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Research Paper

Investigation of Anti-Tumor Effect in Neuroblastoma Cell Line; Amlodipine & Metformin

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Abstract

Neuroblastoma is the most common cranial solid tumor in children. In recent years, scientists have begun to investigate the effect of drugs used to treat other diseases on various types of cancer. Metformin is a popular antihyperglycemic drug. Metformin inhibits glucose uptake by affecting the AMPK metabolism of tumor cells that use glucose for energy. Amlodipine, on the other hand, is a calcium channel blocker and governs Ca^{2+}, which have important role of the cell cycle and metabolism. The purpose of this study; success of amlodipine to increase the effect of metformin against neuroblastoma cell line.

In this study, we prepare the cancer cell line in the proper cell culture medium. Drug doses of metformin (10, 20 and 40 ug), Amlodipine (10 mM), Metformin (10, 20 and 40 ug) + Amlodipine (10 mM) were administered to NBL cancer cell lines for 24 hours. MTT cell viability test, flow cytometry, total oxidant status, and total antioxidant capacity test were performed 24 hours after the application.

The study showed that the combination of high doses of metformin and amlodipine significantly reduced cell proliferation and antioxidant status compared to control and other groups. Apoptosis levels in combination group are higher than the pure MET and AML groups. When amlodipine was administered alone, there was no antitumor activity in the neuroblastoma cell line, however, metformin was determined to increase antitumor activity.

Keywords: Neuroblastoma, Metformin, Amlodipine, Ca^{+2} channel blocker

1. Introduction

Cancer is a serious health problem increasingly worldwide. Chemotherapeutics used in cancer treatment kill cancer cells using different signaling pathways. These drugs target cancer cells, but healthy cells cannot completely free themselves from the effect of cancer drugs. As the secrets of cancer and drugs continue to be resolved, efforts to prevent drug side effects have accelerated, but the cardiovascular, neural, renal or hepatic organ systems of patients using anticancer drugs are still adversely affected [1]. The researchers came to take notice that although there are no anticancer drugs, some drugs that have an effect on cell metabolism may be effective in cancer treatment. Metformin and Amlodipine are among these drug [2].

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Metformin is an antihyperglycemic drug commonly used in the treatment of type 2 diabetes mellitus, a derivative of biguanid. It basically reduces hepatic glucose production and insulin resistance in peripheral tissue. In addition, its effect on cell metabolism is much wider and continues to be elucidated every day [3]. Metformin changes cell metabolism by acting directly on the mitochondria. Metformin inhibits the mitochondrial electron transport system, decreases cellular adenosine triphosphate (ATP) and causes an increase in adenosine monophosphate (AMP)/ATP ratio within the cell. This is a result of the activation of protein kinase (AMPK), which is activated by AMP. AMPK is important in regulating the metabolism of energy-consuming cells. The energy metabolism of cancer cells is largely dependent on the independent oxygen-glycolysis pathway (Warburg Effect) [4, 5]. Dysregulation of these metabolic mechanisms is successful in the fight against cancer cells [6].

In recent years published many meta-analyses have highlighted the good course of cancer patients using metformin, and experimental studies have provided important evidence. There are many studies examining the impact of metformin on cancer types such as breast cancer, stomach cancer, prostate cancer [7]. However, there are not enough studies on the effect of metformin on neuroblastoma (NBL), a childhood disease. Therefore, the neuroblastoma cell line was selected in this study. NBL is the most common extracranial solid tumor in children below the age of 10 and accounts for 15% of all childhood cancer deaths [8, 9]. NBL is a malignant tumor that frequently occurs in the adrenal medulla and sympathetic ganglia and originates from primitive neural crest cells [10, 11]. In the development of neuroblastic tumors; in addition to genetic predisposition, defects differentiation mechanisms of embryonal neural crest cells, post-translational modifications, mutations, and environmental factors have been reported to be effective [12].

Anticancer properties have been studied less than metformin, but another drug that has significant effects on cells is amlodipine. Amlodipine is a calcium channel blocker. Intracellular calcium ions (Ca^{2+}) play a vital role in the cell as well as acting as the second messenger in normal cell physiology to regulate gene transcription, cell migration, proliferation, and death. Amlodipine, a calcium channel blocker, is a lipophilic drug effective on L-type calcium channels. Studies have shown that it is neuroprotective in addition to its effect on cell physiology [13].

Ca^{2+} sensitive receptors are found all the body and in addition to their physiological effects, Ca^{2+} ions affect cell life at the molecular level. In cell biology, Ca^{2+} plays a role in the regulation of cell death patterns such as apoptosis, necrosis and autophagy [14]. The complex mechanism of action has been associated with expression and inhibition of genes (MYBL2, NME2, MCM7, CRABP2, LIF, TP53, MIR17HG, PRMT1, AURKA, MCM8, ODC1, MDM2, LUC7L, BIRC5, TWIST1, RAB5C, H1 F0), enzymes and/or various receptors at the molecular level [10]. Yoshida et al. amlodipine have been reported to induce the expression of p21 Waf1/Cip1 in human epidermoid carcinoma A431 cells, causing inhibition of CDK /cyclin-related kinase activities and stopping G1 cell cycle by a decrease in p1B phosphorylation [15].

In this study; although not chemotherapeutic, the effect of an antihyperglycemic drug metformin and a calcium channel blocker Amlodipine on the NBL cell line has been investigated for the first time.

2. Materials and Methods

2.1. Chemicals and reagents

Metformin was obtained from Sandoz Ltd (Basel, Switzerland). Amlodipine, Dulbecco's Modified Eagles Medium (DMEM), Fetal bovine serum (FBS), Neurobasal medium (NBM), Phosphate buffer solution (PBS), antibiotic-antimitotic solution (100×), L glutamine and trypsin–EDTA were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Cell cultures

Neuroblastoma cell line were taken from the medical pharmacology department of Ataturk University (Erzurum, Turkey). Briefly, the cells in 25 cm^2 flask were treated with trypsin-EDTA and then the cells were seeded in 24-well cell culture plates (Corning, USA) and store at incubator (5% CO_2; 37°C) [15].

2.3.TAS/TOS experiments

The serum TAS (Total antioxidant status) and TOS (Total oxidant status) activities were measured by using the commercial kits which were obtained from Rel Assay Diagnostic® Company (Gaziantep, Turkey).

2.4. Drug administration

After acquire, 90% confluence in 24-well plates, drugs were added. MET (10, 20 and 40 µg) and Amlodipine (10 µM) were added to well plates then incubated for a day (standard incubate in 5% CO_2, 37 °C and 95% moisture).

2.5. MTT assay

At the end of the experiment (24 h exposure time) 10 µL of MTT solution was added to each plate and the plates were incubating for 240 min at 37 °C in a 5% CO_2 incubator. 100 µL of DMSO was added to all well to dissolve formazan crystals. Density of Formazan crystals read at 570 nm wavelength (Multiskan ™ GO Microplate reader, Canada, USA) [15].

2.6. Morphologic determination

We used Leica microscopy (USA) for morphological examination. All experimental groups were photographed after 24 hours (20x magnification) [15].

2.7. Flow cytometry analysis

We used Leica microscopy (USA) for morphological examination. All experimental groups were photographed after 24 hours (20x magnification) [15].

2.8. Statistically analysis

The statistical analysis was performed using Kruskal-Wallis and Mann–Whitney U test comparisons (IBM SPSS 20.0 software). P value < 0.05 was considered as statistically significant.

3. Results

The cells were administrated with 10 µM Amlodipine, 10, 20 and 40 µg Metformin and combinations of these doses for 24 hours. Positive control group containing only DMSO while negative control group containing only medium solution. All tests (MTT, TAS-TOS, and flow cytometry analysis) were performed after exposure time.

3.1. MTT analysis

The survival rate of NBL cells after 1-day drug exposure was evaluated by using the MTT test (Fig. 1-2). According to the results we found, compared to other groups pure AML (10 µg) has the highest viability. The lowest survival rate was observed in the amlodipine + 40 µg MET group. When we looked at combination groups, the lowest viability rate was observed at + 40 µg MET and the highest rate is +10 µg MET. The Amlodipine + 20 µg MET group was found statistically significant when compared with the control group. ($P < 0.001$). In addition, compared to the control groups pure MET had a significant effect also (Table 1). ($P < 0.05$).

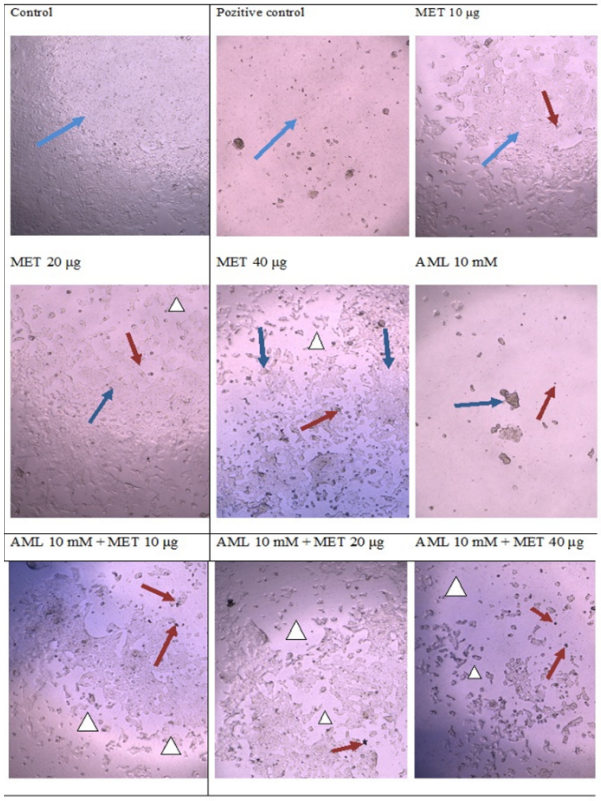

Figure 1. Microscopic View of NBL Cells

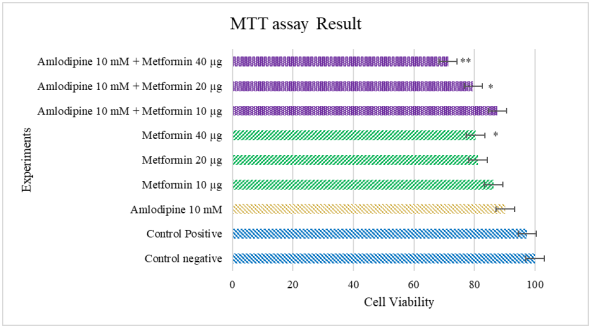

Figure 2. MTT Analysis

3.2. Total oxidant status (TOS)

We measured the TOS test to producer procedure which is based on H_2O_2 equiv/mmol L−1 (Fig. 3). According to our result, both of control groups shows 3.4 and 3.5 H_2O_2 equiv/mmol L−1 respectively. An increase in oxidant levels was observed in pure groups compared to control groups (AML 3.9, MET 4.2, 4.5 and 4.6 respectively). In the combination groups (4.1, 4.8 and 5.5 respectively) there was observed an increase in oxidant levels according to the positive and negative control groups, but the oxidant level was higher in the 40 µg MET group than in the Amlodipine + 10 µg MET group. Amlodipine + 20 µg MET was found statistically significant in groups compared to control groups ($P < 0.05$). There was also no statistically significant difference between the amlodipine, MET (10 and 20 µg) and AML + 10 µg MET groups compared to the control groups (Table 1).

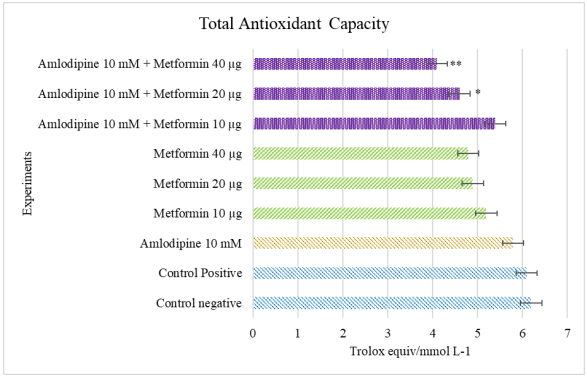

Figure 3. Total Antioxidant Capacity

3.3. Total antioxidant capacity (TAC)

We measured the TAC test to producer procedure which is based on Trolox equiv/mmol L−1 and the all data was compared to control group (Fig. 4). According to our result, the negative control and a positive control group shows 6.2 and 6.1 Trolox equiv/mmol L−1 respectively. A decrease in antioxidant levels (Amlodipine 5.8, MET 5.2, 4.9 and 4.8 respectively) was observed. Antioxidant levels decreased in the combination groups (5.4, 4.6 and 4.1 respectively) compare to control and pure groups. In the Amlodipine given groups 10 mM + 20 µg MET and Amlodipine + 40 µg MET according to the control groups, statistically significant ($P < 0.001$), ($P < 0.05$). Additionally, no statistically significant difference was observed between the Amlodipine, MET (10, 20 and 40 µg) and Amlodipine + 10 µg MET groups compared to the control group (Table 1).

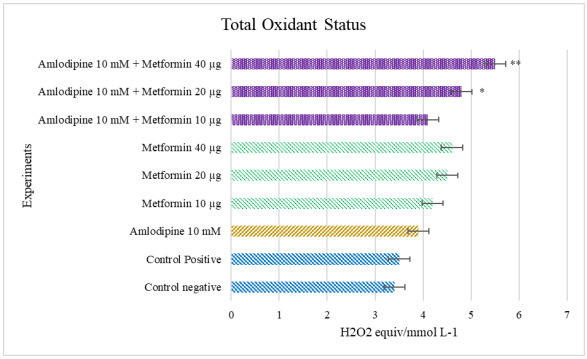

Figure 4. Total Oxidant Capacity

3.4. Flow cytometry analysis

We investigated the progress of apoptosis in the NBL cell line after exposure to MET, AML and the combination of both drugs (Fig. 5). The viability rate of the negative and positive control group was respectively %98.75 and %89.61. Positive control group results of necrosis, early and late apoptosis (respectively %8.43, %0.70) was higher in compare to negative control group. The viability level of pure Amlodipine was %97.70, necrosis %0.14, late apoptosis %0.52, and early apoptosis %2.94. Also, the viability rate (respectively %94.88, %83.96 and %81.78) of the pure MET group was decreased with respect to the control groups, depending on the increasing doses. There was also observed an increase in early apoptosis (respectively %4.76, %7.82 and %6.16) and late apoptosis levels (respectively %0.28, %6.98 and %12.02). The decrease in the viability rate

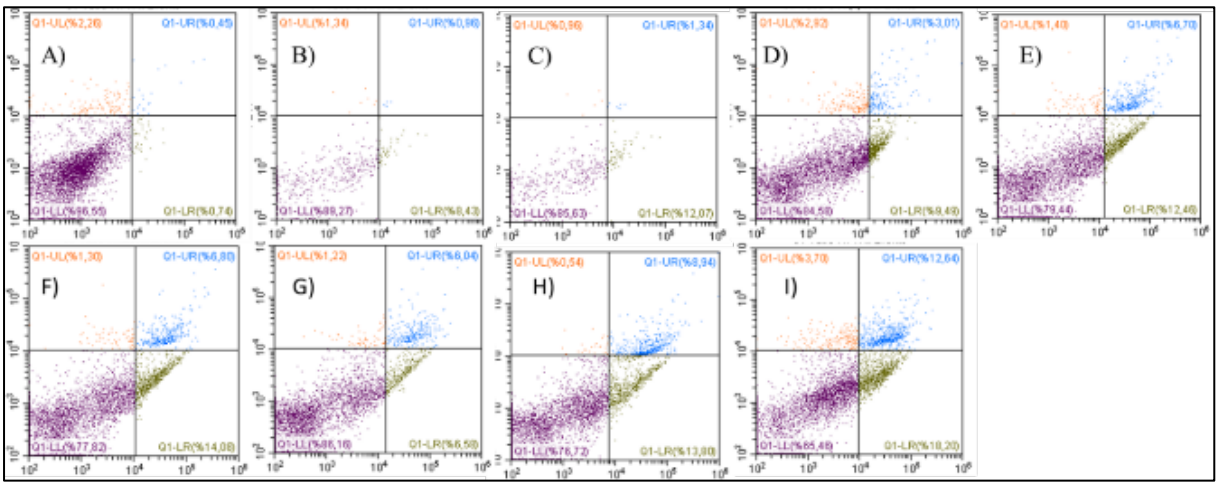

Figure 5. Flow Cytometry Analysis

(respectively %82.22, %80.44 and %78.94) was observed with respect to the control and pure groups of the combination groups. Also, an increase in late

apoptosis (respectively %0.26, %7.46 and %7.58) and early (respectively %17.44, %11.34 and %12.84) apoptosis levels were observed in the combination groups compared to the control groups.

4. Discussion

Neuroblastoma is the most common cranial solid tumor in children. It accounts for approximately 15% of all deaths from childhood tumors [16]. It is rare in adults, high-risk NBL grows mercilessly and rapidly, and low-risk NBL can be treated or re-regulated spontaneously [11, 17]. New approaches with low toxicity are taken attention in the treatment of cancer. Although the effect of metformin on various types of cancer is known, further studies are needed for NBL. Amlodipine, on the other hand, manages calcium, an important ion in the cell cycle and metabolism, and plays an active role in cancer biology. In this study, metformin and amlodipine were used together with a new approach for the treatment of NBL, the effect possible mechanisms on NBL cell line were investigated.

Studies have reported that metformin is effective in many cancers such as lymphoma, pancreatic cancer [18], endocrine tumors [19], colorectal tumors [20], liver cancer [21] and NBL [22]. Mitochondrial mechanisms are the most attention-grabbing mechanisms of action recommended for metformin. After than cell admission of metformin, it causes specific inhibition on the respiratory chain complex 1. Mitochondria, the source of cellular energy, are also the main source of reactive oxygen species (ROS), which can potentially play an important role as signaling molecules in various pathways, potentially causing oxidative damage. ROS production, an important problem in cancer biology, has been shown to reduce Metformin with complex 1 inhibition [23]. In addition, another study reports that metformin promotes neuronal differentiation through reduced cellular proliferation and ROS induction [22]. In accordance with the literature, in our cell culture studies, metformin decreased the number of viable NBL cells compared to control groups. However, the survival rate of NBL cells was lower when administered together with Amlodipine (Amlodipine + 20 µg MET and Amlodipine + 40 µg), which plays a role in intracellular calcium regulation. Research on cancer biology has shown that the changing expression of calcium channels is associated with various types of cancer. Voltage-gated calcium channels have been shown to play an important role in the protection of proliferative signals, inhibition of growth suppression, resistance to cell death, and stimulation of angiogenesis [24].

Table 1. Statistical Analysis Results of MTT, TAS and TOS Measurements

	MTT				TAC				TOS			
	Mean		std	P-Value	Mean		std	P-Value	Mean		std	P-Value
Control Negative	100	±	8,30		6,2	±	0,496		3,4	±	0,272	
Control Positive	97,38	±	7,79		6,1	±	0,488		3,5	±	0,235	
Amlodipine 10 mM	90,17	±	7,21		5,8	±	0,464		3,9	±	0,262	
Metformin 10 µg	86,27	±	6,90		5,2	±	0,416		4,2	±	0,284	
Metformin 20 µg	81,19	±	6,50		4,9	±	0,392		4,5	±	0,225	
METFORMIN 40 µg	80,34	±	6,43	**0,023**	4,8	±	0,384		4,6	±	0,221	
Amlodipine 10 mM + Metformin 10 µg	87,58	±	7,01		5,4	±	0,432		4,1	±	0,24	
Amlodipine 10 mM + Metformin 20 µg	79,58	±	6,37	**0,009**	4,6	±	0,368	**0,045**	4,8	±	0,23	**0,013**
Amlodipine 10 mM + Metformin 40 µg	71,25	±	5,70	**0,001**	4,1	±	0,328	**0,001**	5,5	±	0,255	**0,0000**

Also, Casalà et al. showed that calcium channel blockers significantly reduced the tumorigenic and proliferative capacities of NBL cell lines by causing overexpression of CaSR (a qualitative receptor effective in the differentiation of neuroblastic tumors) [25]. Cellular Ca^{2+} overexpression is known to be highly toxic, causing intense activation of proteases and phospholipases. This toxic role of Ca^{2+} causes cell integrity and exposure to different cell damages and causes the Ca^{2+} activated hydrolyzing enzymes to lead the cell to death (necrosis). However, regular intracellular Ca^{2+} increases have been reported to trigger apoptosis [26]. Cancer-related molecular mechanisms of Ca^{2+} ion are very much and new findings are being added every day. However, although the pathways in NBL progression of Ca^{2+} signals have not been fully elucidated, there is evidence that Ca^{2+} signals are effective in progressing prognosis [10].

However, in this study, only NBL cells treated with amlodipine (10 µM) had high viability and no significant effect compared to control.

5. Conclusion

The anticancer activity of metformin has been known for some time and has attracted the attention of researchers. In this study, we concluded that metformin, which acts on cell metabolism, increases its effect when used with amlodipine. The anticancer activity of metformin has been known for some time and has attracted the attention of researchers. Although amlodipine doesn't give a meaningful result when administered alone, its physiological effect on the cell may increase the effectiveness of anticancer drugs. In this study, we concluded that metformin, which acts on cell metabolism, increases its effect when used with amlodipine. However, more research is needed for to find a place in clinical treatment.

Conflict of Interest

The authors declare no conflict of interest.

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