Original Article

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Anti-Cancer Effect of Aprepitant on Nb4 Leukemic Cells

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ABSTRACT

BACKGROUND AND OBJECTIVE: Genetic studies have demonstrated that the neurokini-1 Receptor (NK1R)is frequently involved in the pathogenesis of wide assortment of human malignancies, including acute promyelocytic leukemia (APL). The activity of this pathway in leukemic cells results in an excessive cell proliferation and evade from apoptosis. In this study, we aimed to investigate the effect of Aprepitant (NK1R antagonist) on the survival rate of APL cells.

METHODS: This experimental study is conducted on APL-derived NB4 cells (Institute Pasteur). To determine the anti-tumor effect of Aprepitant, NB4 cells were divided into 6 groups: control and 1-, 2-, 3-, 4- and 5 µM-drug treated groups. Then the cell viability, metabolic activity, induction of apoptosis and transcriptional alteration of Bax and Bcl-2 genes were investigated after 24 and 36 h treatment using trypan blue assay, MTT assay, Annexin-V/PI staining and RQ-PCR analysis, respectively.

FINDINGS: 36 h treatment with the highest concentration of Aprepitant (5 µM) resulted in an approximately 50% reduction in the viability (assessed by trypan blue) and metabolic activity (assessed by MTT assay) of NB4 cells ($p<0.001$) in comparison with control group. Moreover, Aprepitant is able to increase the proportion apoptotic cells from 1.4% in control group to 10.6% in 5 µM drug-treated cells though up-regulating Bax/Bcl-2 molecular ratio ($p\leq0.05$).

CONCLUSION: Aprepitant exerted both cytotoxic and anti-proliferative effects in NB4 cells.

KEY WORDS: *Acute promyelocytic leukemia, Apoptosis, Aprepitant, NB4 cell line*.

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Introduction

Acute Promyelocytic Leukemia (APL) is one of the most recognized types of hematologic malignancies (1). Although laboratory tests have identified a wide range of genetic abnormalities associated with the disease (2), efforts to find mechanisms involved in the pathogenesis of the disease and the occurrence of resistance to chemotherapy drugs are continuing (3). New studies in the field of neuroscience have defined a new role for neurokinin-1 receptor (NK1R) pathway (Neurokinin1 receptor) and its ligand Substance P in the pathogenesis of cancers (4).

Studies have shown that the addition of Substance P to the neurokinin receptor 1, in addition to adjusting many of the physiological effects associated with the central and peripheral nervous system, is able to target a wide range of subcellular transduction molecules and pathways such as phosphatidylinositide 3- kinases (PI3K) and MAPK mitogen-activated protein kinases) playing a role in the pathogenesis of cancers (6, 5). In addition, many studies on the types of human malignancies, including gilloblastoma, retinoblastoma and pancreatic, clon, lung and leukemia cancers, have explicitly referred to the link between this pathway and the pathogenesis of cancers (7-9).

On the other hand, studies on myeloid malignant blasts have shown that NK1R expression is significantly increased compared to normal blasts, which itself confirms the effect of the NK1R/SP pathway on acute myeloid leukemia (10). Currently, the use of many NK1R inhibitors in the treatment of human malignancies has been heavily taken into account. Aprepitant is one of the most important NK1R inhibitors with very high affinity, which is currently used as an antimycotic and vomiting drug along with many chemotherapeutic drugs (11). The lack of toxicity of this inhibitor has been confirmed by the FDA and studies have shown that the drug also has anticonvulsant, anti-migraine and anti-inflammatory effects in preventing chemotherapy-induced nausea and vomiting. (13, 12).

Studies on cancer cells have shown that Aprepitant can prevent the proliferation of cancer cells and can also induce apoptosis in these cells (14). This drug is capable of inhibiting the adverse effects of NK1R/SP on cell migration, proliferation and inhibition of apoptosis (15).

Studies have shown that inhibition of this pathway by NK1R antagonists in cancers such as breast (16), hepatoblastoma (17) and melanoma (18) can be effective and promising. The aim of this study was to evaluate the cytotoxic and antiproliferative effects of the appritant drug on the APL-derived cell line.

Methods

Cell culture and treatment: In this experimental study, NB4 cells (derived from acute promyelocytic leukemia) (Pasteur Institute) cultured in RPMI 1640 medium with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. They were incubated at 37 ° C and 5% carbon dioxide. To ensure the accuracy of NB4 cells, the mRNA of the PML / RARα gene was evaluated using RT-PCR technique for this category. Treatment of NB4 cells with different concentrations of Aprepitant (1-5 µm) at 24 and 36 hours was performed. To prevent the effects of solvent on the amount of proliferation and survival of the cell line, the cells were treated with negative concentrations of DMSO as negative control. All experiments were performed triplicate to increase the accuracy of the work.

Evaluation of cell absorption by using trypan blue assay: In order to determine the effect of Aprepitant on the cell survival, 4.5×105 cells/ml NB4 cells were incubated in the presence of various doses of drugs for 24 and 36 hours. Then, the treated cells were then mixed at a predetermined time with a ratio of 1: 1 with the essential color of trypianblu 0.4% and the number of cells was counted using a microscope. Then, using the following formula, the viability of the cells was calculated.

Viability (%)=Number of live cells/ Total number of cells × (100)

Evaluation of metabolic activity of cells with MTT Assay: To evaluate the effect of Aprepitant on the metabolic activity of NB4 cells, 5000 cells per well of 96 well plates containing (1-5 µm) and no additive for 24 36 hours were stored in the incubator. After the desired time, MTT 5 mg/ml (Sigma, USA) was added to the wells and placed in 37 C-incubator for 3 hours. Then the plate was centrifuged with round of 1000 g for 10 minutes and after adding 100 µl of DMSO to each well, optical absorption of each well was read by ELISA reader at 570 nm.

Study of apoptosis index using flow cytometry: To evaluate the effect of drug on programmed cell death, 5×105 cells were poured into 12 wells and were treated with Aprepitant for 36 hours at different concentrations.

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After washing the cells with phosphate-saline buffer (PBS) and adding AddinV-FITC (Roche Applied Science, Germany), PI (Roche Applied Science, Germany) and incubation buffer, samples for were incubated 15 minutes in the dark and at room temperature. Cells were evaluated using a flow cytometer (PartecPasIII, Germany) with an excitation wavelength of 488 nm and a reflection of 518 nm. Data analysis was performed by FloMax 2.3 software.

RNA extraction and cDNA synthesis: After treatment with 5 µM Aprepitant for 36 hours, RNA was extracted from the treated cells as well as control samples using trizol. The quantity and purity of the extracted RNA were examined by spectrophotometric method using the NanodropND2000 machine. A reverse transcription reaction was used from the cDNA synthesis kits (TAKARA, Japan). To synthesize cDNA according to the brochure, the kit was incubated for 15 minutes at 37 ° C and 15 seconds at 85 ° C.

Quantitative analysis of Bax and Bcl-2 genes involved in apoptosis: For quantitative analysis of the expression of Bax and Bcl-2 genes RT-PCR assay was performed. For each reaction, 10 µM SYBR green master mix (Amplicon), 2 µm cDNA, 0.5 µm of each primer (10 µmolar) and 7 µm of nuclease-dispersed water were used. The temperature conditions used include an initial activation step at 95 ° C for 30 seconds, followed by 40 cycles for 5 seconds, 95 ° C, and a connecting / rearranging step (20 seconds, 60 °C). To evaluate the product specificity, the melting curve was evaluated; also the relative calculation of the copy number of mRNA multiplied by the formula - ΔΔct 2 was calculated. The sequence of primers used to perform the test was shown in Table 1.

Statistical analysis: All experiments were performed as three independent tests and reported as Mean ± SD values. T-test and SPSS 21 and GraphPad Prism7 software were used for statistical analysis.

Table 1. Primer sequence used in the Real Time Quantitative RT-PCR Assay.

Size(bp)	Forward primer(5'-3')	Reverse primer(5'-3')	Accession number	Gene
111	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA	NM_000194	HPRT
242	CGAGAGGTCTTTTTCCGAGTG	GTGGGCGTCCCAAAGTAGG	NM_138761	Bax
249	CGGTGGGGTCATGTGTGTG	CGGTTCAGGTACTCAGTCATCC	NM_000633	Bcl-2

Results

The Aprepitant resulted in reduction of the survival rate of NB4 cells in a dose and time dependent manner. The results showed that with increasing dosage and time, the absorption of trypane-bubble in NB4 cells increased, indicating a decrease in the vitality of the cells in the face of the drug. The results indicated that despite the fact that doses of 1 and 2 µM of Aprepitant do not significantly affect the survival of NB4 cells, treatment of cells with higher doses of this inhibitor (3, 4 and 5 µM) and within 36 hours, decreased the viability of the cells by 36, 52 and 60%, respectively ($p<0.05$) (Fig 1).

Treatment of NB4 cells with Aprepitant leads to a decrease in proliferation of cells depending on dose and time: Compared to the control group, the treatment of cells with different doses of the Aprepitant caused meaningful reduction of the number of living cells. In confirmation with the results obtained in the study of cell survival, as doses of 1 and 2 µM of the NK1R inhibitor are not able to exert anti-proliferative effects on the NB4 cell line, the treatment of cells with higher doses of this inhibitor prevent from proliferation of cells, so that the Aprepitant at 5 µm dose in 36 hours gives the most inhibitory effect on NB4 cells ($p<0.001$) (Fig 2).

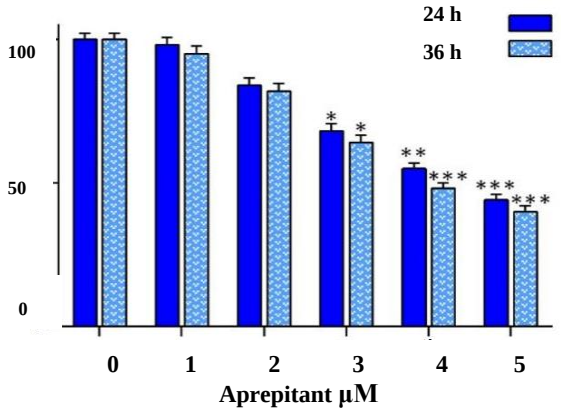

Figure 1. Effect of Aprepitant on the survival rate of NB4 cells. Cells were treated with different doses of Aprepitant and then the cell viability was evaluated by trypan blue test. The mean and standard deviation of the results from the three different work (Mean±SD) were calculated and the obtained p-value ($p<0.05$, $p<0.01$, $p<0.001$) was statistically significant compared to the control sample.

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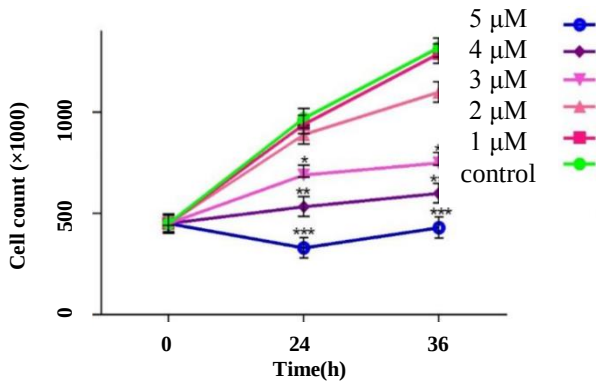

Figure 2. Effect of Aprepitant on cell count in NB4 cells as dose-dependent and time-dependent. The number of 4.5×105 NB4 cells were treated with 1 to 5 µm concentrations of Aprepitant. The mean and standard deviation of the results from the three different work (Mean±SD) were calculated and the obtained p-value (p<0.05, p<0.01, p<0.001) was statistically significant compared to the control sample.

The Aprepitant decreases the metabolic activity of NB4 cells depending on the dose and time. The results of the MTT assay showed that the inhibition of NK1R by Aprepitant resulted in decrease of dose and time -dependent metabolic activity of NB4 cells. The treatment of the cells with the highest dose (5 µM) reduced the metabolic activity after approximately 24 hours (about 50%) (p<0.001) (Fig 3). In addition, the inhibitory effect is more pronounced over time, so that after 36 hours of treatment with 5 µM cells of this inhibitor, the metabolic activity of NB4 cells decreases by 58%. (p<0.001) (Fig 3).

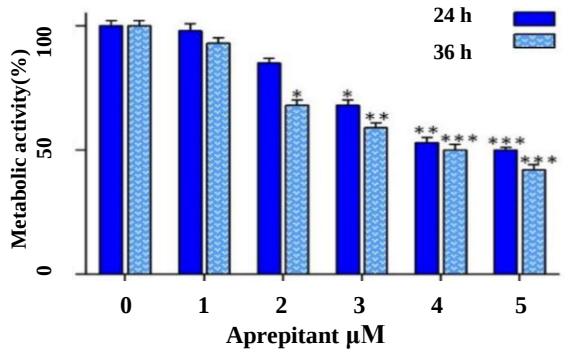

Figure 3. Investigating the Effect of Aprepitant on metabolic activity of NB4 cells. Mean and standard deviation of the results of three different work (Mean±SD) were calculated and the obtained p-value (p<0.05, p<0.01, p<0.001) showed statistically significant results compared with the control sample.

The Aprepitant results in induction of apoptosis in NB4 cells: flow cytometric results suggest that the Aprepitant significantly induces apoptosis in NB4 cells. This drug can increase the percentage of V/PI positive anxin cells at 5µm dose (10µg/dl) (p≤0.01) (Fig 4).

Increase of Bax expression and decrease of Bcl-2 expression following NB4 treatment with Aprepitant: Results showed that treatment of NB4 cells with Aprepitant was associated with increased expression of Bax and decreased expression of Bcl-2 (Fig 4). Bax/Bcl-2 ratio was increased in cells exposed to 5 µm in comparison with control cells due to increased expression of Bax gene and reduction of Bcl-2 expression, which has shown itself by increasing apoptosis (Fig 5).

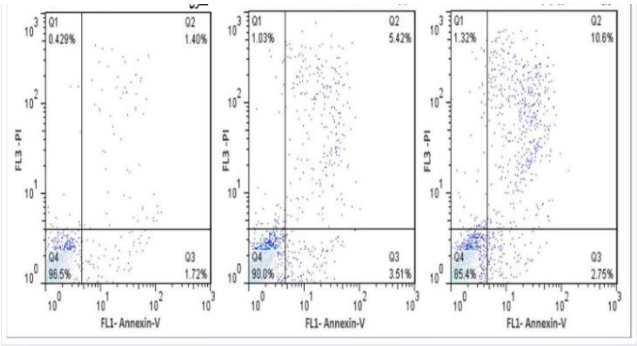

Figure 4. Estimation of the percentage of apoptosis cells after treatment with different doses of Aprepitant. Treatment of NB4 cells with this NK1R inhibitor increases the percentage of V / PI positive anexin cells.

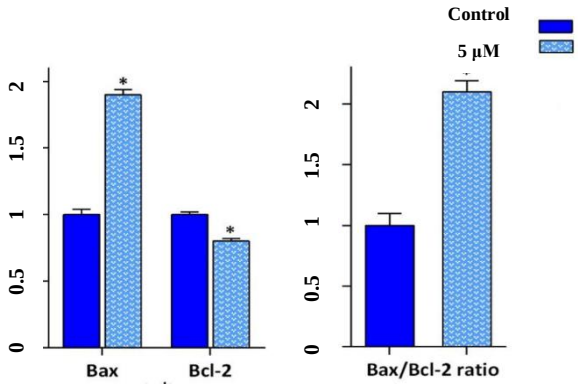

Figure 5. Increasing the transcriptional activity of the Bax propaoptotic gene as well as decreasing the level of mRNA of the Bcl-2 anti-apoptotic gene during 36-hour treatment of NB4 cells with a dose of 5 µm of Aprepitant. The mean and standard deviation of the results from three different processes (Mean±SD) were calculated and the obtained p-value (*p<0.05) indicated significant statistical results compared to the control sample.

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Discussion

The results of this study indicate that treatment of NB4 cells with NK1R inhibitors leads to a decrease in the survival, proliferation, and metabolic activity of the cells, depending on the dose and time. In spite of the absence of significant lethal effects in low doses and short intervals, with increasing dose and time of cells encountering with Aprepitant, the effect of drug significantly increases, so that 36-hour treatment with 5 µm dose of this inhibitor was able to reduce the survival of NB4 cells by more than 50%. Similar to the results, Munoz et al. also found that the NK1R inhibitor was able to reduce the mitogenic effect of substance P on the Hep-G2 cell line, thus preventing the proliferation and survival of these cells 19).

In another study on melanoma cells, it was found that Aprepitant at concentrations between 10 and 60 µm can decrease the growth rate of the myeloma cells (18). From this comparison, it can be seen that the effect of this drug on APL cells is greater than that of melanoma cells, which is probably due to the different nature of these two cells and their differences in NK1R expression rates. On the other hand, two other studies that have taken place on different cell lines have shown that even high concentrations of Aprepitant have no deleterious effect on normal cells. Berger and colleagues showed that despite high concentrations of Aprepitant is capable of inducing sell death in malignant cells, the drug cannot induce cell death in fibroblast cells up to a concentration of 40 µm (17). In another study by Munoz and colleagues, it was also reported that Aprepitant at concentrations greater than 90 µm can reduce the survival rate of normal embryonic kidney cells (20).

From the results of both studies, it can be deduced that the concentration of the Aprepitant used in this study has no deleterious effect on healthy cells. As a result, it would be hoped that the results of the current study, due to the effect on tumor cells, could be used after further examination in clinical trials. Various mechanisms have been proposed in the pathogenesis of cancers, among which escape from apoptosis is one of the most important mechanisms.

Disrupting the equilibrium of the expression of apoptotic precursor proteins and inhibitors of this phenomenon by increasing the ratio of the expression of the anti-apoptotic genes to the pro-apoptotic genes is one of the most important and major way of escaping apoptosis (22, 21). Studies have shown that excessive activity of the NK1R pathway in cancer cells can prevent apoptosis by influencing and activating a wide range of molecules and substrates (23). In this study, the treatment of cells derived from leukemia-activated promyelocyte leukemia with an NK1R antagonist Aprepitant not only induced apoptosis in this cell line, but also increased the transcriptional activity of pro- and anti-apoptotic genes of the Bcl-2 family, altered the balance of proteins involved in the apoptosis process, and thus applied its cytotoxic effect to NB4 cells.

In this regard, another study has shown that inhibition of the pathway of NK1R by Aprepitant induces apoptosis by increasing expression of pro-apoptotic genes in acute lymphoblastic leukemia cells (24). Similar findings are found in solid tumor cells in breast cancer. Munoz and colleagues showed that Aprepitant could also induce apoptosis in breast cancer cells (25). The results of this study indicated that Aprepitant probably induces apoptosis in APL cells by altering the transcriptional activity of pro- and anti-apoptotic genes of the Bcl-2 family, thereby decreases the survival and proliferation of these cells.

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