Reduced Anticancer Potency of Carboplatin in the Presence of Sialic Acid in SH-SY5Y Cells

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Abstract

Background: Neuroblastoma (NB) is the most common solid childhood tumor with aggressive behavior and a high mortality rate. Multiple therapeutic approaches have been developed and applied to treat neuroblastoma, but resistance to therapies is inevitable, leading to treatment failure and cancer relapse. Therefore, perceiving the mechanisms of reduced drug sensitivity seems appropriate and promising in NB treatment to determine synergistic effects strategies like combination therapy.

Objectives: The present study aimed to investigate the modulation effect of the therapeutic efficacy of carboplatin (a chemotherapeutic agent) by co-administration of sialic acid (an alpha-keto acid sugar with a 9-carbon backbone located at the outermost end of N-linked and O-linked carbohydrate chains and in lipid-associated glycoconjugates showing aberrant expression in tumor cells causing tumor-genesis) in NB cells.

Methods: In the present study, the effects of sialic acid, carboplatin, and Sia-Carbo (sialic acid in combination with carboplatin) were evaluated on viability and apoptosis using the determination of EC50 and IC50. Also, the capability of metastasis and apoptosis were assessed using real-time PCR. Moreover, the expression of MRP-1 encoded by ABCC1 (as a target for therapeutic suppression in high-risk neuroblastoma) was studied in different treatment groups.

Conclusions: Regarding IC50 increasing carboplatin in the presence of sialic acid, the results showed that sialic acid treatment significantly modulated carboplatin's effect on cell apoptosis and induced ABCC1 expression. These findings show that sialic acid metabolic engineering can be a good approach to neuroblastoma therapy. It was suggested that targeting aberrant sialylation in combination with carboplatin exerts more profound apoptotic and anticancer effects on the neuroblastoma SH-SY5Y cell line than carboplatin monotherapy.

Keywords: Sialic Acid (N-acetyl Neuraminic Acid: NANA), SH-SY5Y Neuroblastoma Cell Line, Multidrug Resistance-Associated Protein 1 (MRP-1), Multidrug Resistance-Associated Protein 2 (MRP-2), Carboplatin

1. Background

Neuroblastoma (NB) is a pediatric solid tumor with heterogeneous features, aggressive behavior, and a high mortality rate. The prevalence of this malignancy is 0.8% to 10% of all common cancers in children (1, 2). Multiple therapeutic approaches have been taken and applied in clinics to cure neuroblastoma, including surgery, radiotherapy, chemotherapy, immunotherapy, stem cell transplant, and retinoids (3-5). Nevertheless, resistance to therapies, being somehow inevitable, occasionally leads to treatment failure and cancer relapse (6). Hence, understanding the mechanisms of minimization of drug sensitivity that leads to therapy resistance and discovering reversal strategies is crucial. These experiments might provide evidence for the application of combinatorial therapy for treatment and prolonged survival of patients with NB, as to the application of chemotherapeutic agents with combination regimens for target therapy.

According to the Epidemiology data, the incidence of NB has been reported higher in high-income countries (HICs) than in low- and middle-income countries (LMICs); though, the limitations of diagnosis and management must be considered in such reports (7). Regarding the heterogeneous nature of NB, scientists suggested to determine the molecular mechanisms underlying tumor progression, metastasis, and drug resistance, further studies must be carried out to understand the tumor biology (7). Based on the scientific data, the increase in the incidence of cancer and neurological disorders, decreased drug sensitivity, and enhancement of treatment side
3.1. Chemicals

Sialic acid (N-acetyl neuraminic acid: NANA) and all other chemical reagents were purchased from Qiagen company (Hilden, Germany), Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and Merck Company (Darmstadt, Germany).
used for total RNA extraction. RNA quality and quantity were analyzed by electrophoresis on 1% agarose gel and NanoDrop instrument (Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometers). Then, cDNA was synthesized using 2 µg of total RNA by M-MLV RT kit (Yekta Tajhiz Azma). The expression of ABCC1, BAX, and BCL2 was measured using qRT-PCR. The sequence of the primers is listed in Table 1. The RT-PCR reactions were up to 10 µl including 5 µl SYBR Green Master Mix (Yekta Tajhiz, Tehran, Iran), 2 µl cDNA, and 0.8 µl forward and reverse primers (10 µM). The q-RT PCR program was 1 cycle of 94°C for 1 minute, followed by 40 cycles of denaturation and annealing/extension at 94°C for 10 seconds and 60°C for 30 seconds, respectively. The ACTB was used as the housekeeping endogenous control for RT-data normalization. One-step Real-Time PCR Equipment (Applied Biosystems) was utilized for PCR. The calculations were performed by the 2^\(\Delta\Delta C_{t}\) (Livak) formula.

### 3.5. Statistical Analysis

All experiments were repeated three times. The data from treated and untreated cells in three independent experiments were presented as mean ± standard deviation. The statistical analyses were performed by the paired-student t-test. Differences with P < 0.05 and P < 0.01 were considered significant (*) and more significant (**).

### 4. Results

#### 4.1. Cancer Cell Viability

In our study, the half-maximal effective concentration (EC50) of sialic acid was determined depending on the time and concentration manner that reflects the metastatic potency of sialic acid. MTT assessment revealed that sialic acid treatment could significantly increase the cell viability of the neuroblastoma cell line. According to the MTT results, the present study showed that the anticancer effect of carboplatin was in a time- and concentration-dependent manner, modulated with a combination of sialic acid treatment. The enhanced concentration-dependent manner, modulated with a combination of sialic acid, carboplatin, and the combinatorial effect of sialic acid, carboplatin, and the combinatorial effects of sialic acid, carboplatin, and a combination of sialic acid + carboplatin in the effective concentration in the enhanced concentration + half maximal Inhibitory concentration, respectively, on SH-SY5Y cell line were defined as EC50 = 116.1 µM, IC50 = 623.4 µM for sialic acid (Figure 1A1 and B1), IC50 = 87.21 µg/mL for carboplatin (Figure 1C1) and IC50 = 218.8 µg/mL for carboplatin in combination with sialic acid treatment for 48 h (Figure 1D1). Cell viabilities are illustrated by *, **, **, respectively, also, the cytotoxic activities of carboplatin alone and in combination with sialic acid are demonstrated by bidirectional arrow) and EC50 = 64.21 µM, IC50 = 427.6 µM for sialic acid (Figure 1AII and BII), IC50 = 45.76 µg/mL for carboplatin (Figure 1CII) and IC50 = 91.50 µg/mL for carboplatin in combination with sialic acid treatment (Figure 1DII) for 72 h (S; cell viabilities are illustrated by *, **, **, respectively. Also, the cytotoxic activities of carboplatin alone and in combination with sialic acid are demonstrated by bidirectional arrow). It is important to note that our observations are in concordance with other data indicating that sialic acid promotes cell viability and survival and has no significant cytotoxicity effect on cell lines. It is worth noting that IC50 is merely determined based on a little decrease in absorbance and no significant change in that.

#### 4.2. Gene Expression Assay

To better understand the metastatic and apoptosis effects of sialic acid, carboplatin, and the combinatorial effect of sialic acid and carboplatin in 2*EC50 of sialic acid and IC50 of carboplatin, we studied the expression of BAX as pro-apoptotic and BCL2, as anti-apoptotic gene expression in these groups. Notably, some research has reported that the BAX/BCL2 ratio, a prognostic marker, determines cell susceptibility to apoptosis. Furthermore, multidrug resistance protein 1 (MRP-1) expression encoded by ABCC1 was studied in these groups.

#### 4.2.1. The Effect of Sialic Acid on the BAX/BCL2 Ratio

After being treated with sialic acid, the BAX expression has remained unchanged. Besides, the BCL2 expression was induced approximately 16-fold. Compared with the untreated cells, a noticeable difference in BCL2 expression was observed in SH-SY5Y treated cells. This increase is associated with other data about cell proliferation and viability verified by MTT assay (Figure 2).

#### 4.2.2. The Effect of Carboplatin on the BAX/BCL2 Ratio

Carboplatin treatment induced the expression of BAX about 11 times. Also, in the case of BCL2, the expression induction was about 9 times higher. The ratio change was 1.74 compared with the control group, proving carboplatin’s apoptotic effect (Figure 2).

#### 4.2.3. The Effect of Sia-Carbo (CBDCA) on the BAX/BCL2 Ratio

Carboplatin treatment in sialic acid’s presence induced BAX’s expression about 46.36 times higher. This expression induction for BCL2 was about 52.17 times. The BAX/BCL2 ratio, which is an indicator of apoptosis, changed from 1/1 in control cells to 46/52.17 in the treated
**Figure 1.** Half maximal effective concentration (AI, II); the inhibitory concentration of sialic acid (BI, II); carboplatin treatment (CI, II); and sialic acid-carboplatin (DI, II) on the viability of SH-SY5Y cell line after 48 and 72 h treatment.
Table 1. Primer Sequences Used For Real-Time PCR

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cells, which proved the anti-apoptotic effect and resistance to apoptosis of SH-SYSY cells reflecting the metastatic and anti-apoptotic effect of the sialic acid. In the case of treatment with carboplatin, this ratio was 11/9, which, by comparing the ratio of 46/52.17, i.e., 1.22 to 0.88, decreased the apoptotic index in the presence of sialic acid. An important case is to examine the expression at the protein level for further confirmation (Figure 2).

4.2.4. The Effect of Sialic Acid, Carboplatin, and Sia-Carbo Treatment on ABCC-1 mRNA Expression

Real-time PCR was used to measure the differences in the ABCCI mRNA expression level in sialic acid, carboplatin, and Sia-Carbo-treated (treated by sialic acid in combination with carboplatin) neuroblastoma compared to the untreated cells. As shown in Figure 3, the ABCCI mRNA expression level in the treated cells was approximately 2.5, 40, and 90 times more than in untreated cells (Figure 3).

5. Discussion

One of the biggest obstacles in cancer treatment is drug resistance. Scientists have suggested that intrinsic resistance might cause the initial response to therapy, such as specific cell membrane transporter proteins. The acquired genetic and epigenetic modifications in cancer cells could impart in drug resistance development (6). One of the main intrinsic factors deregulated in the cancer cell membrane is the sialic acid involved in the interplay between the tumor microenvironment and cancer cells, physically and chemically (18). Sialic acid can mask cell membranes and antigens, evading immune response (19). It can also trigger the underlying mechanisms involved in cell proliferation, angiogenesis, and metastasis, especially with the upregulation of CDCP1 as a metastatic marker (manuscript under revision). Having a significant role in tumor genesis and cell proliferation, metastasis induction, immune evasion, and drug resistance, hypersialylation is a beneficial target for cancer therapy, i.e., the development of sialyltransferase blocking and strategies to block Siglecs and selectins as its receptors (20).

It has been well documented that ST6Gal-I sialyltransferase activity blocked cell death induced by cisplatin in ovarian tumor cells (21). Recently, overcoming drug resistance and combating tumor progression and metastasis in cancer cells using combination therapy has attracted great attention, especially regarding the whole exome sequencing and transcriptome data through targeting various mechanisms contributing to cell proliferation, progression, metastasis, and drug resistance (22).

Depending on the type of cancer, carboplatin can be used by itself or in combination with other chemotherapy drugs. Although multidrug transporters, especially in childhood neuroblastoma, the ATP-binding cassette, subfamily C (ABCC) transporters, have been reported to play an important role in cytotoxic drug efflux and mediating drug resistance. It is noted that decreasing the apoptotic ratio (BAX/BCL2 ratio as pro-apoptotic/anti-apoptotic ratio) can cause drug resistance (23).

In the present study, we compared the metastatic, anticancer activity and drug sensitivity modulation with a 48 hours treatment of the sialic acid (200 uM), carboplatin (90 uM), And Sia-Carbo (200 - 90 uM) combination
Figure 2. The expression analysis in NBs by qRT-PCR for the BAX and BCL2 gene. Bars were performed in sialic acid, carboplatin, and sialic acid-carbo-treated cells, compared to the untreated cells as control (200 μM and 90 μg/mL as effective concentration and inhibitory concentration of sialic acid and carboplatin, respectively, and 2*EC50 of sialic acid/IC50 of carboplatin on NBs) (*, **, statistical significance and more significance; P < 0.05 and P < 0.01, respectively).

Figure 3. The expression analysis in NBs by qRT-PCR for the ABCC1 gene. Bars were performed in sialic acid, carboplatin, and sialic acid-carbo-treated cells, compared to the untreated cells as control (200 μM and 90 μg/mL as effective concentration and inhibitory concentration of sialic acid and carboplatin, respectively, and 2*EC50 of sialic acid/IC50 of carboplatin on NBs) (*, **, statistical significance and more significance; P < 0.05 and P < 0.01, respectively).
in SH-SYSY cells and possible involved mechanisms. The results revealed that the treatment of sialic acid caused a significant decrease in the BAX/BCL2 ratio in neuroblastoma, which agrees with the other studies that showed the metastatic effect of sialic acid in different cancer cells. Furthermore, our study showed a sialic acid reduction in the anti-cancer activity of carboplatin on SH-SYSY cells by induction of viability and metastatic and anti-apoptosis induction, which was shown by an increase in IC50 of carboplatin. These facts suggest that sialic acid can be a major drug response challenge and cause drug failure.

Reduction of drug sensitivity and anti-apoptosis are important mechanisms of therapy failure in various cancer cells. The results revealed that the reduction and modulation of apoptosis rate of SH-SYSY cancer cells was increased following the treatment with Sia-Carboplatin, significantly more than carboplatin. This reflects sialic acid’s neutralizing effect and chemotherapy toxicity prevention. Previous studies have reported that sialic acid can induce drug resistance in various cancer cells. One of the main factors involved in drug resistance is P-glycoprotein transporters. MDRI is a primary active transporter that pumps various compounds out of the cells using ATP hydrolysis. Its activity impacts clinical outcomes and toxicity. Providing resistance to cancer chemotherapeutic agents, multidrug resistance protein 1 is considered a major cause of treatment failures. As MDRI transports a wide range of chemotherapy drugs, such as anthracyclines, vinca alkaloids, taxanes, etoposide, mitoxantrone, bisantrene, and the histone deacetylase inhibitor depsipeptide (24, 25), targeting inhibition of MDRI could theoretically improve the clinical outcomes for many cancers. Early studies identified MDRI expression as a significant prognostic indicator in several childhood cancers (26), and more recent studies have linked MDRI expression to poor outcomes in leukemias and breast cancer. By the decrease of sensitized NB to carboplatin, the ABC transporter inducer increases proliferation and metastasis. Therefore, we evaluated the mRNA expression of the MDRI gene in SH-SYSY with sialic acid, carboplatin, and Sia-Carbo. The results showed that sialic acid treatment can upregulate ABCCI expression, which is more upregulated in carboplatin treatment and might reflect drug resistance induction. As demonstrated, sialic acid, in combination with carboplatin, can cause an evaluation of ABCCI expression significantly, and synergistically, drug resistance is increased. We can demonstrate that the combinational treatment of Sia-Carbo neutrally favored the anti-apoptotic pattern of BCL2 and BAX expression. According to the results, the apoptotic, anti-metastatic activity and the efficacy of chemotherapy significantly decreased in carboplatin treatment in combination with sialic in SH-SYSY cells. Our results are consistent with the previous reports about the role of hyper-sialylation in promoting cancer development.

5.1. Conclusion

This study demonstrated that sialic acid, by inducing intrinsic anti-apoptotic pathways and SH-SYSY proliferation, decreased the anticancer effects of carboplatin. It might be suggested that targeting aberrant sialylation could be beneficial for treating patients with Neuroblastoma. Basically, it recommended that further protein-level research need to be carried out to evaluate and support these in vitro outcomes. However, further studies are needed to clarify the molecular mechanisms and pharmacokinetics behind the drug-resistance effects of this compound.

Footnotes

Authors’ Contribution: HG and MS, conceived and designed the study, obtained funding, developed methodology, performed statistical analyses and interpretation of data, and reviewed and revised the manuscript; DA, cultured NB cells, treated them with sialic acid, carboplatin, and Sia-Carbo, and performed cellular and molecular techniques; DA, wrote the original draft; HG and MS, reviewed and edited the manuscript. Coauthors read and accepted the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of Interests: The authors declare no conflict of interest.

Data Reproducibility: The dataset presented in the study is available on request from the corresponding author during submission or after publication.

Ethical Approval: All procedures performed in studies involving human neuroblastoma cell line is in accordance with the ethical standards of the research committee (Committee of the Shahid Chamran University of Ahvaz (Code: SCU.SBU1401.90).

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