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A Comparative Study of the Antiproliferative and Apoptotic Effects of Some Chemotherapeutic Drugs on Neuroblastoma Cells

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Abstract
In this study, it was aimed to investigate the antiproliferative and apoptotic effects of nivolumab, cetuximab and gemcitabine used in the treatment of different cancer types as well as cisplatin and cyclophosphamide used in the treatment of neuroblastoma on SH-SY5Y neuroblastoma cells. The effect of each chemotherapeutic on cell viability and the individual half-maximal inhibitory concentration (IC50) values were determined by the crystal violet method. To determine their apoptotic effects, RT-PCR and Annexin V-FITC apoptosis detection technique were used. The results indicated that all the used chemotherapeutic drugs showed dose-dependent cytotoxic effects and induced apoptosis in SH-SY5Y cells. The IC50 values of cisplatin, cyclophosphamide, nivolumab, cetuximab, and gemcitabine were calculated as 10.91 µM, 0.54 µM, 30.26 µM 4.74 µM and 0.036 µM, respectively. After IC50 dose treatment of cisplatin, cyclophosphamide, nivolumab, cetuximab, and gemcitabine, apoptotic cell rates were found as 21%, 12%, 16%, 10% and 39% respectively. It was determined that statistically significant changes in mRNA expression levels in almost all apoptosis-related genes occurred after chemotherapeutic drugs treatment. In conclusion, gemcitabine showed more antiproliferative and apoptotic effects on neuroblastoma cells than the other chemotherapeutics. It is clear that further studies that will elucidate the mechanism of action of gemcitabine may contribute to the treatment of neuroblastoma.

1. Introduction

Neuroblastoma is an extracranial solid tumor of the autonomic nervous system that is frequently encountered in children [1]. It constitutes 7% of pediatric neoplasms and 10% of all pediatric deaths caused by cancer [2]. When evaluated in terms of incidence rate among pediatric cancers, it ranks third after leukemia and brain tumor [3].

Neuroblastoma is quite diverse, ranging from incidental tumors without symptoms to diffuse metastases with systemic signs. The biological variability of neuroblastoma causes it to exhibit various clinical behaviors with outcomes ranging from spontaneous regression or progression to metastasis and mortality despite extensive treatment [4]-[6]. The etiology of neuroblastoma is not known exactly, but the early diagnosis age and heterogeneity of the disease show that the main cause of neuroblastoma cases is that the development of irregular neural crest cells may cause tumors in the adrenal glands or sympathetic ganglia [7]-[9]. Familial neuroblastoma can generally result from mutations in various genes and has been associated with a poor prognosis [2], [10]. However, DNA methylation changes also appear to contribute to neuroblastoma biology and clinical behavior [11].

The age of the patient at the time of diagnosis, the stage of the disease, the tumor's histology, and the ploidy of the tumor cells are some of the factors used for stratifying the risk of the disease. Based on these variables and clinical and biological standards,

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patients are divided into low, medium, and high-risk groups [6], [12]. Patients in the high-risk group receive intensive, multimodal treatment including chemotherapy, surgery, immunotherapy with antibodies, radiotherapy, autologous stem cell transplantation, and myeloablative chemotherapy [6], [12], [13]. However, the disease can often exhibit a resistant picture and relapse [14], [15].

Chemotherapy for neuroblastoma usually involves a combination of drugs. Various drugs such as cyclophosphamide, cisplatin, vincristine, doxorubicin, etoposide, and topotecan are used in the treatment of the disease, but resistance to these chemotherapeutic drugs may develop [16]. Therefore, patients need new drugs to overcome chemoresistance, but drug development phases are multifaceted and complex, and there is a risk that the drug will not be successful even after many resources have been invested [17]. Developing drugs related to nervous system diseases, in particular, presents a series of difficulties that complicate the process due to the complex nature of the nervous system [18]. Therefore, there is an urgent need for *in vitro* drug screening with clinically approved drugs for the treatment of different types of cancer.

It has been established that high-risk neuroblastoma patients who develop metastatic neuroblastoma also have an immune resistance mechanism mediated by programmed death ligand 1 (PD-L1). [19]. Blocking the PD-1/PD-L axis seems important in a combined immunotherapy approach. In the treatment of neuroblastoma, nivolumab, a PD-1 inhibitor immunotherapeutic drug, stands out in terms of *in vitro* cytotoxicity. However, it is known that epidermal growth factor receptor (EGFR) expression is high in neuroblastoma tissues, suggesting that it is possible to develop treatment strategies for neuroblastoma by targeting EGFR [20]. Cetuximab is an anti-cancer agent that works by inhibiting the growth and survival of tumor cells that express EGFR. Gemcitabine, the deoxycytidine analog, works by a different mechanism than the drugs used in the treatment of neuroblastoma. In addition, it has been shown that gemcitabine is not a substrate for P-glycoprotein and some proteins associated with multidrug resistance in neuroblastoma [21].

Most of the currently used anticancer drugs direct cancer cells to apoptosis by acting on different signaling pathways. To avoid apoptosis, cells use different signal transduction pathways. A better understanding of these apoptotic signaling pathways could increase the effectiveness of cancer therapy. Therefore, in this study, it was aimed to comparatively investigate the antiproliferative and apoptotic effects of nivolumab, cetuximab, and gemcitabine used in the treatment of different cancer types as well as cisplatin and cyclophosphamide used in the treatment of neuroblastoma on SH-SY5Y neuroblastoma cells.

2. Material and Method

2.1. Cell Culture

The human neuroblastoma cell line SH-SY5Y (ATCC CRL-2266) was used in the experiments. DMEM-F12 medium supplemented with 1% antibiotic and 10% heat-inactivated fetal bovine serum (FBS) was used to grow cells at 37°C in a humidified incubator with 5% CO_2. By changing the medium every two days, cells were produced in a monolayer and grown in cell culture dishes. After the cells adhered to the culture dishes in a single layer, they were separated from the surface with Trypsin-EDTA, the cell mixture/trypan blue (1:1) was counted on the Thoma slide and made ready for cultivation [22]. All the chemicals were purchased from Sigma-Aldrich (Germany).

2.2. Determination of Cytotoxic Activity

At a density of 2×10^3 cells/ml in the culture medium, SH-SY5Y cells were seeded in 96-well plates. Cells were exposed to various chemotherapeutic drug concentrations (ranging from 0.0025 μM to 100 μM) after a 24-h incubation period prior to treatment. Cisplatin, cyclophosphamide, nivolumab, cetuximab, and gemcitabine used in the study were obtained from MCE (Sweden). Cells were incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere, either with chemotherapeutic drugs or as controls. Crystal violet solution (0.5% concentration, in 50% methanol) was added to the medium after incubation. The plates were incubated for 10 min at room temperature, washed with water, and the adsorbed dye was eluted with Na-citrate (0.1 M Na-citrate in 50 percent ethanol, pH 4.2). At 600 nm, the absorbance, a measure of cell viability, was taken. Viable cell was expressed as the percentage of viable cells compared to control cells [23].

2.3. RNA Isolation and RT-PCR

The innuPREP RNA Mini Kit 2.0 (Analytic Jena GmbH, Germany) was used to extract RNA from SH-SY5Y cells in accordance with the manufacturer's recommendations. The NanoDrop 1000 spectrophotometer (MaestroNano Micro-volume Spectrophotometer, USA) was used to measure the quantity and quality of RNA. The Easy Script cDNA

Synthesis Kit (ABM, Canada) was utilized to create cDNA. The gene-specific primer sequences used in the study are given in Table 1. RT-PCR was carried out using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Dubai) and KiloGreen 2X qPCR Master Mix (ABM). β-actin was used as a control gene [24].

Table 1. Primer sequences of human genes associated with apoptosis

Gene	Primer sequence	
ACTB	F	TCCTCCTGAGCGCAAGTACTC
	R	CTGCTTGCTGATCCACATCTG
Bax	F	AGAGGATGATTGCCGCCGT
	R	CAACCACCCTGGTCTTGGATC
Bcl-2	F	ATGTGTGTGGAGAGCGTCAACC
	R	TGAGCAGAGTCTTCAGAGACAGCC
p53	F	ATCTACAAGCAGTCACAGCACAT
	R	GTGGTACAGTCAGAGCCAACC

2.4. V/PI Staining for the Identification of Apoptotic Cells

2×10^5 cells were plated in 6-well dishes to identify apoptotic cells. After 24 h of incubation, the cells were exposed to the half-maximal inhibitory concentration (IC50) of the tested drugs. Cells were harvested after 24 h and then treated with Annexin V-EGFP Apoptosis Detection Kit (BioVision, USA) in accordance with the manufacturer's instructions. Counting of cells was done using a NanoEnTek (USA) Arthur Novel Fluorescence Cell Counter. Calculations were made to determine the proportion of necrotic and apoptotic cells relative to the total cell population. A positive control for apoptosis was hydrogen peroxide (H_2O_2) [25].

2.5. Statistical Analysis

Minitab 13 statistical software was used to conduct the statistical analysis. The mean and standard deviation (SD) of independent experimental sets were used to express all results. The value needed for statistical significance was set at $p < 0.05$ when making comparisons between groups using the Student's t-test.

3. Results and Discussion

Neuroblastoma, a clinically heterogeneous pediatric cancer of the sympathetic nervous system, is the most common childhood tumor [14]. Although neuroblastoma has a high morbidity and mortality rate, it can sometimes disappear spontaneously [6]. Therefore, it shows a heterogeneous malignancy ranging from long-term survival to a high risk of death [26]. Mortality analyzes performed in high-risk groups have shown little success despite intensive multimodal therapy [27]. This lack of success can be explained by the fact that the etiology of the disease is not fully explained and it has a significant heterogeneity in its pathophysiology [28]. The high-risk group has the worst prognosis, and the disease may metastasize to various organs [27]. Chemotherapy and immunotherapeutic drugs are used in the treatment of the disease. All these drugs show beneficial effects on neuroblastoma symptoms. However, the treatment of the disease is limited due to the side effects of these drugs and the development of resistance to the drugs used [29], [30]. Thus, it is essential to identify novel therapeutics that can be applied to the management of neuroblastoma and to investigate their outcomes. Since drug development stages are challenging and risky processes, it is important to test the usability of various clinically approved therapeutics for the treatment of neuroblastoma. In the present study, the cytotoxic and apoptotic effects of five different drugs approved by the US Food and Drug Administration (FDA), which are used in various cancer treatments, on neuroblastoma cells were comparatively investigated. Cisplatin and cyclophosphamide were used as references because these drugs are the backbone of current clinical protocols for the treatment of neuroblastoma.

Investigation of drug candidates and/or drugs on cancer cells has become the primary strategy for discovering anti-cancer agents. For this reason, the effects of these drugs, which are effective on various cancer cells, on the viability in SH-SY5Y cells were determined and IC50 values were calculated in the present study. In all chemotherapeutic drug groups, the cell viability seemed to decrease with increasing drug concentration. The IC50 values of cisplatin and cyclophosphamide used in the treatment of neuroblastoma were determined as 10.91 μM and 0.54 μM, respectively (Table 2). These values are in agreement with the ones reported by several studies in the literature. For instance, in a study conducted to investigate the intracellular mechanisms of neurotoxicity of platinum drugs, the effect of cisplatin on SH-SY5Y neuroblastoma cell line was examined, and the IC50 value of cisplatin was calculated as 15 μM [31]. In another study investigating the effect of cyclophosphamide on the cell viability and tumor progression of neuroblastoma cell line, it was shown that the IC50 value was 0.602 μM and that the drug caused antiproliferative effects [32]. Based on the IC50 value results in the present study, it can be suggested that cyclophosphamide has more toxic effect on SH-SY5Y cells compared to cisplatin.

The IC50 values of nivolumab, cetuximab, and gemcitabine, which are clinically approved for the treatment of other cancer types, were calculated as 30.26 μM, 4.74 μM, and 0.036 μM, respectively (Table 2). It was observed in a study that the inhibitory effect of nivolumab on ovarian cancer cells increased in a dose-dependent manner and that nivolumab at a concentration of 20 μM could play a synergistic antitumor role with cisplatin in ovarian cancer cells [33]. However, in the literature, there is no study regarding the antiproliferative activity of nivolumab in SH-SY5Y cells. On the other hand, in a study on the efficacies of several drugs, including cetuximab, and their combinations in eight different lung cancer cell lines with different genetic characteristics, the IC50 value of cetuximab in these cell lines was found at concentrations ranging from 0.05 μM to 12 μM [34]. In another study, the IC50 value of cetuximab in four different rhabdomyosarcoma cell lines was determined to be at concentrations ranging from 4.7 μM to 9.1 μM [35]. In a study investigating the effect of disulfiram and copper complex, which is used as a radiosensitizing anticancer agent, on cell cycle regulation, it was shown that approximately 50% of the SH-SY5Y cells treated with 40 nM gemcitabine did not survive [36]. Taken together, among the chemotherapeutic drugs tested in the present study, gemcitabine exhibited the most cytotoxic effect on neuroblastoma cells.

Apoptosis plays a strategic role in cancer treatment because one of the most important distinguishing features of cancer is avoidance of apoptosis. Mutations in various genes can occur in cancer cells. For this reason, it is important to know through which pathway chemotherapeutic drugs designed to induce apoptosis act in order to destroy cancer cells. In general, the pathway inhibited in cancer cells is the intrinsic pathway. Overexpression of Bcl-2 and loss of Bax are ways that cancer cells avoid apoptosis. In addition, these cells ensure that the tumor suppressor gene p53, which regulates Bax, is inhibited [37].

Table 2. The cytotoxic effects of five different drugs on neuroblastoma cell line (SH-SY5Y)

Drug	**IC50 (μM), mean ± SD**
Cisplatin	10.91 ± 2.23
Cyclophosphamide	0.54 ± 0.13
Nivolumab	30.26 ± 6.27
Cetuximab	4.74 ± 1.09
Gemcitabine	0.036 ± 0.009

The mRNA expressions of Bcl-2, Bax and p53 genes in SH-SY5Y neuroblastoma cells treated with cisplatin, cyclophosphamide, nivolumab, cetuximab, and gemcitabine were normalized with β-actin mRNA expression. As compared to the control group, treatment of SH-SY5Y cells with the chemotherapeutic drugs examined in the current study increased the expression of p53 mRNA; the increase in expression level was statistically significant for cisplatin, cyclophosphamide, cetuximab, and gemcitabine (4.53-fold, 4.57-fold, 3.43-fold, and 5.40-fold, respectively) but not for nivolumab (Table 3). The highest increase in p53 mRNA expression was observed after gemcitabine treatment. In contrast, all the chemotherapeutic drugs tested in the present study decreased the Bcl-2 mRNA expression compared to the control group; the decrease in expression level was statistically significant for cisplatin, cyclophosphamide, nivolumab, and gemcitabine (3.60-fold, 2.08-fold, 4.00-fold, and 3.95-fold, respectively) but not for cetuximab (Table 3). In addition, compared to the control group, the relative Bax mRNA level was statistically increased in SH-SY5Y cells treated with cisplatin, cyclophosphamide, nivolumab, cetuximab, and gemcitabine (3.07-fold, 5.58-fold, 2.23 fold, 4.47-fold, and 6.02-fold, respectively) (Table 3).

Table 3. Expression levels of Bax, Bcl-2 and p53 genes after five different drugs treatment

Drug	**Bax**	**Bcl-2**	**p53**
Cisplatin	3.07 ± 0.28*	−3.60 ± 0.03*	4.53 ± 0.83*
Cyclophosphamide	5.58 ± 0.72*	−2.08 ± 0.08*	4.57 ± 0.12*
Nivolumab	2.23 ± 0.24*	−4.00 ± 0.14*	1.94 ± 0.04
Cetuximab	4.47 ± 0.45*	−1.74 ± 0.02	3.43 ± 0.50*
Gemcitabine	6.02 ± 0.68*	−3.95 ± 0.62*	5.40 ± 0.65*

*Significantly different from respective control value for each gene ($p < 0.05$).

The percentage of apoptotic cells over the total cell population was calculated in the Novel Fluorescence Cell Counter after the neuroblastoma cells were stained with Annexin V/PI to determine whether they underwent apoptosis after being treated with drug. Apoptotic cell rates were 21%, 12%, 16%, 10%, and 39% after IC50 dose treatment of cisplatin, cyclophosphamide, nivolumab, cetuximab, and gemcitabine, respectively (Figure 1). Among all drug groups, the greatest increase in apoptosis was seen in the cells treated with gemcitabine compared to the control group.

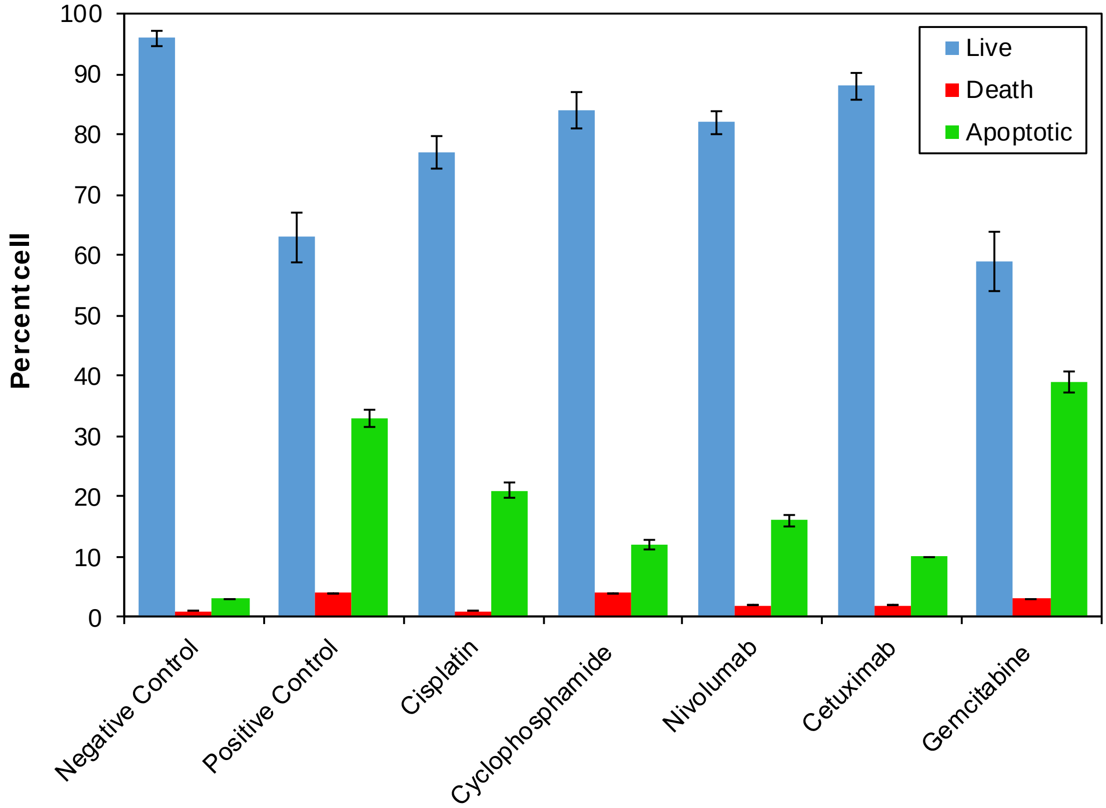

Figure 1. Apoptosis assay in SH-SY5Y cells after cisplatin, cyclophosphamide, nivolumab, cetuximab, and gemcitabine treatment. H_2O_2 was used as a positive control for cells

In a study investigating possible mechanism of cisplatin and nivolumab on platinum-resistant ovarian cancer cells, it was shown that these two drugs decreased Bcl-2 protein expression level but increased Bax protein expression level and that 50 µM cisplatin and 50 µM nivolumab induced apoptosis at a rate of 42.67% and 40.73%, respectively [33]. It was reported that p53 had an important role in cisplatin-induced apoptosis in neuroblastoma and renal tubular cells [38], [39]. Álvarez-León et al. showed that cyclophosphamide exhibited ~35% apoptotic induction and caused an increase in the apoptotic index Bax/Bcl-2 ratio, which is an indicator of caspase pathway activation in neuroblastoma cell line [32]. Moreover, gemcitabine was reported to induce p53-dependent apoptosis associated with proapoptotic proteins such as PUMA and Bax in pancreatic cancer cells [40], and cetuximab was revealed to promote apoptosis in head and neck squamous cell carcinoma cell lines [41].

4. Conclusions

Neuroblastoma, a disease with a high morbidity and mortality rate, originates from neural crest cells and is classified as an embryonal neuroendocrine tumor. Due to the serious side effects of the chemotherapeutic and immunotherapeutic drugs used in the treatment and the development of resistance to the drugs used, scientists and pharmaceutical companies have started to search for new drugs. However, due to the complex nature of the nervous system and the difficulty of drug development stages, it has revealed the necessity of *in vitro* drug screening with clinically approved drugs in the treatment of various cancer types. In conclusion, in the present study, it was determined that some cancer drugs (cisplatin, cyclophosphamide, nivolumab, cetuximab, and gemcitabine) have antiproliferative and apoptotic effects on SH-SY5Y neuroblastoma cells.

Gemcitabine was found to be more effective than the other four drugs in reducing cell viability and tending to apoptosis in neuroblastoma cells. However, it is recommended that further studies that will elucidate the mechanism of action of gemcitabine are necessary to contribute to the treatment of neuroblastoma.

Conflict of Interest Statement

There is no conflict of interest regarding the study.

Statement of Research and Publication Ethics

The study is complied with research and publication ethics.

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