Antitumoral Action of the Neurokinin-1-Receptor Antagonist L-733,060 and Mitogenic Action of Substance P on Human Retinoblastoma Cell Lines

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Purpose. Activation of the neurokinin-1 receptor is known to induce proliferation in tumor cells, but it is as yet unknown whether this applies to retinoblastoma. This was an in vitro study of the growth inhibitory capacity of the potent and long-acting neurokinin-1 receptor antagonist L-733,060, at concentrations ranging from 7.5 to 20 μM, against the human retinoblastoma line WERI-Rb-1 and from 10 to 25 μM against the human retinoblastoma line Y-79. The ability of substance P (an neurokinin-1 stimulator) to activate the cell growth of these retinoblastoma cell lines was also determined.

Methods. A cell counter was used to determine the number of viable cells, followed by application of the tetrazolium compound WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4 nitrophenyl))-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, colorimetric method to evaluate cell viability in this cytotoxicity assay.

Results. Nanomolar concentrations of substance P increased the growth of both cell lines and micromolar concentrations of L-733,060 inhibited the growth of the two cell lines studied, with and without previous administration of substance P. L-733,060 inhibited the growth of the WERI-Rb-1 and Y-79 cell lines in a dose-dependent manner. IC_{50} was 12.15 μM for 49 hours for WERI-Rb1 and 17.38 μM for 40 hours for Y-79.

Conclusions. The findings demonstrate that substance P is a mitogen and also indicate that the neurokinin-1 receptor antagonist L-733,060 acts on both human retinoblastoma cell lines as an antitumoral agent. (*Invest Ophthalmol Vis Sci.* 2005; 46:2567–2570) DOI:10.1167/iovs.04-1530

Retinoblastoma arises from the photoreceptor elements, poorly differentiated neuroblasts, of the inner layer of the retina.[1] This tumor comprises 3% of tumors recorded in children younger than the age of 15 years and represents one of the most common malignant tumors of childhood, with an incidence of 1 in 15,000 live births and an increased frequency in patients with a positive family history of retinoblastoma.[2,3] This malignancy leads to metastatic disease and death in 50% of children worldwide but in less than 5% of children in developed nations with advanced medical care.[4] Several new methods are currently being developed to improve the success rates of eye preservation (60%) with useful visual functions. It is expected that in the near future newer and more effective anticancer drugs that are less toxic to normal tissues will become available for the treatment of retinoblastoma.[5]

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-γ are derived from the preprotachykinin (PPT-1) A gene, whereas neurokinin B (NKB) is derived from the preprotachykinin (PPT-2) B gene. The biological action 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 NK1 receptor, SP regulates many biological functions,[6–8] and this neuropeptide has also been implicated in neurogenic inflammation, pain, and depression.[9] Moreover, SP is known to have a widespread distribution in both the central and peripheral nervous systems and the undecapeptide is released from primary sensory nerve fibers. It is also known that activation of the NK1 receptor induces mitogenesis in several tumoral cells.[10–13] Moreover, SP immunoreactivity has been demonstrated, using an indirect immunofluorescence technique, in retinoblastoma cells.[14]

L-733,060 is a selective, potent, and long-acting central nonpeptide tachykinin NK1 receptor antagonist showing high affinity for the human NK1 receptor in vitro.[15] It is known that administration of L-733,060 produces analgesia[15] and antidepressive effects.[16,17] Furthermore, L-733,060 has been used in the treatment of a broad range of anxiety and mood disorders[18] and in inflammatory liver disease; its action is most probably due to an inhibition of the effects of SP.[19] In addition, we have recently reported that L-733,060 shows antitumoral activity against the human neuroblastoma, glioma, and melanoma cell lines.[13,20]

Several SP antagonists have been shown, in vitro and in vivo, to inhibit the growth of both small cell lung cancer and glioma.[21–25] However, to our knowledge, no study has been performed on the effect of the potent and long-acting NK1 receptor antagonist L-733,060 on human retinoblastoma cell lines (WERI-Rb-1, Y-79). It is also unknown whether SP exerts a mitogenic action on these tumor cell lines. Thus, the purpose of this study was to demonstrate, using a WST-8 colorimetric method to evaluate cell viability, the antitumoral action of the NK1 receptor antagonist L-733,060. A further intent was to study the role of SP on the induction of retinoblastoma WERI-Rb-1 and Y-79 cell proliferation.

Materials and Methods

Cell Cultures

We used the human retinoblastoma cell lines WERI-RB-1 and Y-79 (DSMZ [Deutsche Sammlung von Mikroorganismen und Zellkulturen], Braunschweig, Germany). These cell lines were maintained in RPMI 1640 (Invitrogen-Gibco, Barcelona, Spain) supplemented with 10% and

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20% heat-inactivated fetal bovine serum respectively according to the culture conditions suggested by the manufacturer. Cell lines were subcultured in 75-cm^2 tissue culture flasks (Falcon, Heidelberg, Germany) and the medium was renewed once a week. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5%CO_2.

Drug Treatments

The NK1 receptor antagonist (2*S*,3*S*)3-([3,5-bis (trifluoromethyl) phenyl]methoxy)-2-phenylpiperidine, MW 438.9 (L-733,060; Sigma-Aldrich, Madrid, Spain) was dissolved in distilled water containing 0.2% dimethylsulfoxide (DMSO) before treatment of the samples. To determine the 50% inhibition concentration (IC_{50}), different concentrations (7.5, 10, 15 and 20 μM for WERI-RB-1; 10, 15, 20 and 25 μM for Y-79) of L-733,060 were evaluated. SP acetate salt (Sigma-Aldrich, Madrid, Spain) was dissolved in distilled water. To determine SP-induced cell proliferation, different concentrations of SP (10, 50, and 100 nM) were evaluated. The most effective SP concentration for each cell line was incubated 1 hour before the addition of L-733,060.

Proliferation Assays

Cell proliferation was evaluated using the tetrazolium compound WST-8 ([2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl))-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) according to the manufacturer's instructions (Cell Counting Kit-8 [CCK-8]; Dojindo Laboratories, Kumamoto, Japan). At the time of the assay, cells cultured for 4 to 5 days were harvested by trypsinization, and cell viability was evaluated by trypan blue exclusion. Cells were quantified (Coulter counter; Beckman-Coulter, Fullerton, CA) and 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 treated with L-733,060, and control wells treated with different concentrations of SP (with or without L-733,060). The plates were inoculated with L-733,060 (7.5, 10, 15, and 20 μM for WERI-RB-1 and 10, 15, 20, and 25 μM for Y-79) and were incubated for 49 and 40 hours, respectively. The plates were also inoculated with SP (10, 50, and 100 nM), with (10 μM: WERI-RB-1 and 15 μM: Y-79) and without L-733,060 for their first doubling times (49 or 40 hours). For the proliferation assays, 10 μL of the CCK-8 reagent was added to each well 90 minutes before reading the samples on a multiscanner microplate reader (Multiskan Spectrum, Thermo Labsystems, Barcelona, Spain) at 450 nm. The quantity of product, as measured by optical density, is directly proportional to the number of living cells. Each experimental condition (blank wells, control wells, and control wells treated with different concentrations of L-733,060 or SP) was assayed in duplicate, and all experiments were performed at least three times. The IC_{50} of L-733,060 was calculated with a curve-fitting parameter.

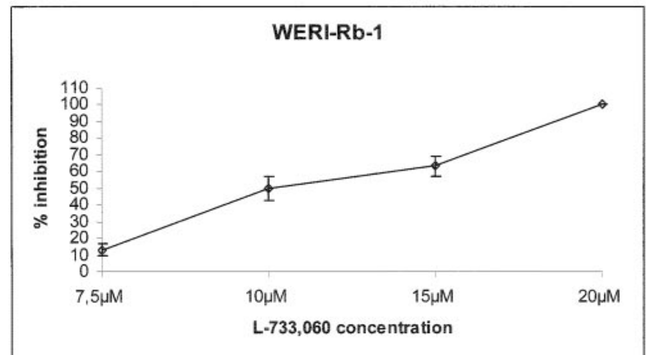
Statistical Analyses

The data were evaluated statistically using Student's *t*-test, taking $P \leq 0.05$ or ≤ 0.01 as the level of significance.

Results

Growth inhibition of the WERI-Rb-1 and Y-79 retinoblastoma cell lines by L-733,060 was observed after the addition of increasing concentrations of L-733,060 (Figs. 1A, 2A). Moreover, treatment of both cell lines with L-733,060 resulted in a concentration-dependent cytotoxicity (Figs. 1A, 2A). Thus, the concentrations required for a 50% reduction in optical density (IC_{50}) observed in the control cultures treated with L-733,060 were 12.15 μM for 49 hours for WERI-Rb1 and 17.38 μM for 40 hours for Y-79. Maximum inhibition was observed when the drug was present at a concentration of 20 (WERI-Rb-1) and 25 (Y-79) μM during the culture periods. At the first doubling time, a strong decrease in the number of the two lines studied was found at intermediate concentrations and no remaining

A



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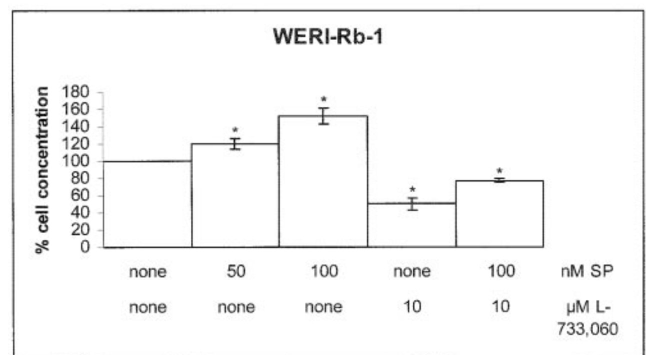

FIGURE 1. (**A**) Percentage of growth inhibition of WERI-Rb-1 cells at 49 hours in in vitro cultures after the addition of increasing concentrations (7, 5, 10, 15, or 20 μM) of L-733,060. The percentage of inhibition for the first doubling time of incubation is plotted on a linear graph. Values are the means ± SD (bars). (**B**) Induction of cell proliferation of WERI-Rb-1 cells by SP at several nanomolar concentrations (10, 50, and 100 nM). The NK1 receptor antagonist L-733,060 was added (10 μM) in the presence (100 nM) or absence (none) of SP for 49 hours. In both cases, L-733,060 inhibited WERI-Rb-1 cell proliferation. Student's *t*-test showed a significant difference between each group (10-none, 50-none, 100-none, none-10, 100 to 10) and the control group (none-none) was found. $^*P \leq 0.01$.

living cells were observed at the maximum concentrations. A lower inhibition of growth of the two lines was observed in the presence of low dosages of L-733,060 for each line. The figures also show that the standard deviations in the two cell lines studied were small, pointing to total agreement among the results obtained at the three times the experiments were performed.

Growth of the WERI-Rb-1 and Y-79 cell lines was observed after the addition of SP, and it was observed that certain nanomolar concentrations of SP induced cell proliferation compared with the control cells (Figs. 1B, 2B). SP stimulation was evident at 10 nM, the maximum level being reached at 100 nM for WERI-Rb-1 and Y-79 (Figs. 1B, 2B). This indicates that the activation of SP receptors leads to mitogenesis in the WERI-Rb-1 and Y-79 human cancer cell lines. Thus, the percentage of cell proliferation of both cellular lines increased from 3% to 50% in WERI-Rb-1 and from 12% to 35% in Y-79, depending on the dose of SP administered (Figs. 1B, 2B).

Treatment with L-733,060 at 10 μM for WERI-Rb-1 and 15 μM for Y-79 partially inhibited the growth of both cell lines (Figs. 1B, 2B). To explore whether the NK1 receptor antago-

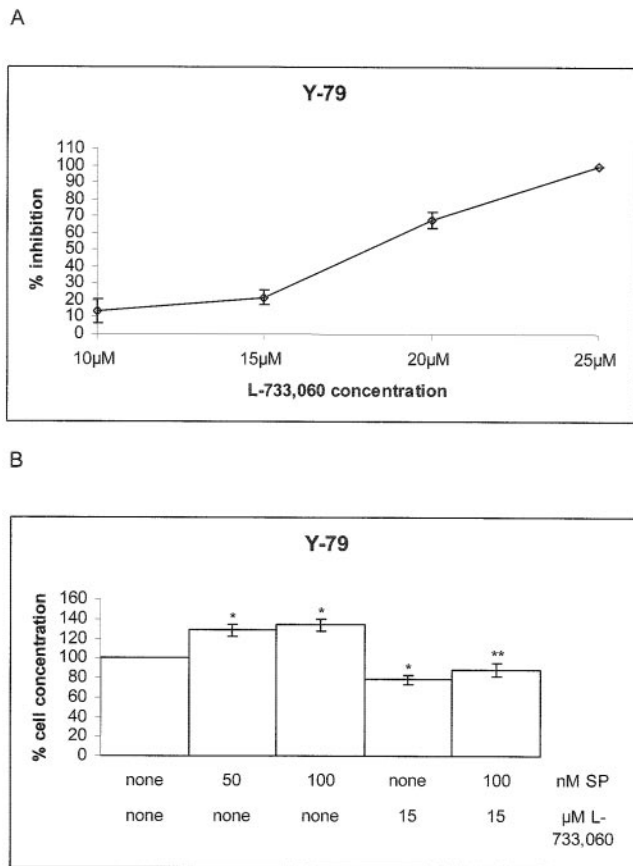

FIGURE 2. (**A**) Percentage of growth inhibition of Y-79 cells at 40 hours in in vitro cultures after the addition of increasing concentrations (10, 15, 20, or 25 μM) of L-733,060. The percentage of inhibition for the first doubling time of incubation is plotted on a linear graph. Values are the mean ± SD (bars). (**B**) Induction of cell proliferation of Y-79 cells by SP at several nanomolar concentrations (10, 50, or 100 nM). The NK1 receptor antagonist L-733,060 was added (15 μM) in the presence (100 nM) or absence (none) of SP for 40 hours. In both cases, L-733,060 inhibited Y-79 cell proliferation. Using Student's *t*-test, a significant difference between each group (10-none, 50-none, 100-none, none-15, 100 to 15) and the control group (none-none) was found. Level of significance: * $P \leq 0.01$; ** $P \leq 0.05$.

nist L-733,060 inhibited cell proliferation through an interaction with its own receptor, we used the specific NK1 receptor agonist SP in competition experiments. Thus, the cellular concentration at 10 (WERI-Rb-1) and 15 (Y-79) μM of L-733,060 and 100 nM (WERI-Rb-1 and Y-79) of SP was higher than that observed with L-733,060 alone for WERI-Rb-1 (Fig. 1B) and Y-79 (Fig. 2B). These results indicate that L-733,060 blocks SP mitogen stimulation, since L-733,060-induced growth inhibition was partially reversed by the administration of a nanomolar dose of exogenous SP.

DISCUSSION

We have demonstrated in vitro for the first time a potent growth inhibition on the human retinoblastoma WERI-Rb-1 and Y-79 cell lines after administration of the nonpeptide NK1 receptor antagonist L-733,060. This is in agreement with previous studies, in which the use of SP antagonists other than L-733,060 inhibited, both in vitro and in vivo, the growth of small-cell lung cancer.[21–24] Our findings are also in agreement with recent reports showing that L-733,060 exerts antitumoral activity against other human cancer cell lines, such as neuroblastoma, glioma, and melanoma.[13,20] It should be noted that in cell lines as different as human neuroblastoma, glioma, melanoma, and retinoblastoma the same NK1 receptor antagonist (L-733,060) elicits growth inhibition. This observation suggests the possibility of a common mechanism for cancer cell proliferation mediated by SP and NK1 receptors. Were this so, it would mean that NK1 receptor antagonists (e.g., L-733,060) could inhibit a large number of tumor cell types in which both SP and NK1 receptors are expressed,[14,26–29] and could be a candidate as a broad-spectrum antineoplastic drug.

We have demonstrated that treatment of the retinoblastoma cell lines with L-733,060 produces growth inhibition and cell death. Currently, we do not know whether the cell death observed here was due to a general toxic effect of L-733,060 or to a specific action of this substance. Despite this, there are data suggesting the second possibility. Thus, the action on these cell lines by the NK1 receptor antagonist L-733,060 is probably related to the ability of this antagonist to block the NK1 receptor in such tumors. The blockade of NK1 receptors in both retinoblastoma cell lines by L-733,060 could inhibit both DNA synthesis and cell proliferation through the mitogen-activated protein kinase (MAPK) pathway.[10] L-733,060 could induce retinoblastoma cell death by apoptosis, as has been reported previously in vitro in lung cancer cell lines.[23] In this sense, it is known that an SP antagonist (other than L-733,060) induces apoptosis in lung cancer and causes a concentration-dependent loss of cell viability,[23] as we observed using L-733,060, since the antitumoral activity of L-733,060 reported herein was dose-dependent. These data therefore suggest that the antiproliferative action of the NK1 receptor antagonist L-733,060 could involve a signal transduction pathway for apoptosis. However, further studies in this field are necessary to know whether the cell death observed in our study was due to a general toxic effect or to a specific action associated with the ability of the L-733,060 to block NK1 receptor.

All the above data suggest that treatment with antagonists of the NK1 receptor in cancer cell lines expressing the NK1 receptor could improve cancer treatment. In this sense, it would be very interesting to know whether the treatment of cancer cell lines with the NK1 receptor inhibitors that have been studied in humans[30] might produce the same growth-inhibiting action that we found with L-733,060. At present, there are more than 30 compounds that act as NK1 receptor antagonists.[31]

We also demonstrated for the first time that SP increases the growth of retinoblastoma cell lines. This is consistent with the findings of previous studies, since it has been reported that the activation of NK1 receptors by SP induces mitogenesis in several cancer cell types[10–13] and that the NK1 receptor is overexpressed in breast cancer and metastasis.[28] Moreover, substance P-immunoreactivity has been observed in retinoblastoma cells[14] and NK1 receptors have been described in the retina.[32]

Finally, our results indicate that L-733,060 blocks SP mitogen stimulation, since L-733,060-induced growth inhibition was partially reversed by the administration of exogenous SP. This observation suggests that the NK1 receptor may play an important role in the growth of the retinoblastoma cell lines studied (Figs. 1B, 2B), since after treatment with SP and L-733,060 increases in the cellular concentration (27.36% and 9.65%) were observed respectively in the WERI-Rb-1 and Y-79 cell lines (Figs. 1B, 2B) with respect to the results when the antagonist was administered alone. However, in the future, more studies should be performed to demonstrate definitively that the antitumoral action of L-733,060 on both retinoblastoma cell lines is through the NK1 receptor.

Summarizing our results, the antitumoral activity of the NK1 receptor antagonist L-733,060 on retinoblastoma human cell

lines is described for the first time. In addition, we demonstrate that SP is a mitogen of both cancer cell lines studied. These in vitro observations suggest that the inhibition of the SP/NK1 receptor system may be a useful novel treatment for the management of retinoblastoma disease.

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