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PRECLINICAL STUDIES

Human acute myeloid leukemia cells express Neurokinin-1 receptor, which is involved in the antileukemic effect of Neurokinin-1 receptor antagonists

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Summary
The substance P/neurokinin-1 receptor system has been implicated in tumor cell proliferation. Neurokinin-1 receptor has been identified in different solid tumors but not frequently in hematopoietic malignant cells. We investigated the presence of the Neurokinin-1 receptor in acute myeloid leukemia cell lines (KG-1 and HL-60), demonstrating that acute myeloid leukemia cell lines overexpress the truncated Neurokinin-1 receptor isoform compared with lymphocytes from healthy donors. Using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) method, we demonstrated that substance P induced cell proliferation in both acute myeloid leukemia cell lines. We also observed that four different Neurokinin-1 receptor antagonists (L-733,060, L-732,138, CP 96–345 and aprepitant) elicited inhibition of acute myeloid leukemia cell growth lines in a concentration-dependent manner, while growth inhibition was only marginal in lymphocytes; the specific antitumor action of Neurokinin-1 receptor antagonists occurs via the Neurokinin-1 receptor, and leukemia cell death is due to apoptosis. Finally, administration of high doses of daily intraperitoneal fosaprepitant to NOD scid gamma mice previously xenografted with the HL60 cell line increased the median survival from 4 days (control group) to 7 days (treated group) ($p = 0.059$). Taken together, these findings suggest that Neurokinin-1 receptor antagonists suppress leukemic cell growth and may be considered to be potential antitumor drugs for the treatment of human acute myeloid leukemia.

Keywords Acute myeloid leukemia · Neurokinin-1 receptor · Substance P (SP) · Xenograft, Aprepitant · Fosaprepitant · L-733,060 · L-732,138, CP 96–345

Introduction

Acute myeloid leukemia (AML) results in the lethal overgrowth of myeloid progeny in bone marrow. AML is one of the most clinically challenging hematologic malignancies. In this regard, the long-term disease-free survival of AML patients remains poor, with minimal improvement over the past several decades [1]. Thus, the development of new therapeutic approaches is an unmet medical need for these patients.

In recent decades, the secretion of peptides and expression of specific receptors by tumors have attracted increasing attention. In particular, the substance P/neurokinin-1 receptor (SP/NK1R) system is known to play an important role in the development of cancer [2–4].

SP belongs to the tachykinin family of peptides that also includes Neurokinin A and B (NKA, NKB) and hemokinin 1, which are encoded by *TAC* genes [5]. The tachykinins are expressed throughout the nervous and immune systems and regulate several physiological processes [6]. Neurokinin receptor NK1R shows preferential affinity for SP [7], which exerts proliferative and anti-apoptotic effects through the

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ERK and Akt pathways [8, 9]. In this regard, it has been reported that SP induces mitogenesis in several tumor cell lines [10]; thus, NK1R is overexpressed in several human tumor cells [2]. Nowicki et al. documented that human IM-9 B-lymphoblasts expressed NK1R three-fold more per cell than healthy human T lymphocytes [11].

Regarding hematopoiesis and the SP/NK1R system, a major regulatory mechanism of hematopoiesis involves bidirectional crosstalk with the neural system [12]. SP and NKA are present in bone marrow through their release by either innervating tachykinin-positive nerve fibers within bone marrow [13] or resident cells from the bone marrow stroma [14]. SP and NKA mainly interact, but not exclusively, with NK1R and NK2R, respectively, which involves networks formed by stimulatory or inhibitory cytokines and intracellular crosstalk between the two receptors to modulate hematopoiesis [15, 16]. In the presence of SP, NK1R mediates hematopoietic stimulation and increases the proliferation of bone marrow progenitors [17], whereas NKA acts as a negative feedback factor by exerting opposing hematopoietic effects [14].

Regulatory mechanisms might be altered in leukemic cells in which predominant expression of *TAC1* transcripts has been found to produce only SP without its regulatory NKA neuropeptide [11, 18, 19]. In fact, disruption of this bone marrow homeostasis and upregulation of SP stimulatory effects have been linked to hematologic disorders, such as leukemia and bone marrow fibrosis [19, 20]. In addition, the autocrine role of SP in the proliferation of basophilic leukemia cells has been described [21].

In this context, there is increasing evidence supporting NK1R antagonists as being useful for treating hematologic malignancies [18, 19, 22]. In this regard, the antitumor effect of NK1R antagonists on tumor cell lines expressing NK1R have been demonstrated [23].

To the best of our knowledge, scant information is available regarding the role of the SP/NK1R system in human AML. Here, we analyzed whether the expression of NK1R in human AML in its truncated splice variant was linked to its up-regulation. We demonstrated the effects of SP and four different NK1R antagonists in acute myeloid leukemia cell proliferation in vitro and the effect of fosaprepitant in a xenograft mouse model.

Materials and methods

Cell culture

In vitro studies were conducted using two different human AML cell lines: human bone marrow KG-1 [American Type Culture Collection (ATCC) CCL-246; Manassas, VA, USA]; human peripheral blood acute promyelocytic leukemia HL-60 (ATCC CCL 240; Manassas, VA, USA).

For in vivo assays, we used human primary cells from healthy donors. T lymphocytes were isolated from peripheral blood by positive immunomagnetic separation using human CD3-MACS microbeads (AutoMACS CD3 Reagent; Miltenyi Biotec). The mononuclear fraction from bone marrow was extracted using Ficoll-Hypaque (Amersham Biosciences) by density gradient centrifugation.

The cell lines were cultured in RPMI-1640 (GIBCO BRL) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin as recommended by the supplier. The cell lines were cultured in 75-cm^2 tissue culture flasks (Falcon, Heidelberg, Germany) and were maintained at 0.3–1.0 × 10^6 cells/ml. The cells were fed twice weekly and were incubated at 37 °C in a humidified atmosphere (95% air/5% CO_2). For human primary cells, the FBS concentration was up to 20%.

Drug treatment

Four different NK1R antagonists were used in this study: L-733,060 (Sigma-Aldrich, Madrid, Spain); L-732,138 (Sigma-Aldrich); CP 96–345 (Sigma-Aldrich) and aprepitant (kindly supplied by Merck Research Laboratories, Madrid, Spain). L-733,060 and L-732,138 were dissolved in distilled water containing 1.25% and 7.5% dimethylsulfoxide (DMSO), respectively; CP 96–345 was dissolved in distilled water, and aprepitant was dissolved in distilled water containing 0.1% acetonitrile before the sample treatment.

SP and acetate salt (Sigma-Aldrich) were dissolved in distilled water containing 1% BSA (bovine serum albumin) at a low pH (0.05 M acetic acid), and different concentrations (10–100 nM) were used. In experiments investigating competing effects, the AML cell lines were incubated with SP for 1 h before the addition of each NK1R antagonist.

Western blot analyses

Total protein was prepared from cell cultures of the KG-1 and HL-60 cell lines. The protein concentrations were determined using a protein assay kit from BIO-RAD according to the manufacturer's instructions. From each sample, 15 μg of each protein sample was separated by electrophoresis on 10% SDS-polyacrylamide gels, followed by electroblotting onto PVDF membranes. The blots were incubated in blocking solution (5% non-fat milk in PBS, 0.1% Tween-20 [PBS-T]), followed by an overnight incubation with a primary antibody, IgG polyclonal antibody Anti-Substance P Receptor (1:2000 dilution; Sigma-Aldrich), and polyclonal Anti-Tubulin antibody (1:1000 dilution; Sigma-Aldrich). The membranes were then washed with PBS-T and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Cell Signaling Technology, United States) for 2 h at room temperature (1:3000 dilution). Antibody detection was performed

using an enhanced chemiluminescence system (ECL Western blotting detection; Amersham Life Science, United Kingdom). Image J densitometry software (Version 1.6; National Institutes of Health, Bethesda, MD) was used for gel band quantitative densitometric analysis. Selected bands were quantified based on their relative intensities.

RNA extraction, reverse transcription and real-time reverse transcription–polymerase chain reaction (qRT-PCR)

From cultured cells (KG1 or HL60), total RNA was isolated using the RNeasy Mini Kit (QIAGEN) and was treated with a RNeasy MinElute Clean Up kit (QIAGEN) to remove contaminating DNA. Reverse transcription was performed according to the manufacturer's instructions using the iScript™ cDNA Synthesis kit (Bio Rad). cDNA products were amplified in a Roche Light Cycler with a fluorogenic detection system (SYBR green) using sequence-specific primers under the following conditions: 95 °C for 7 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 30 s, followed by a final extension cycle at 72 °C for 7 min for *TAC1R* and 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s, followed by a final extension cycle at 72 °C for 7 min for the TAC1R full-length and -truncated products. The primer sequences for the human truncated *TAC1R* isoforms were *TAC1R-truncated-forward* (GGCTGTGGCCTTTGATAGGT) and *TAC1R-truncated-Reverse* (GTCCCCACTTGTCCCTCTTG). The primer sequences for the human full length *TAC1R* isoforms were *TAC1R-full-length-Forward* (AACCCCATCATCTACTGCTGC) and *TAC1R-full-length-Reverse* (ATTTCCAGCCCCTCATAGTCG). The primer sequences for *beta actin* were *BACTIN-Forward* (CGGCATCGTCACCAACTG) and *BACTIN-Reverse* (CACGCAGCTCATTGTAGAAGGT). The amplification products were visualized by electrophoresis on 2% agarose gels stained with ethidium bromide. The data were normalized using data from ACTIN in each sample by the $-2^{(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001).

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 or DMSO, control wells treated with SP, control wells treated with NK1R antagonists, and control wells treated with the highest mitogenic SP concentration (nM) and NK1R antagonists (μM) (50% inhibitory concentration (IC_{50}) of each antagonist for their first doubling times). The IC_{50} was calculated using the regression straight-line function based on the least squares technique. For the proliferation assay, 20 μl of 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.

In vitro analysis of apoptosis: DAPI staining

Briefly, after treatment with each NK1R antagonist for the first doubling time, 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 1:1000 (1 mg/ml) for 15 min in the dark. The cells were then analyzed using a fluorescence microscope (Zeiss, Oberkochen, Germany). Apoptotic cells were defined by chromatin condensation and nuclear fragmentation. We counted the number of apoptotic cells located in five different sequential fields, repeating the counts on three different slides.

In vivo AML xenograft

HL-60 leukemia cells (5×10^6) were transplanted into NOD/scid/IL-2R gamma null (NSG) 8-to 12-week-old mice via tail-vein injections after sublethal irradiation as conditioning (250 cGy). Three weeks after leukemic cell transplantation, the percentage of human leukemic cells in mouse bone marrow was determined by flow cytometry (FACS-Cantoll-BD) using species-specific antibodies: Pacific Blue™-conjugated human CD45 and APC-conjugated mouse CD45 antibodies. Mice with more than 20% human cells, according to the OMS 2008 AML definition, were eligible for randomization into two experimental groups. The treatment group received daily intraperitoneal (i.p.) fosaprepitant dimeglumine, as previously reported in breast cancer [24]. Fosaprepitant was dissolved in a physiological saline solution (15 mg of fosaprepitant/ml) and was administered at doses of 80 mg/kg of aprepitant. The control group received the same volume of placebo, which consisted of the vehicle (physiological saline solution) without fosaprepitant dimeglumine.

Statistical analyses

Statistical analysis was performed with SPPS statistical software for Microsoft Windows, release 18.0 (Professional Statistic, Chicago). The Shapiro–Wilk test was used to test the normality of the data. The relative quantitative expression levels of HL-60, KG-1 and control mRNA were analyzed by the Mann–Whitney U test. The results of the descriptive analysis of the proliferation assays were subjected to the non-parametric Wilcoxon test for paired samples. Kaplan–Meir survival curves were compared by the log-rank (Mantel–Cox) test. The data were expressed as the means ± SD

(standard deviation) or ± SEM (standard error of mean). The criteria for significance were $*p < 0.05$ and $**p < 0.01$ for all comparisons.

Results

Expression of NK1R in human KG-1 and HL-60 AML

To evaluate the expression of NK1R in human AML cell lines (KG-1 and HL-60), we performed both western blot and RT–PCR analyses. First, using western blotting, we observed strong expression of two isoforms of NK1R, at 60 and 50 kDa, in both cell lines. The expression of the 50-kDa isoform was twofold higher than that of the 60-kDa isoform in both KG-1 and HL-60 cells, representing the truncated and full-length splice variants, respectively. By contrast, no expression was found for the 50-kDa truncated variant in control cells. The expression levels of the 60-kDa isoform in KG-1 and HL-60 cells were 20 and 12-fold higher, respectively, compared with that in control cells (bone marrow cells from healthy donors) (Fig. 1a).

Next, RT–PCR assays were carried out as previously described to differentiate between full-length and truncated NK1R transcripts, the latter of which is an important splice variant described for NK1R signaling in cancer [25]. We compared the expression levels in KG-1 and HL-60 with that observed in human lymphocytes and observed no increase in the expression of full-length *TAC1R* in KG-1 and HL-60 cells compared with that in lymphocytes (ratio: 1.01 and 0.94, respectively) (Fig. 1b). By contrast, the AML cell lines markedly overexpressed the truncated splice variant with increased expression levels of 3.8- and 4.8-fold in KG-1 and HL-60 cells versus lymphocytes, respectively ($p = 0.016$ and 0.009, respectively) (Fig. 1c). Therefore, the expression of NK1R mRNA correlated with the protein levels analyzed by western blotting.

Anti-proliferative effect of NK1R antagonists (L-733,060, L-732,138, CP 96–345 and aprepitant)

We assessed the effects of different NK1R antagonists on the proliferation of both the KG-1 and HL-60 cell lines using the MTS assay. We found that incubation with four different

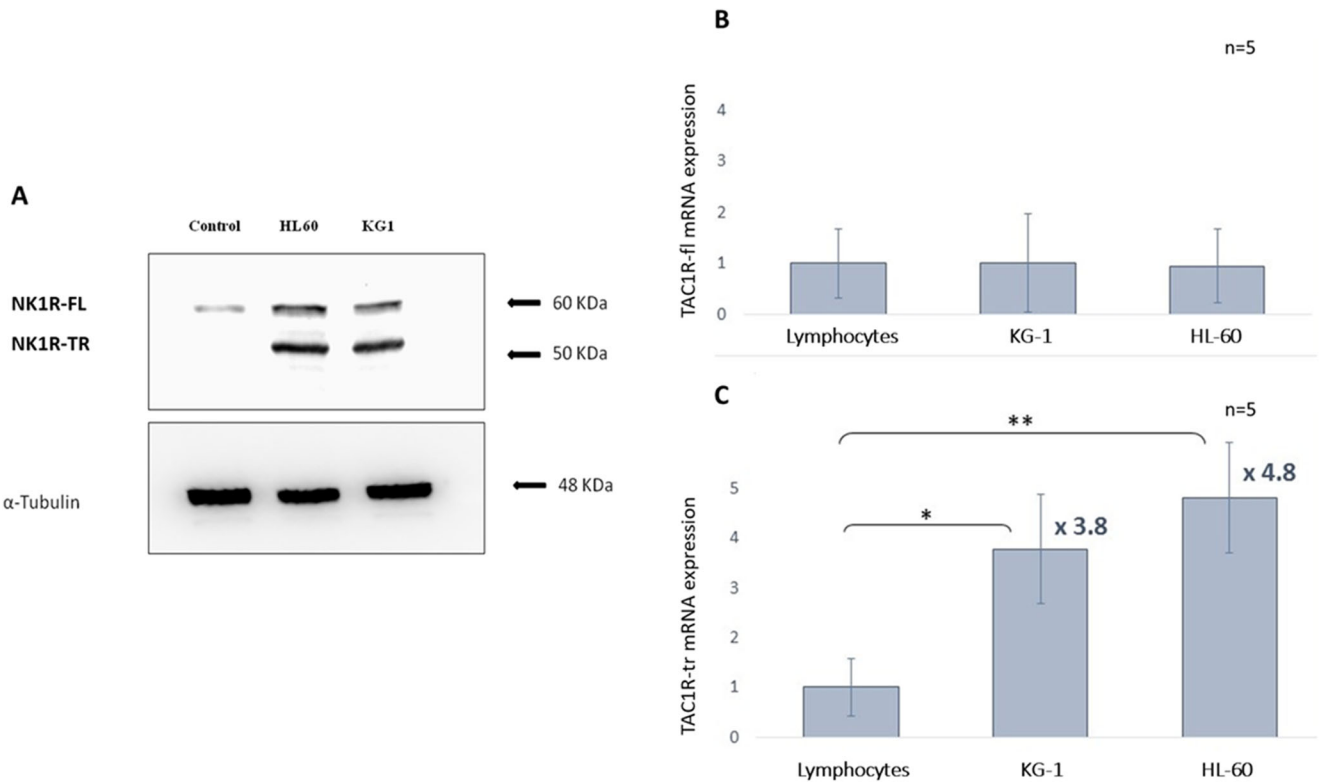

Fig. 1 Expression pattern of NK1R in the human AML cell lines KG-1 and HL-60. (**a**) Western blot analysis was performed using specific antibodies against NK1R without fl- and tr-NK1R discrimination; we observed strong expression of NK1R at 50 kDa and 60 kDa in both AML cell lines and slight expression at 60 kDa in healthy bone marrow cells. Tubulin served as an internal loading control. (**b** and **c**) The mRNA expression levels of NK1R from KG-1 and HL-60 are shown as the fold expression of the two NK1R isoforms (fl, full length (**b**); tr, truncated (**c**)) relative to lymphocytes. Analysis was carried out by RT–PCR, and normalization was performed using the housekeeping gene β-actin. The data are shown as the means ± standard deviation. $*p < 0.05$; $**p < 0.01$

NK1R antagonists (L-733,060, L-732,138, CP 96–345 and aprepitant) significantly reduced the proliferation of both AML cell lines compared with that of untreated cells at 48 h. We observed concentration-dependent growth inhibition in KG-1 and HL-60 cells, (Fig. 2a-b). The concentrations required for 50% growth reduction (IC_{50}) are shown (Table 1). L-733,060 and aprepitant showed the strongest antiproliferative effects. Finally, we tested the effect of aprepitant in lymphocytes from healthy donors. Strikingly, lymphocyte proliferation remained unaffected upon treatment with aprepitant even at the highest dose tested (Fig. 2c).

NK1R antagonists block substance P-induced mitogen stimulation

The mitogenic potential of SP was identified in several cancer cell lines. We therefore stimulated KG-1 and HL-60 cells with SP in the nanomolar range for 48 h. We observed increased cell growth up to 20.6% with concentrations of 25 nM for KG-1 (Fig. 3a), up to 31.6% with 50 nM for the HL-60 cell line (Fig. 3b), and up to 28.3% with 100 nM for lymphocytes (Fig. 3c) ($p = 0.043$, $p = 0.028$ and $p = 0.14$, respectively, compared with untreated samples).

To examine whether the NK1R antagonists inhibited cell proliferation via their interaction with the receptor, we performed competitive inhibition cell growth assays. For this purpose, we stimulated cell proliferation with SP at the optimal concentration, as previously specified, with increasing concentrations of aprepitant (10–60 μM). When KG-1 and HL-60 cells were treated with increasing concentrations of aprepitant, the addition of SP reversed the anti-proliferative effect of the NK1R blockade in a concentration-dependent manner, while this effect was not observed in lymphocytes. We observed increased cell growth inhibition with increasing concentrations of aprepitant in both cell lines (Fig. 4a and b). By contrast, we showed that lymphocyte cell growth was not significantly affected by increasing doses of aprepitant in the presence or absence of SP (Fig. 4c).

Finally, we repeated the competitive inhibition assay for four different NK1R antagonists. We stimulated cell proliferation with the same concentration of SP plus the IC_{50} of every antagonist. In all cases, exposure to the antagonist inhibited cell proliferation, and this inhibition was partially reversed in the presence of SP (Fig. 5).

After confirming the substantial growth inhibition of AML cells with NK1R antagonists, we also assessed whether this effect could be mediated via apoptotic mechanisms. For this

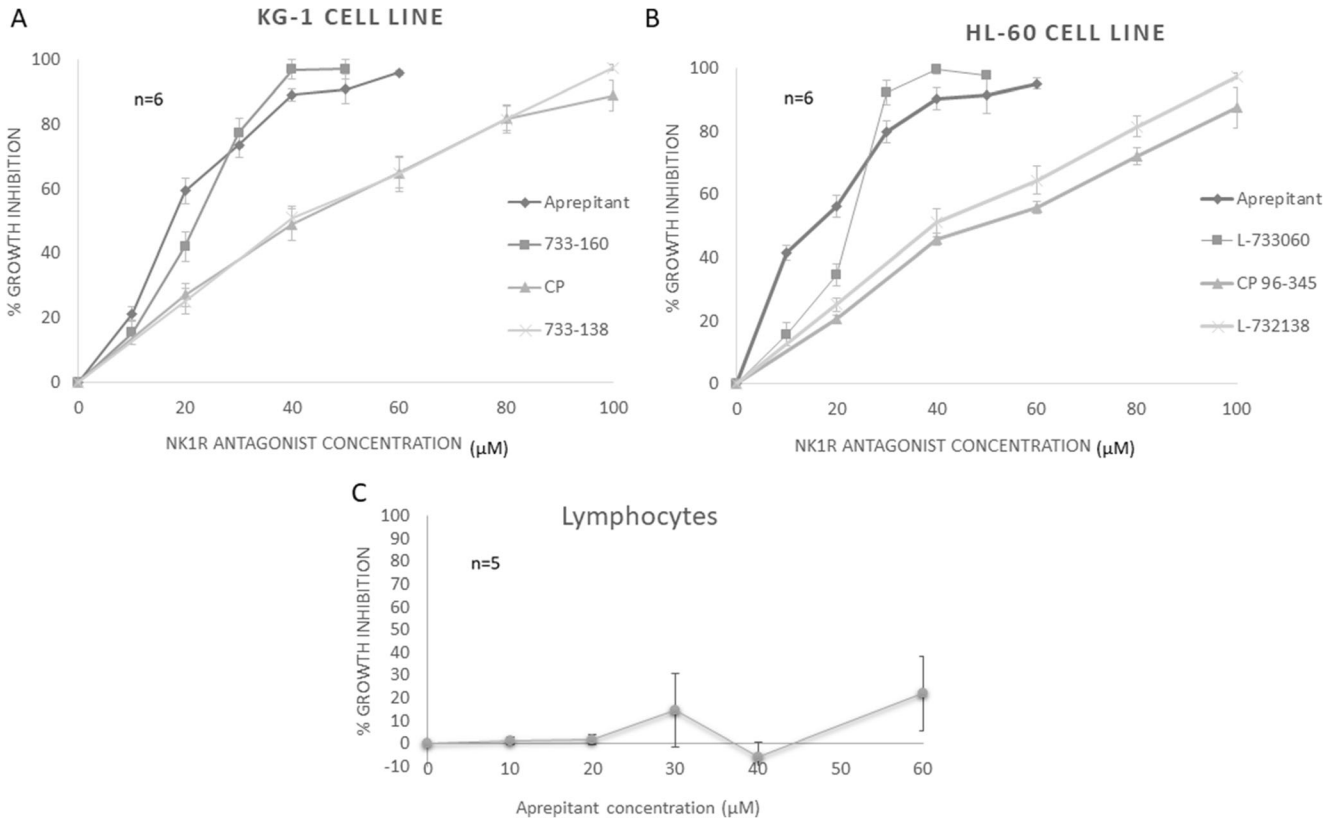

Fig. 2 Percentage of growth inhibition induced by NK1R antagonists in AML cell lines. MTS assays to determine cell growth after the addition of increasing concentrations of L-733,060, L-732,138, CP 96–345 or aprepitant for 48 h are shown for the cell lines KG-1 (**a**) and HL-60 (**b**). The values are expressed as the means ± SEM (bars). Cell survival is shown after the addition of increasing concentrations of aprepitant to lymphocytes at 72 h in in vitro cultures (**c**)

Table 1 Based on the MTS assay, the IC_{50} and IC_{100} (μM) of the four antagonists were calculated for KG-1 and HL-60 cells as well as the IC_{50} and IC_{100} (μM) of aprepitant for lymphocytes

KG1 IC_{50}	21.85	47	47.07	18.8
KG1 IC_{100}	42.7	98	104.14	60
HL60 IC_{50}	21.33	46.48	53.4	14.8
HL60 IC_{100}	37	98.65	111.6	60
Lymphocytes IC_{50}				176.2
Lymphocytes IC_{100}				344.72

purpose, AML cells were treated for 48 h with NK1R antagonists at doses of the IC_{50} and IC_{100}. The cells were subsequently stained with DAPI. The percentage values of apoptotic cells in both the KG-1 and HL-60 cell lines in DAPI-stained cultures are shown (Table 2).

AML xenograft mouse model

After observing a robust anti-leukemic effect in vitro, we next analyzed the effect of the NK1R antagonist in vivo using a preclinical model of a human AML xenograft in immunodeficient NOD/SCID mice (NSG). For this purpose, we xenografted the HL-60 cell line in NSG mice and treated them with intraperitoneal (i.p.) fosaprepitant.

The median percentage values of leukemic engraftment in bone marrow at the time of starting treatment, including placebo use, were 52% (22.9–93.4%) for the control group and 53% (20–96.7%) for the fosaprepitant group. A trend toward prolonged survival following fosaprepitant administration was observed, with a median survival of 4 days (IC95%: 3–7) for the control group versus 7 days (IC95%: 5–9) for the fosaprepitant group (log rank $p = 0.059$).

Discussion

We demonstrated, for the first time, the presence of two isoforms of NK1R in the KG-1 and HL-60 AML cell lines. In addition, we showed that exogenous nanomolar concentrations of SP exerted a proliferative effect on AML cell lines, in agreement with previous data from solid tumors [26] and B cell leukemia [22]. Moreover, previous reports showed that SP was expressed in the cytoplasm of human blast cells [11] and in the sera of patients with leukemia and myelofibrosis [18, 19]. A direct interaction between the nervous system and leukemic cells might occur through the previously described neuro-immune-hematopoietic axis [27, 28], allowing central modulation of leukemic cells. In this regard, we observed that AML cells overexpressed NK1R and that the neuropeptide SP produced a strong mitogenic action on AML cells. In line with this finding, in our competition experiments, exogenous SP cell

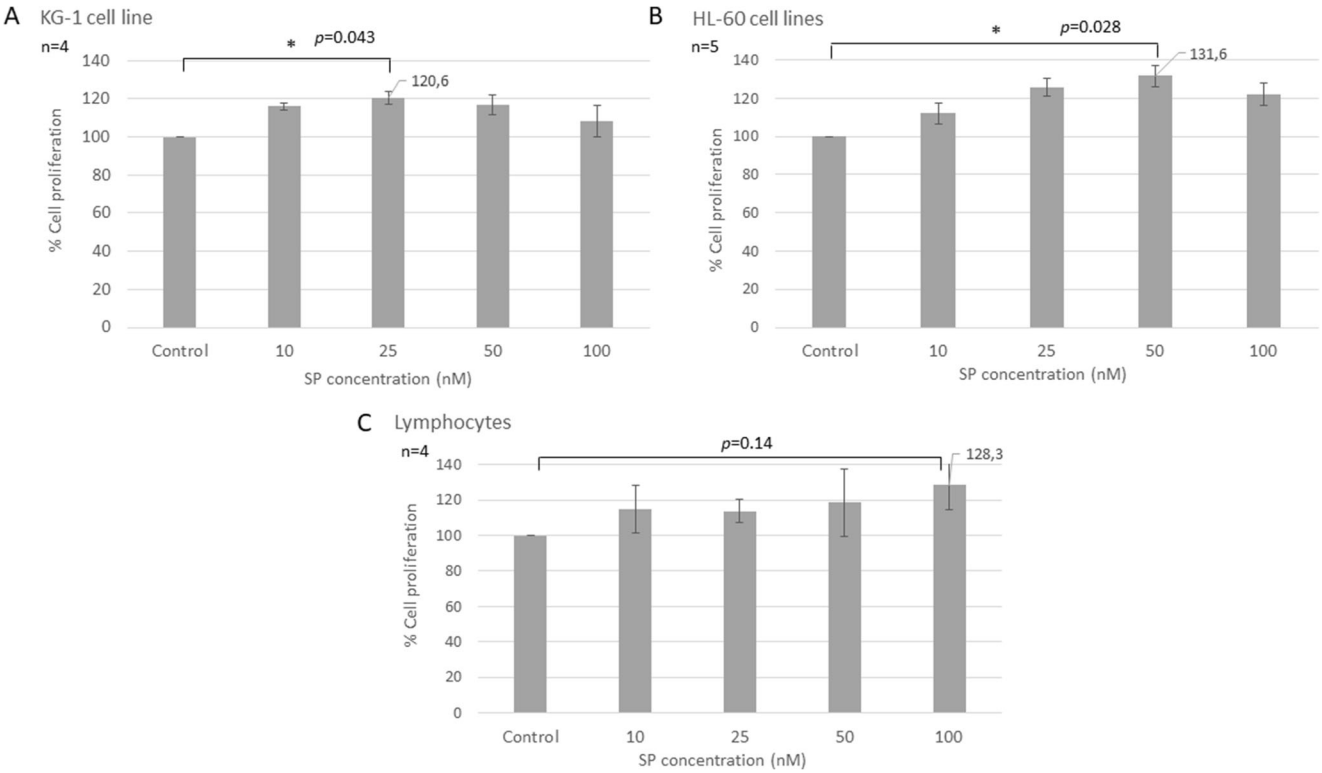

Fig. 3 SP induces cell growth in AML cell lines. KG-1 (**a**) and HL-60 (**b**) cell lines and lymphocytes (**c**) were stimulated with different nanomolar concentrations of SP to demonstrate its mitogenic potential in leukemic cells ($n = 5$)

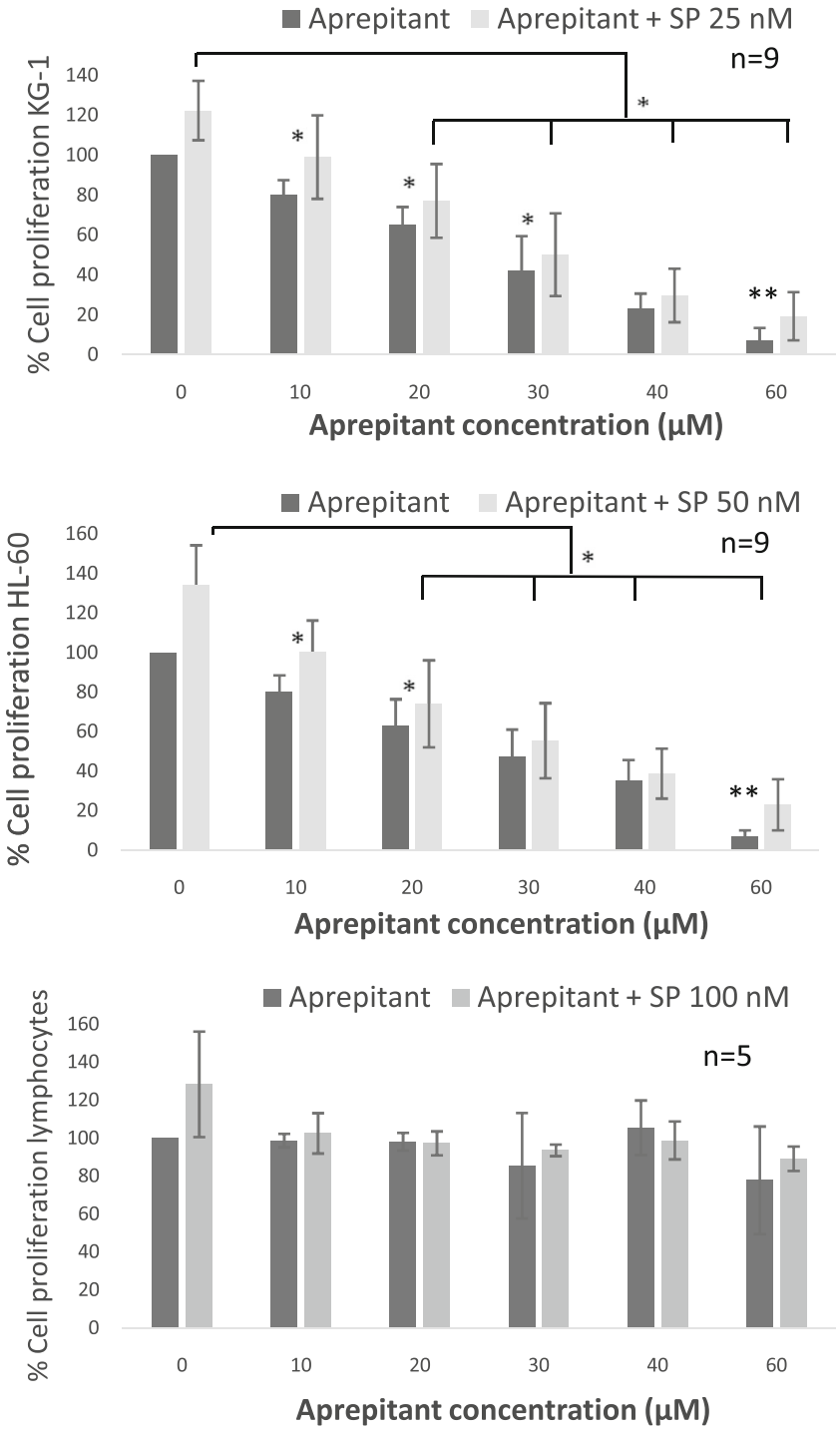

Fig. 4 Treatment with aprepitant reduces AML cell line proliferation in a dose-dependent manner. Competitive inhibition assays with aprepitant and SP. Analysis of cell proliferation using the MTS assay in KG-1 and HL-60 cells after treatment with aprepitant at the indicated increasing doses for 48 h in the presence or absence of the highest mitogenic SP concentration. The data represent the means ± SEM. Statistical significance, *$p < 0.05$; **$p < 0.01$ with respect to control conditions (untreated) and the aprepitant concentration. (C) Analysis of cell proliferation in lymphocytes treated with aprepitant using the MTS assay. Lymphocytes were treated with increasing doses of aprepitant in the presence or absence of 100 nM SP for their first doubling time (24 h). There were no significant differences between the control and control-DMSO or acetonitrile

proliferation was partially reversed in both AML cell lines by the administration of NK1R antagonists, suggesting the specificity of the NK1R blockade of the SP proliferative action.

On the other hand, we demonstrated that aprepitant reduced AML cell proliferation in a dose-dependent manner, while growth inhibition was only marginal for lymphocytes. In fact, the IC_{50} for lymphocytes was found to be approximately ten-fold higher than that for AML. Thus, lymphocytes are significantly more resistant to NK1R antagonists than AML cell lines, a finding that agrees with previous data from monocytes with 10 μM aprepitant [29] and from fibroblasts [30]. Recently, growth inhibition acute promyelocytic leukemia cells was also reported with aprepitant, but with a low IC_{50} (5 μM) [31] compared with our results on HL-60 (IC_{50}: 14.8 μM).

Remarkably, we demonstrated by RT–PCR and western blot assays that the truncated NK1R (NK1R-tr) splice variant is over-expressed in AML cell lines compared with that in

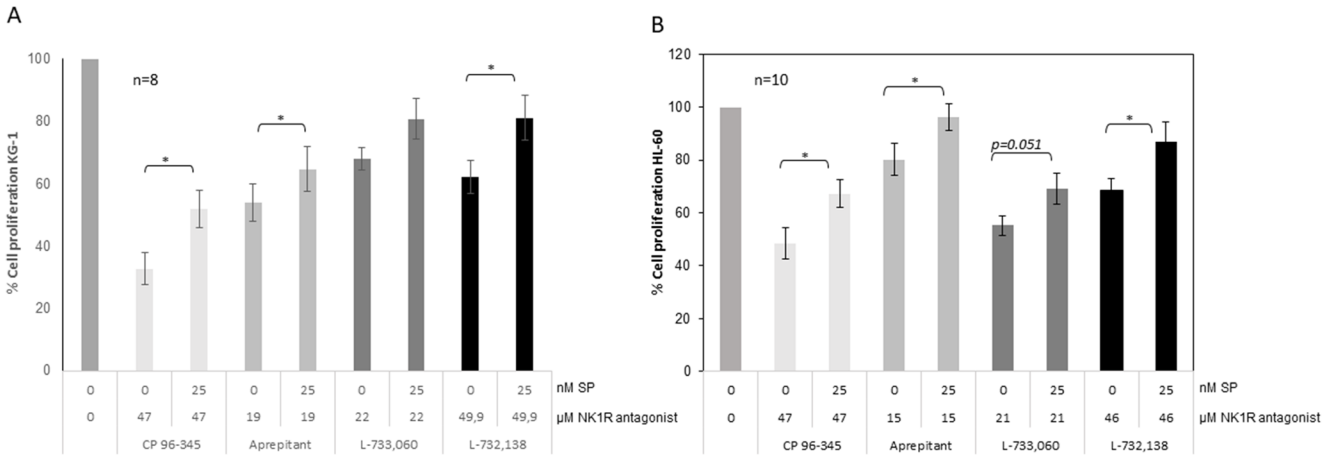

Fig. 5 Treatment with four different NK1R antagonists reduces AML cell proliferation. AML cells were treated with the IC_{50} concentration (μM) of the four NK1R antagonists for their first doubling time in the presence or absence of the highest mitogenic SP concentrations (25 nM for KG-1 and 50 nM for HL-60 cells), and the effects were analyzed using the MTS proliferation assay and were shown graphically. The values are expressed as the means ± SEM (bars) analyzed by the Wilcoxon signed-rank test. *$p < 0.05$; **$p < 0.01$

lymphocytes. This finding might explain the different effects of aprepitant on AML cell lines compared with that on normal lymphocytes. NK1R-tr shows a missing COOH terminal region that corresponds to the place where signals arrest, limiting receptor internalization [32, 33]. Expression of this NK1R isoform may have implications under pathological conditions where selective upregulation of the NK1R-tr was observed [25, 30]. Additionally, we confirmed that the growth inhibition of AML cell lines occurred not only with aprepitant but also with three other NK1R antagonists with different chemical compositions: piperidine (L-733,060), L-tryptophan (L-732,138), quinuclidine (CP 96–345) and morpholine (aprepitant). This result indicates that the antitumor action of NK1R antagonists is related to their stereochemical features (receptor affinity) and not to their chemical structures.

Finally, we observed a trend toward increased survival in our experimental animal model using fosaprepitant, despite high bone marrow infiltration (>50%), at the time of starting treatment, which translated into a short median survival in the control group. However, the control group received only a physiological saline solution vehicle, without the dimeglumine salt of fosaprepitant, which is known to produce inflammation (phlebitis) [34]. An in vivo anticancer effect for NK1R antagonists has previously been reported in a malignant glioma cell line [35], human breast cancer [36], brain tumors [37], human osteosarcoma [38] and hepatoblastoma cell lines [30] in accordance with our findings.

Interestingly, NK1R antagonists have progressed to human clinical trials, but not as anti-neoplastic agents. Importantly, no significant toxic side effects have been reported [39, 40]. Aprepitant has been used at 375 mg for 14 days in HIV clinical trials, obtaining a peak drug concentration of approximately 14 μM, [41] which is close to the value obtained from our in vitro data on AML cell lines for aprepitant (IC_{50}: 18.8 and 14.8 μM for KG-1 and HL-60 cells, respectively).

In summary, we describe, for the first time, the presence of two isoforms of NK1R in the human KG-1 and HL-60 AML cell lines and, specifically, an important splice of the NK1R-tr isoform. We report that SP is mitogenic in both AML cell lines. We also demonstrate the antiproliferative effects of four NK1R antagonists in human AML cell lines; this effect is specifically mediated through NK1R, and antagonists induce apoptosis. The current study identifies NK1R as a promising therapeutic target of AML and NK1R antagonists (e.g., aprepitant) as a novel antileukemic drug for the treatment of AML.

Table 2 DAPI staining of the human AML cell lines

	KG-1			HL-60		
	Control	IC_{50}	IC_{100}	Control	IC_{50}	IC_{100}
CP 96–345	3.7	40.21	76.66	3.93	42.66	79.21
Aprepitant		49.43	79.78		41.45	80.27
L-733,060		47.12	72.7		49.23	87.5

The average percentage of apoptotic cells by DAPI staining is shown in relation to all cells per power field in the KG-1 and HL-60 cell lines using the IC_{50} and IC_{100} of three different NK1R antagonists and the absence of an antagonist as the control

Compliance with ethical standards

Conflict of interest Authors: A Molinos-Quintana, P Trujillo-Hacha, JI Piruat, JA Bejarano-García, E García-Guerrero and JA Pérez-Simón declare that they have no conflict of interest.

M Muñoz declares that he has a conflict of interest: USPTO Application no. 20090012086 'Use of non-peptidic NK-1 receptor antagonists for the production of apoptosis in tumor cells'.

Ethical approval All applicable international, national and institutional guidelines for the care and use of animals were followed. All procedures

performed in studies involving human material or animal samples were approved by the Ethical Committee for Clinical Research of the University Hospital Virgen del Rocío (Seville, Spain) and were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent All samples from volunteer healthy donors were obtained from the regional blood donation center of the University Hospital Virgen del Rocío, and informed consent was obtained from all individual participants included in the study.

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