antitumor action of NK-1 receptor antagonists (L-733,060; L-732,138 and aprepitant)

Growth inhibition of the BE-13 and SD-1 cell lines by different NK-1 receptor antagonists was observed after the addition of increasing concentrations of these (Fig. 2a, b). Moreover, treatment of both cell lines with the three antagonists resulted in a concentration-dependent cytotoxicity (see Fig. 2a, b). From this figure, the IC_{50} (50% inhibitory concentration) at the first doubling times can be calculated. Thus, the concentrations required for a 50% reduction in optical density (IC_{50}) observed in the controls treated with L-733,060 were 15.4 μM for BE-13, and 18.4 μM for SD-1; with L-732,138, they were 63.9 μM for BE-13, and 49.7 μM for SD-1, and with aprepitant they were 19.5 μM for BE-13, and 29.4 μM for SD-1 (Table 1). Maximum inhibition was observed when the drug was present at a concentration of 40 μML-733,060; 124 μML-732,138 or 45.6 μM aprepitant (BE-13), and 50 μML-733,060; 103.5 μML-732,138 or 59.2 μM aprepitant (SD-1) during the culture periods. At the first doubling time, a strong decrease in the number of the two cell lines was found at intermediate concentrations and with the maximum concentration no remaining living cells were observed. A lower inhibition of growth of the two cell lines was observed in the presence of low doses of each antagonist.

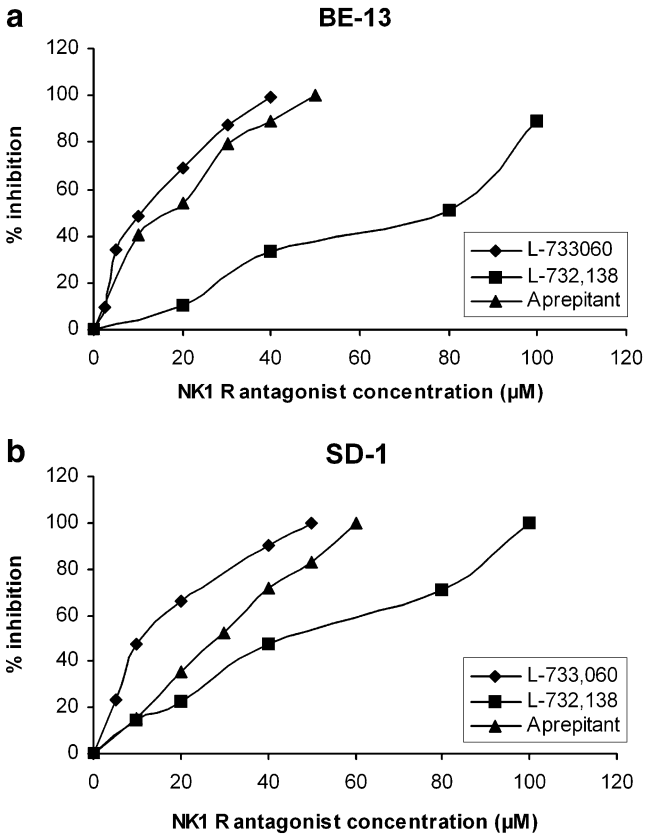

Fig. 2 **a** Percentage of growth inhibition of human T-ALL BE-13 cells at 48 h in in vitro cultures following the addition of increasing concentrations (2.5–40 μM) of L-733,060, (20–100 μM) of L-732,138, or (10–50 μM) of aprepitant. The percentage of inhibition for the first doubling time of incubation is plotted on a linear graph. Level of significance: * $p \leq 0.05$. Values are means±SD (bars). The regression line is indicated, as well as the equation used to obtain the IC_{50}. **b** Percentage of growth inhibition of human B-ALL SD-1 cells at 32 h in in vitro cultures following the addition of increasing concentrations (5–50 μM) of L-733,060; (10–100 μM) of L-732,138, or (10–60 μM) of aprepitant. The percentage of inhibition for the first doubling time of incubation is plotted on a linear graph. Level of significance: * $p \leq 0.05$. Values are means±SD (bars). The regression line is indicated, as well as the equation used to obtain the IC_{50}

NK-1 receptor antagonists block substance P-induced mitogen stimulation

Growth of the BE-13 and SD-1 ALL cell lines was noted after the addition of SP, and nanomolar concentrations of SP were seen to induce cell proliferation as compared to the controls (Fig. 3). SP stimulation was evident at 5 nM and the maximum level was reached at 50 nM for BE-13 and 10 nM for SD-1 (Fig. 3). This indicates that the activation of SP receptors leads to mitogenesis in the BE-13 and SD-1 human ALL cell lines. Thus, the percentage of cell proliferation of both cell lines increased from 14.5% to 77.5% in BE-13 and from 17.25% to 49.30% in SD-1, depending on the dose of SP administered (Fig. 3).

Treatment with L-733,060, L-732,138 or aprepitant at 10 μM for BE-13 and SD-1 partially inhibited the growth of both cell lines (Fig. 3). In order to examine whether the NK-1 receptor antagonists inhibited cell proliferation via an interaction with the NK-1 receptor, we used the specific NK-1 receptor agonist SP in competition experiments. Thus, the cellular concentration at 10 μM (BE-13 and SD-1) of L-733,060, 40 μM (BE-13 and SD-1) of L-732,138 and 20 μM and 30 μM (BE-13 and SD-1 respectively) of aprepitant with 50 nM (BE-13) and 10 nM (SD-1) of SP was higher than that observed with NK-1 receptor antagonist alone for BE-13 (Fig. 3a–c) and SD-1 (Fig. 3d–f). These results indicate that L-733,060, L-732,138 and aprepitant block SP mitogen stimulation, since NK-1 receptor antagonist-induced growth inhibition was partially reversed by the administration of a nanomolar dose of exogenous SP. This indicates the specificity of tachykinin NK-1 receptor activation in the growth of the human T-ALL and B-ALL cell lines, since an increase in the cellular concentration (47.50% and 34.60% in the case of L-733,060, for example) was respectively observed in the BE-13 and SD-1 cell lines with respect to the values found when the antagonist was administered alone (Fig. 3). There were no significant differences between the control and the control-DMSO (data not shown).

Table 1 Results of half inhibition (IC_{50}) and maximum inhibition (IC_{100}) experiments in leukemia cell lines following the administration of NK-1 receptor antagonists

Leukemia cell lines	L-733,060		L-732,138		Aprepitant	
	IC_{50} μM	IC_{100} μM	IC_{50} μM	IC_{100} μM	IC_{50} μM	IC_{100} μM
BE-13	15.4	36.3	63.9	124	19.5	45.6
SD-1	18.4	45.7	49.7	103.5	29.4	59.2

Apoptosis

After the administration of L-733,060, many apoptotic cells were found in both the BE-13 and the SD-1 ALL cell lines (Fig. 4). In fact, in DAPI-stained cultures we observed 59.24%±4.038 SD and 52.312%±5.630 SD of apoptotic cells respectively in the BE-13 and SD-1 cell lines after administration of IC_{100} L-733,060; and 55.5%±2,8 SD and

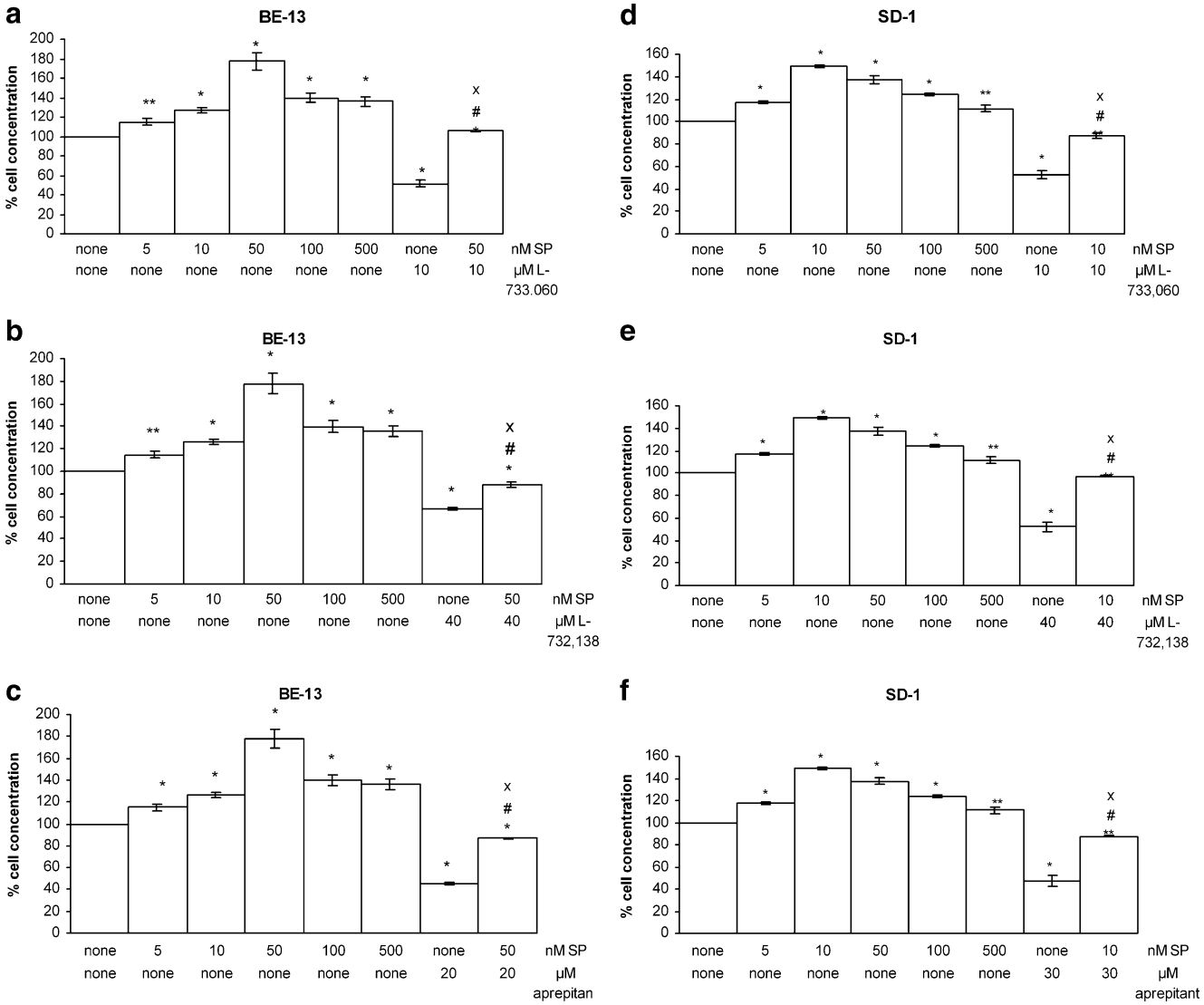

Fig. 3 **a–c** Induction of cell proliferation of human T-ALL BE-13 cells by SP at several nanomolar concentrations (5, 10, 50, 100 and 500 nM). The NK-1 receptor antagonists L-733,060, L-732,138 or aprepitant were added (10 μM, 40 μM and 20 μM respectively) in the presence (50 nM) or absence (none) of SP for their first doubling time. In both cases, each NK-1 receptor antagonist inhibited BE-13 cell proliferation. **d–f** Induction of proliferation of human T-ALL SD-1 cells by SP at several nanomolar concentrations (5, 10, 50, 100 and 500 nM). The NK-1 receptor antagonists L-733,060, L-732,138 or aprepitant were added (10 μM, 40 μM and 30 μM respectively) in the presence (10 nM) or absence (none) of SP for their first doubling time. In both cases, each NK-1 receptor antagonist inhibited SD-1 cell proliferation. Using the ANOVA test, a significant difference between each group and the control group (none-none) was found. Level of significance: * $p \leq 0.01$. ** $p \leq 0.05$. # indicates the value of significance of IC_{50}-most mitogenic SP concentration vs. IC_{50}-none, $p<0.05$, and x indicates IC_{50}-most mitogenic SP concentration vs. none-most mitogenic SP concentration, $p \leq 0.01$. Vertical bars indicate SD

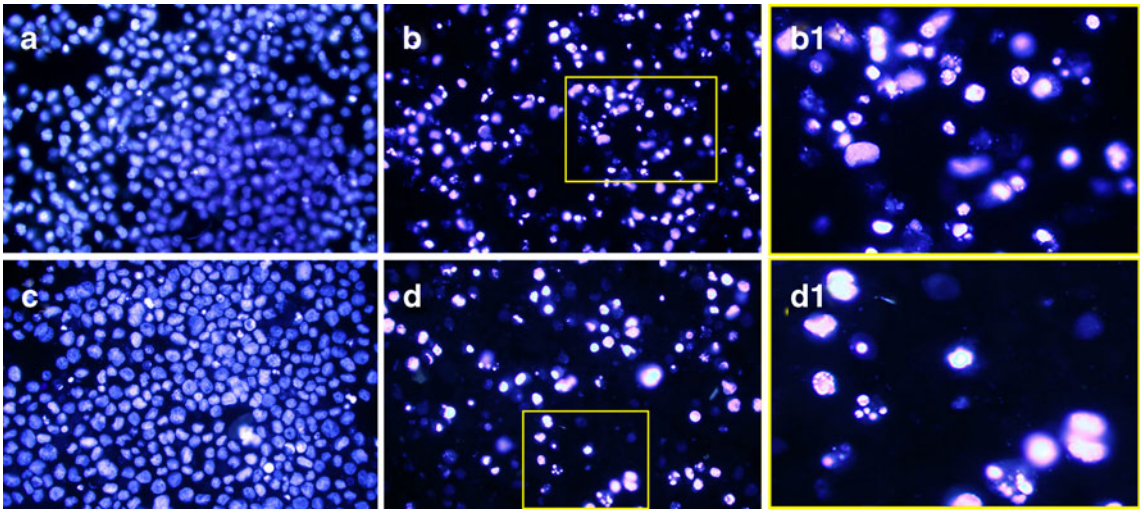

Fig. 4 Human T-ALL BE-13 (**a**, **b**) and B-ALL SD-1 (**c**, **d**) cell lines. **a** and **c** culture cells not treated with the NK-1 receptor antagonist (x 20). **b** and **d** culture cells treated with L-733,060 (x 20). **b1** and **d1** higher-power magnifications of the regions delimited, respectively, in the rectangles shown in **b** and **d** (x 40). Note in **b1** and **d1** that many nuclei of the treated cells show apoptotic figures: chromatin condensation and nuclear fragmentation

41.7%±4.7 SD of apoptotic cells in the BE-13 and SD-1 cell lines after the administration of IC_{100} L-732,138. Furthermore, we observed 36.4%±2.7 SD and 48.7%± 4.2 SD of apoptotic cells in the BE-13 and SD-1 cell lines after the administration of IC_{100} aprepitant.

PCR and RT-PCR quantitative analyses

From the PCR analyses, we also observed that the BE-13 and SD-1 tumor cell lines expressed mRNA for the tachykinin NK-1 receptor (Fig. 5). NK-1 mRNA expression was detectable in these cell lines as a product of the expected size of 186 bp, corresponding to only one band because the primers used in our PCR were designed for both the short and long isoforms.

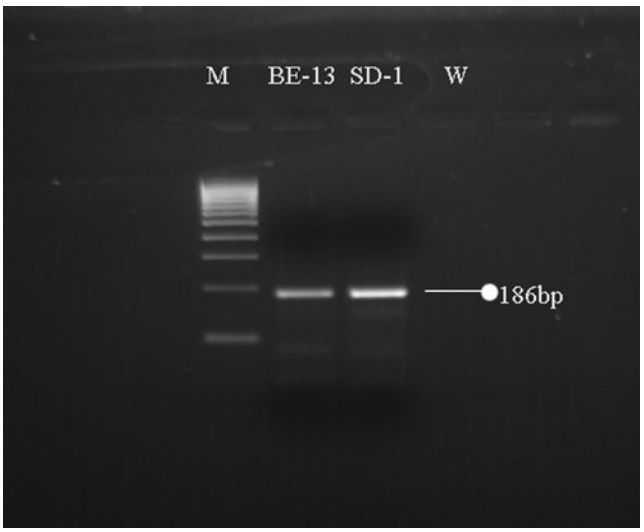

Fig. 5 Agarose gel showing the expression of *TAC1-R* cDNA in human leukemia cell lines. Equal aliquots of each cDNA were amplified by PCR for 40 cycles (target genes) with specific primers

Real-time quantitative RT-PCR was performed to analyze NK-1 receptor expression (Fig. 6a) using beta-actin (Fig. 6b) as a control. The mean NK-1 receptor/beta-actin ratio was 1.23±4.2 for the BE-13 tumor cell line and 3.2±0.8 for the SD-1 tumor cell line. However, the ratio was 0.01±3.9 in the HEK 293 normal cell line. Thus, the NK-1 receptor mRNA level was approximately 30-fold higher in leukemia cell lines than in the normal cell line.

Knockdown gene-silencing method (siRNA)

The knockdown method was carried out in the leukemia cell lines. Thus, after 72 h we found 9.7×10^5 siRNA-negative control BE-13 cells and 5.4×10^5 siRNA *TAC 1* cells (Fig. 7a). Moreover, after the administration of siRNA *TAC 1* to both cultured cell lines, many apoptotic cells were found in all wells studied. In fact, in the DAPI-stained cultures we observed means of 25±1.7 (SD)% of apoptotic cells. However, in the siRNA-negative control cells, we observed means of 4±1.5 (SD)%. Regarding SD-1 cells, after 72 h we found 5×10^5 siRNA-negative control cells and 2×10^5 siRNA *TAC 1* cells (Fig. 7b). As before, after the administration of siRNA *TAC 1* to SD-1 cultured cells many apoptotic cells were found. Thus, in the DAPI-stained cultures we observed means of 29±0.5 (SD)% of apoptotic cells, but in the siRNA-negative control SD-1 cells, we observed means of 4±0.8 (SD)%. All these data show that NK-1 receptors play an important role in the viability of such tumor cells.

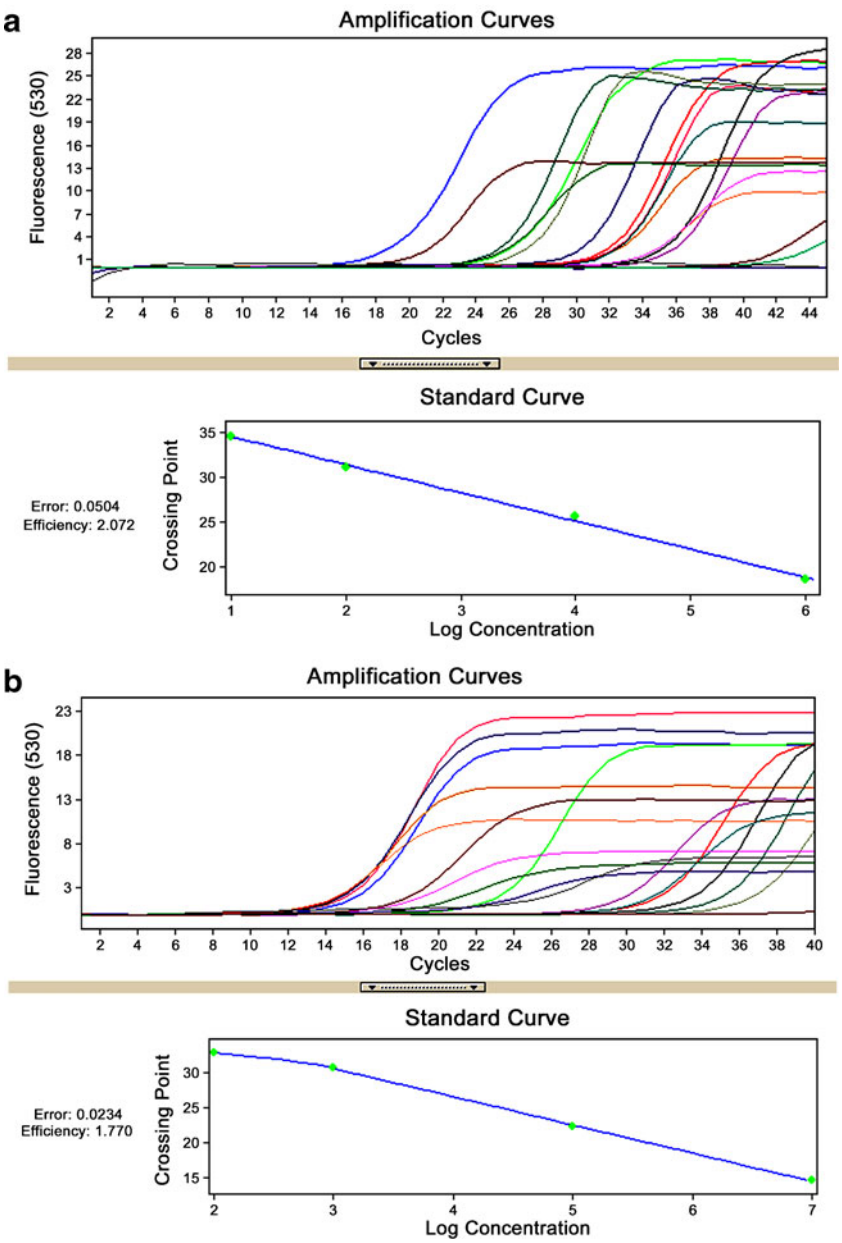

Fig. 6 Amplification plot obtained by real-time quantitative RT-PCR for NK-1 receptor cDNA (**a**) and beta-actin cDNA (**b**). Curves represent the fluorescence of SYBR green for the amplification of the *TAC1R* fragment in standard and experimental samples. The negative control is also shown

Discussion

In the present work, we have demonstrated for the first time the presence of isoforms of the NK-1 receptor in the BE-13 (33 and 58 kDa) and SD-1 (33, 58 and 75 kDa) ALL cell lines, although the functional roles of these NK-1 receptor isoforms are currently unknown. The data described here are in agreement with those reported in previous studies, in which the presence of isoforms of the NK-1 receptor in neuroblastoma, glioma, retinoblastoma, melanoma, pancreatic, larynx, colon and gastric carcinoma cell lines was reported [6, 8, 10, 11, 20, 26]. Moreover, we observed that *TAC1R* cDNA was present in human ALL cell lines by real-time quantitative RT-PCR, with the highest levels in ALL cells and the lowest ones in normal cells. Our data are in agreement with previous studies, since it is known that NK-1 receptors are overexpressed in primary glioblastomas, breast cancer and its metastasis, retinoblastoma, and larynx, pancreatic, gastric and colon carcinomas [11, 15–18, 26]. Additionally, it is known that human blood T-lymphocytes and cultured human IM-9 B-lymphoblasts express 7,000–10,000 and 25,000–30,000 NK-1 receptors per cell, respectively [21]; that NK-1 receptor expression is increased 25–36-fold in human pancreatic cancer cell lines in comparison with normal controls, and that tumor samples from patients with

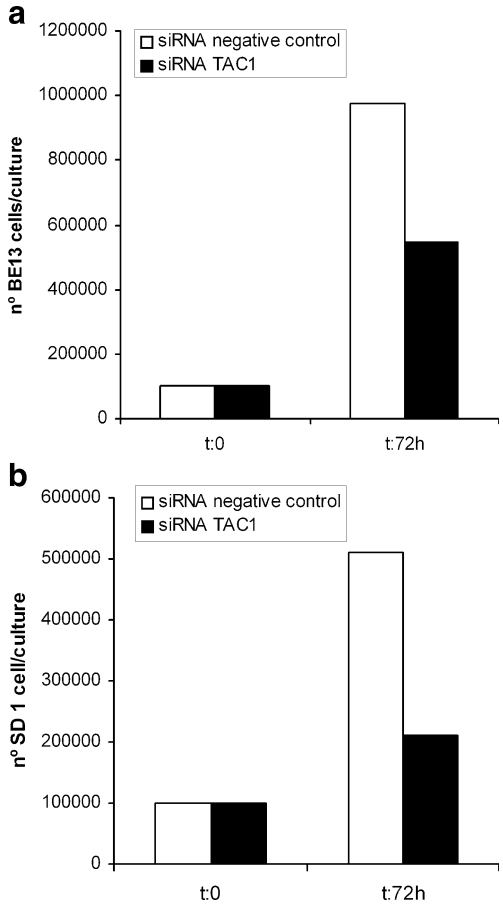

Fig. 7 Viability of BE-13 (**a**) and SD-1 (**b**) leukemia cells. The number of siRNA-negative control cells is compared with the number of siRNA *TAC1R* cells. At time 0, 100,000 BE-13 and 100,000 SD-1 cells were seeded. In both cases, at 72 h the decrease in the number of siRNA *TAC1R* cells was significant in comparison with the number of siRNA-negative control cells

advanced tumor stages exhibit significantly higher NK-1 receptor levels [17]. In addition, following the use of a knockdown method our data indicate that the NK-1 receptor plays a crucial role in the viability of the tumor cells studied here, as has been reported previously in human melanoma cell lines [20].

We have also demonstrated for the first time that, at nanomolar concentrations, SP induces mitogenesis in human ALL cell lines, this being consistent with previous studies in which it has been reported that SP exerts a mitogenic action on several cancer cell lines [3, 5–12, 20]. Moreover, it is known that in ALL SP is expressed in the cytoplasm of human blast cells [22]. Thus, SP could be released from these cells, stimulating their own mitogenesis via an autocrine/paracrine mechanism, although the peptide could also be released from nerve terminals and a potential role of bone marrow stroma cells as a potential source of SP should not be discarded. The demonstration here that SP stimulates ALL cell proliferation at low (nanomolar) concentrations in vitro suggests a new mechanism for the regulation of ALL activity through SP. These findings also suggest that a direct interaction between the nervous system and leukemia cells might occur, through the SP/NK-1 receptor system. Thus, SP and NK-1 receptors may be important in the development of ALL, since we observed that ALL cells expressed NK-1 receptors and that after binding to the NK-1 receptor SP exerted a strong mitogenic action, because in our competition experiments exogenous SP cell proliferation was partially reversed in both ALL cell lines by the administration of NK-1 receptor antagonists. This indicates the specificity of NK-1 receptor blockade by NK-1 receptor antagonists. Moreover, L-773,060-, L-732,138- and aprepitant-induced growth inhibition was gradually reversed by increasing concentrations of SP. Therefore, the antitumor action of these NK-1 receptor antagonists on these ALL cell lines is probably related to their ability to block the NK-1 receptor. This suggests that NK-1 receptor antagonists block the NK-1 receptors expressed by tumor cells and that the structurally very different molecules piperidine (L-733,060), L-tryptophan (L-732,138) and morpholine (aprepitant) exert the same antitumor action (these molecules only have their specificity for the NK-1 receptor in common). In this sense, it seems that the antitumor action of NK-1 receptor antagonists is related to stereochemical features and not to the chemical structures of the molecules. In sum, the data suggest the possibility of a common mechanism for cancer cell proliferation mediated by SP and the NK-1 receptor.

In addition, the findings of the present study demonstrate that treatment of the two ALL cell lines with the NK-1 receptor antagonists 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 [35], rhabdomyosarcoma [36], neuroblastoma, retinoblastoma, glioma and larynx, gastric and colon carcinomas [3, 10, 11, 26, 27, 37] and melanoma cell lines [20]. The blockade of NK-1 receptors in ALL cell lines by NK-1 receptor antagonists could inhibit both DNA synthesis and cell proliferation through the mitogen-activated protein kinase [5]. Moreover, it has been reported that NK-1 receptor antagonists decrease the basal phosphorylation of Akt, indicating the presence of a constitutively active form of the NK-1 receptor for producing apoptosis in tumor cells, and that they also cause the cleavage of caspase-3 and the proteolysis of poly (ADP-ribose) polymerase [37].

Neoangiogenesis, a hallmark of tumor development, has been associated with increased tissue innervation and expression of NK-1 receptors [16]. The effect of SP on the growth of capillary vessels in vivo has been reported, and it has been demonstrated that NK-1 receptor agonists also induce neoangiogenesis. It is also known that the proliferation of endothelial cells increases in a

concentration-dependent manner. These findings indicate that this peptide can stimulate the process of neoangiogenesis directly, probably through induction of the endothelial cell proliferation NK-1 receptor pathway [4]. In addition, in a large majority of tumors NK-1 receptors have been found in intra- and peritumoral blood vessels [15, 17], and intratumoral microvessel density is significantly increased in ALL patients in comparison with controls [38]. As previously mentioned, both autocrine/paracrine mechanisms via SP might be related to neoangiogenesis, given that SP is located in the cytoplasm of ALL cells and that the peptide could also be released from nerve terminals [22].

The active migration of tumor cells is a crucial requirement for the development of infiltration and cancer progression. It is known that SP induces the migration of tumor cells to specific organs by binding to NK-1 receptors in cancer cells, where it can be blocked by NK-1 receptor antagonists [14]. Moreover, a recent study carried out on HEK 293 cells has reported that the activation of the NK-1 receptor by SP induces a rapid change in cellular shape, including blebbing. This is not associated with apoptosis, but is rather a consequence of the Rho-activated ROCK system, leading to an increase in the phosphorylation of the myosin regulatory light chain (MLC) [39]. Membrane blebbing is important in cell movement, cell spreading, and cancer cell infiltration [40]. It has also been reported that aprepitant and L-733,060 block rapid SP-induced changes in cellular shape, including blebbing [39]. Moreover, treatment failure in children with ALL has been reported to be closely related to the expression of SP observed at the start of treatment, and a connection between the presence of SP-positive blasts and leukemia relapse has been suggested [41]. In addition to the results reported here, the administration of NK-1 receptor antagonists has been described to inhibit tumor cell growth in vitro and in vivo [7–9, 24, 25, 27, 42], and it is also known that aprepitant crosses the blood-brain barrier. This is quite important for the possible treatment of infiltration in the central nervous system.

All the above data suggest that the use of NK-1 receptor antagonists could improve human ALL treatment because the drug could exert an antitumoral action through three mechanisms: 1) an antiproliferative effect, due to the inhibition of tumor cell growth, inducing cell death by apoptosis; 2) an inhibition of angiogenesis in the tumor mass; and 3) an inhibition of tumor cell migration (infiltration). Moreover, aprepitant has been used in a placebo-controlled trial in patients with depression, with the observation that the safety and the tolerability of this drug are generally similar to the placebo [28]. Recently, we have reported the lack of toxicity of aprepitant against human fibroblast cells (HEK 293). Thus, the IC_{50} for HEK 293 cells was found to be approximately three-fold higher than the IC_{50} for tumor cells [30], as occurred in the ALL cell lines studied here. In addition, in the host NK-1 receptor antagonists exert beneficial dose-dependent actions such as antiemetic, antidepressant, anxiolytic, anti-inflammatory, analgesic, hepatoprotector and neuroprotector effects [3].

In sum, we describe for the first time the presence of several isoforms of the NK-1 receptor in the human BE-13 and SD-1 ALL cell lines and report that SP is mitogenic in both ALL cell lines. We also demonstrate the antitumor action of three NK-1 receptor antagonists (L-733,060, L-732,138 and aprepitant) against human ALL cell lines; that the antitumor action of these antagonists occurs through the NK-1 receptor, and that the antagonists induce apoptosis in ALL cell lines. Finally, we also report that NK-1 receptors play an important role in the viability of tumor cells; that the above-mentioned ALL cell lines express mRNA for the NK-1 receptor, and that *TAC1* gene is overexpressed in ALL cell lines. All these observations suggest that the NK-1 receptor could be a new target candidate in the treatment of human ALL and that NK-1 receptor antagonists (e.g., aprepitant) could be novel and promising antitumor drugs in the treatment of human ALL.

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