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REVIEW

The NK-1 receptor antagonist aprepitant as a broad spectrum antitumor drug

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Summary Aprepitant is a selective high-affinity antagonist of human substance P (SP)/Neurokinin-1 (NK-1) receptors. Until now this drug has been used as anxiolytic, antidepressant and antiemetic. It has been demonstrated that SP induces cell proliferation and NK-1 receptor antagonists different to aprepitant inhibit growth in several human cancer cell lines, where NK-1 receptors are overexpressed. The purpose of this study is to demonstrate the antitumor action of aprepitant. We performed an in vitro study of the growth inhibition capacity of the NK-1 receptor antagonist aprepitant against glioma, neuroblastoma, retinoblastoma and pancreas, larynx, gastric and colon carcinomas cell lines. Coulter counter was used to determine viable cell numbers followed by application of the MTS colorimetric method. Furthermore, a DAPI method was applied to demonstrate apoptosis. We have demonstrated: aprepitant at (5–70 μM) concentration elicits growth cell inhibition in a concentration dependent manner in all tumor cell line studied. Maximum inhibition (100%) was observed when the aprepitant was administered at a concentration of ≥70 μM in all tumor cell lines studied. The specific antitumor action of aprepitant occurs through the NK-1 receptor and tumor cells death was by apoptosis pathway. These findings reported here for the first time indicate that aprepitant is a new and promising broad spectrum antitumor drug in the treatment of cancer.

Keywords NK-1 receptor antagonist · Aprepitant · Apoptosis · Antitumor · Drug

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Introduction

The past two decades has witnessed an exponential increase in research into cancer. This has led to a considerable increase in investment in the field. This effort, however, has not been translated in better perspectives as regards the problem, although several fields of research have been promising, such as the human genome project, gene therapy, the search for new cytostatic agents and stem cell research. The short- and medium-term perspectives are not very promising and hence now pathways must be opened up to offer future hope to oncologic patients. In this sense, it is expected that in the near future newer and more effective and safe anticancer drugs will become available for the treatment tumors [1].

Substance P (SP) is an undecapeptide that belongs to the tachykinin family of peptides. It is known that SP, neurokinin A (NKA), neuropeptide K and neuropeptide Gamma (the two latter elongated forms of NKA) are derived from the preprotachykinin A gene (PPT-1), whereas neurokinin B (NKB) is derived from the preprotachykinin B gene (PPT-2). The biological actions of SP, NKA and NKB are mediated by three 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 [2], and this neuropeptide has also been implicated in neurogenic inflammation, pain and depression [3]. 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 [4–11]. In addition, SP a main mediator on the growth of capillary vessels in vivo and on the proliferation of cultured endothelial cells in vitro, as it has been demonstrated that NK-1 receptor agonists also induced neoangiogenesis [12]. Also, the active migration of tumor cells, a crucial requirement for invasion and metastasis development is regulated by signal SP [13].

Conversely, the NK-1 receptor antagonist blocking the biological actions of SP. At the present, there are more than thirty compounds that act as NK1 receptor antagonists [14]. Moreover, it has been reported that the NK-1 receptor antagonist L-733,060 a piperidine derivative, produces analgesia [15] and antidepressive effects [16]. In addition, it has been used in the treatment of a broad range of anxiety and mood disorders [17] and in inflammatory liver disease; its action is most likely to be due to an inhibition of the effects of SP [18]. Additionally, in vitro and in vivo studies have demonstrated that SP antagonists (synonymous of the NK-1 receptor antagonist) inhibit the growth of both small cell lung cancer and glioma [19, 20]. Moreover, we have also demonstrated that L-733,060 shows antitumor activity against human neuroblastoma, glioma, melanoma, retinoblastoma and pancreas, larynx, gastric and colon carcinoma cell lines that overexpress NK-1 receptors [5, 7, 8, 10, 11, 21]. Likewise, other NK-1 receptor antagonist L-732,138 (L-tryptophan derivative) also shows antitumor activity [9, 10, 22]. The aprepitant drug is a selective high-affinity antagonist of human SP/NK-1 receptors. Until now this drug has been used as anxyolitic, antidepressant and antiemetic [16, 17, 23]. However, to our knowledge no study has been carried out on the antitumor effect of the NK-1 receptor antagonist aprepitant. Thus, the aims of this study were: (1) To demonstrate, using a MTS colorimetric method to evaluate cell viability, the antitumor action of the NK-1 receptor antagonist aprepitant against human glioma, neuroblastoma, retinoblastoma, pancreatic, larynx, gastric and colon carcinoma cell lines and to show that this antitumor action occurs through the NK-1 receptor and (2) To know whether the NK-1 receptor antagonist aprepitant produces, or not, apoptosis in all these tumor cell lines.

Materials and methods

Cell cultures

We used the human glioma (GAMG); neuroblastoma (SKN-BE(2), IMR-32, KELLY); retinoblastoma (Y-79, WERI-Rb-1); pancreatic carcinoma (PA-TU 8902, CAPAN-1); larynx carcinoma (HEp-2); gastric carcinoma (23132-87) and colon carcinoma (SW-403) and Human Embryonic Kidney (HEK 293) cell lines, these cell lines were purchased to DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), ATCC (American Type Culture Collection) and ICLC (Interlab Cell Line Collection), these cell lines were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 and according to the manufacturer's instruction.

Drug treatments

The NK-1 receptor antagonist 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) (was supplied by Merck Research Laboratories, Madrid, Spain) was dissolved in distilled water containing acetonitrylo before sample treatment. In order to determine the IC_{50}, different concentrations (5 to 70 μM) of aprepitant were evaluated. SP, acetate salt (Sigma-Aldrich, Madrid, Spain), was dissolved in distilled water and different concentrations of SP (5, 10 and 100 nM) were evaluated. The most mitogenic nanomolar SP concentration for each cell line was incubated 1 h before the addition of aprepitant.

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. Cells were cultured in 96-well plates: each well contained 10^4 cells in a total volume of 100 μl. Each assay included one plate The plate included blank wells (0 cells/0.1 ml), control wells (10^4 cells /0.1 ml), control wells with acetonitrylo, control wells treated with aprepitant and control wells treated with the most effective SP concentration and aprepitant. The plates were inoculated with aprepitant (5–70 μM for tumor cell lines) and were incubated for the first doubling time specific for each tumor cell line. The plates were also inoculated the most mitogenic exogenous SP nM concentration with the fifty-percent inhibition concentration (IC_{50}) of aprepitant μM concentration approximately and without aprepitant for their first doubling times respectively. For the proliferation assay, 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. The quantity of product, as measured by optical density, is directly proportional to the number of living cells. Each experimental

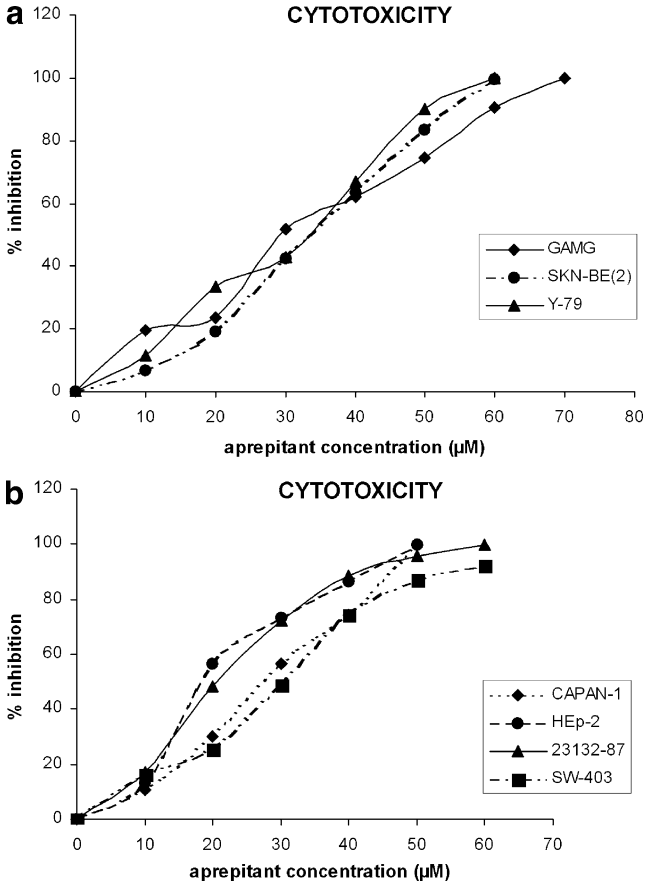

Fig. 1 **a** Percentage of growth inhibition of human GAMG glioma, SKN-BE(2) neuroblastoma and Y-79 retinoblastoma cells at first doubling time in in vitro cultures following the addition of increasing concentrations (10 to 70 μM) of aprepitant. Level of significance: *$p \leq 0.05$. **b** Percentage of growth inhibition of human CAPAN pancreas, HEp-2 larynx, gastric 23132-87 and SW-403 colon carcinoma cells at first doubling time in in vitro cultures following the addition of increasing concentrations (10 to 70 μM) of aprepitant. Level of significance: *$p \leq 0.05$

condition was assayed in duplicate and all experiments were performed at least three times. The IC_{50} of aprepitant 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 13.0 (Professional Statistic, Chicago, IL, 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 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 antagonist aprepitant used here, DAPI staining was performed. Briefly, after treatment with aprepitant for their first doubling times approximately, the cells were fixed in 4% paraformaldehyde. Following a second wash in PBS, cells were incubated in DAPI solution (Sigma-Aldrich) at a dilution of 1/1,000=1 μg/ml for 30 min in the dark. The cells were then observed through a fluorescence microscope (Zeiss, Oberkochen, Germany). Apoptotic cells were defined by the chromatin condensation and nuclear fragmentation. We counted the number of apoptotic cells. In each case, the count was repeated in three different slides. Finally, in each slide, we counted the number of apoptotic cells located in five different sequentially fields.

Results

Antitumor action of NK-1 receptors aprepitant

Growth inhibition of the all tumor cell lines by aprepitant was observed after the addition of increasing concentrations of aprepitant (Fig. 1). Moreover, treatment of all tumor cell lines with aprepitant resulted in a concentration-dependent cytotoxicity (see Fig. 1). The aprepitant concentration IC_{50} and IC_{100} growth inhibition of tumor cell lines were shown (Table 1). Maximum inhibition was observed when the drug was present at a concentration of 70 μM during the culture periods. At the first doubling time, a strong decrease in the number of the all cell lines studied was found at intermediate concentrations and no remaining living cells were observed at the maximal concentration. A lower

Table 1 The results of half inhibition (IC_{50}) and maximum inhibition (IC_{100}) experiments in the tumor cells after administration of NK-1 receptor antagonist aprepitant

Tumor	Cell line	Aprepitant	
		IC_{50} μM	IC_{100} μM
Glioma	GAMG	33.1	66.2
Neuroblastoma	SKN-BE(2)	24.6	48.8
	IMR-32	19.6	45.1
	KELLY	27.7	49.5
Retinoblastoma	Y-79	30.4	59
	WERI-Rb-1	23	53.1
Pancreas carcinoma	PA-TU- 8902	31.2	63
	CAPAN-1	27.4	52
Larynx carcinoma	HEp-2	22.7	46.5
Gastric carcinoma	23132-87	24.2	52.5
Colon carcinoma	SW-403	30.5	60.5

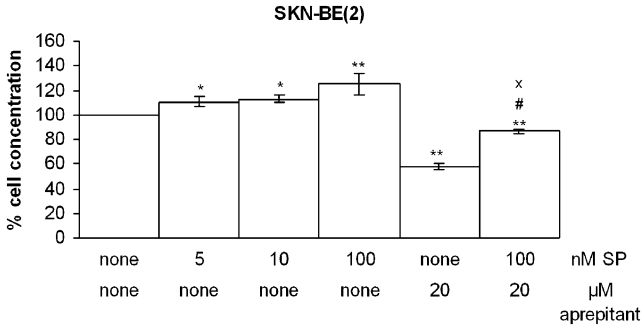

Fig. 2 Induction of cell proliferation of human neuroblastoma SKN-BE (2) cells by SP at several nanomolar concentrations (5, 10 and 100 nM). The NK-1 receptor antagonist aprepitant was added (20 μM) in the presence (100 nM) or absence (none) of SP for first doubling time. In both cases, aprepitant inhibited SKN-BE(2) cell proliferation. Using the ANOVA test, a significant difference between each group and the control group (none–none) was found. Level of significance: *$p \leq 0.05$ and **$p \leq 0.01$. *Number sign* indicates the value of significance of 100–none vs 100–20 and (*plus sign*) 100–20 vs none–20 (*number sign*) and (*plus sign*) $p \leq 0.05$. *Vertical bars* indicate SD

inhibition of growth of the all cell lines was observed in the presence of low doses of aprepitant.

The NK-1 receptor antagonist aprepitant blocks substance P-induced mitogen stimulation

As previously our group has reported all these tumor cell lines were observed to growth after the addition different doses of SP [1, 6–11]. In order to examine whether the NK-1 receptor antagonist aprepitant 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 IC_{50} μM of aprepitant and nM of SP was lower than that observed with different doses of SP alone for all tumor cell lines studied. These results indicate that aprepitant blocks SP mitogen stimulation, and on the other hand aprepitant-induced growth inhibition was partially reversed by the administration of the most mitogenic nanomolar dose of exogenous SP, thus we observed cellular concentration higher than with aprepitant alone (see e.g. Fig. 2). This indicates the specificity of NK-1 receptor activation in the growth of all tumor cell lines by SP, since an increase in the cellular concentration was observed in these cell lines studied with respect to the values found when the antagonist was administered alone. There were no significant differences between the control and the control-acetonitrylo (data not shown).

NK-1 receptor antagonist aprepitant against human embryonic kidney 293 cells

In order to examine the safety of aprepitant, we used the NK-1 receptor antagonist aprepitant against HEK 293 cells, The concentrations required for an IC_{50} observed in the HEK 293 cells treated with aprepitant were >90 μM data not shown.

Apoptosis

After administration of NK-1 receptor antagonist aprepitant a great number of apoptotic cells were found in all tumor cell lines (Fig. 3). In fact, we observed in DAPI-stained cultures respectively as means 25.5±4.88 (SD) % at IC_{50} concentration and 44.1±6.01 (SD) % at IC_{100} concentration of apoptotic cells in all tumor cell lines studied after administration of aprepitant. Conversely, in tumor cells control (non-treated with NK-1 receptor antagonist aprepitant) we observed as means 0.9±0.95 (SD) % apoptotic cells in all tumor cell lines studied.

Discussion

We have demonstrated for the first time in vitro a potent growth inhibition on human glioma, neuroblastoma, retinoblastoma, pancreatic carcinoma, larynx carcinoma, gastric carcinoma and colon carcinoma cell lines through use of the NK-1 receptor antagonist aprepitant. This is in agreement with previous studies reported by our group, in which the use NK-1 receptor antagonists L-733,060/L-732,138 other different of aprepitant exert antitumor action on glioma, neuroblastoma, retinoblastomas, pancreas, larynx, gastric and colon carcinoma

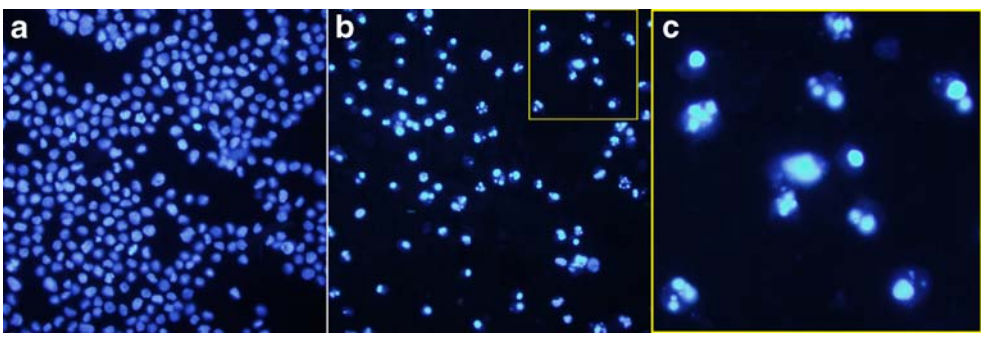

Fig. 3 **a** Culture SW-403 colon carcinoma cells non-treated. **b** Culture SW-403 colon carcinoma cells treated with NK-1 receptor antagonist aprepitant. Apoptotic figures: the chromatin condensation and the nuclear fragmentation can be observed (**c**) high power magnification

cell lines same than aprepitant [1, 6–11, 21]. Moreover, SP analogue antagonists (synonymous of NK-1 receptor antagonist) inhibited, both in vitro and in vivo, the growth of small cell lung cancer [19] and other NK-1 receptor antagonists inhibit the growth of glioma and breast carcinoma in vivo [20, 24]. It should be noted that in tumor cell lines as different as those mentioned above the same NK-1 receptor antagonist aprepitant 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 (e.g., aprepitant) could inhibit a large number of tumor cell types in which both SP and NK-1 receptors are overexpressed [11, 22, 25–28]. Moreover, it has been demonstrated that the NK1 receptor expression is increased 25- to 36-fold in human pancreatic cancer cell lines in comparison with normal controls, and that tumours samples of patients with advanced tumour stages exhibit significantly higher NK1 receptor levels [26]. Thus, the NK-1 receptor antagonist aprepitant could be candidate for a broad-spectrum antineoplastic drug. Indeed, previously we have reported that all tumor cell lines studied express the NK-1 receptor and its isoforms by western blot analysis [6, 8–11, 22].

We have demonstrated that treatment of the all tumor cell lines with NK-1 receptor antagonist aprepitant produces growth inhibition and cell death. We have also demonstrated the cell death observed here was due to a specific toxic effect of aprepitant and not to a non-specific action of this drug. In our competition experiments, exogenous SP cell proliferation was partially reversed by the administration of aprepitant, suggesting the specificity of NK-1 receptor blocking on all tumor cell lines by aprepitant, which supports the specific effect. Thus, the action on these cell lines by the NK-1 receptor antagonist aprepitant is probably related to the ability of this antagonist to block the NK-1 receptor in such tumor cell lines. In addition, the findings of the present study demonstrate that treatment of all tumor 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 [29], rhabdomyosarcoma [30], neuroblastoma, retinoblastoma, larynx, gastric and colon carcinoma cell lines [9–11, 22]. In this sense, it is known that NK-1 receptor antagonists above mentioned (other than aprepitant) induces apoptosis in tumor cells and causes a concentration-dependent loss of cell viability [9–11, 22, 29]. Likewise, as we observed using aprepitant, the antitumor activity of aprepitant reported here was dose-dependent. The blockade of NK-1 receptors in all tumor cell lines by aprepitant could inhibit both DNA synthesis and cell proliferation through the mitogen-activated protein kinase (MAPK) pathway [4].

It is known, SP has been implicated in neurogenic inflammation, on the growth of capillary vessels in vivo, as well as it has been demonstrated that it also induced neoangiogenesis and that the proliferation of endothelial cells increased in a concentration-dependent manner. These findings indicate that SP can directly stimulate the process of neoangiogenesis, probably through induction of the endothelial cell proliferation NK-1 receptor pathway [12]. Conversely, SP analogue antagonists, synonymous of the NK-1 receptor antagonist, inhibit tumor growth in pancreatic cancer via a dual mechanism involving both antiproliferative and the antiangiogenic properties [31]. Additionally, The active migration of tumor cells is a crucial requirement for metastasis development and cancer progression. It is also known that SP induces the migration of tumor cells to specific organs by binding to the NK-1 receptor in cancer cells, where it can be blocked by NK-1 receptor antagonists [13]. These data suggest that SP and NK-1 receptors could play an important role in the development of metastasis.

All the data mentioned above suggest that treatment with the NK-1 receptor aprepitant in cancer cell lines expressing the NK-1 receptor could improve cancer treatment because, it exert antitumor action through three mechanisms: (1) An antiproliferative effect due to the inhibition of tumour cell growth, inducing cell death by apoptosis; (2) An inhibition of angiogenesis in the tumour mass; and (3) An inhibition of the migration of tumour cells (invasion and metastasis).

Moreover, SP also has been implicated in pain and depression [3, 32] and in this sense the NK-1 receptor antagonist aprepitant has been used as antidepressive drug [16]. Thus, for example, it has been indicated that the pathogenesis of depression could be due to an alteration in the SP/NK-1 receptor system and in depression an increase in SP production has been observed [16]. All these data suggest that depression could induce tumor cell proliferation by activating the SP/NK-1 receptor system and that treatment with NK-1 receptor antagonists could be useful not only for the depression, but also for the treatment of tumor cells. Moreover, it is also known that SP is expressed in the limbic system (e.g., hypothalamus, amygdala) of the central nervous system (CNS), and this system has been implicated in emotional behavior. Thus, these brain regions could regulate both the progression of cancer, since all the above data indicate that emotional behaviour (e.g., depression...) [16] and cancer might be related through alterations in the SP/NK-1 receptor system. Indeed, the use of NK-1 receptor antagonist aprepitant improve both depression and tumor proliferation by blocking NK-1 receptors expressed in tumor cells and limbic system of the CNS. Moreover, aprepitant was also used in a placebo-controlled trial in patients with depression, showing that the safety and the tolerability of this drug were generally similar to placebo [16]. Indeed, we demonstrated here the safety of NK-1 receptor antagonist aprepitant against HEK 293 cells, the IC_{50} for HEK 293 was > 90 μM three times more than IC_{50}

all tumor cells approximately and higher than IC_{100} of all tumor cells studied (see Table 1).

Thus, In the 21st century, the era of "molecularly targeted" anticancer therapy, the Paul Ehrlich's concept of the "Magic Bullets" for cancer cells, the NK-1 receptor antagonists are a new and promising antineoplastic agents, and could be considered as new magic bullets called "Intelligent Bullets" concept goes beyond because they are to attack the tumor cells invaders antitumor action, but in addition, in the host they present/display beneficial effects such as: anti-inflammatory [18], analgesic [15], anxyolitic [17], antidepressant [16], antiemetic [23], hepatoprotector [18], and neuroprotector [33].

In summary, we have reported for the first time the antitumor action of the NK-1 receptor antagonist aprepitant against human glioma, neuroblastoma, retinoblastoma, pancreas, larynx, gastric and colon carcinoma cell lines. We also demonstrated that the antitumor action of aprepitant is trough the NK-1 receptor and finally that aprepitant induces apoptosis in such tumor cell lines. All these observations suggest that the NK-1 receptor aprepitant could be a new and promising broad spectrum antitumor drug in the treatment of human cancer and that the NK-1 receptor antagonists aprepitant could improve cancer treatment.

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