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CARBOPLATIN AND SODIUM BUTYRATE, SEPARATE – YES, BUT COMBINED – NEVER

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Abstract: With the object of improving the effectiveness of a malignant melanoma's treatment and a patients' quality of life, there is a serious need to identify new anticancer compounds, for example, among naturally derived compounds such as sodium butyrate. The aim of this study was to assess the combined impact of carboplatin (C) and sodium butyrate on the B16 melanoma viability by *in vitro*. B16 cell line was exposed to various concentrations of carboplatin (0.001–10 μmol/L) and sodium butyrate (1 to 100 mmol/L) for 24 h. LC_{10}, LC_{50} and LC_{90} values were calculated. The influence of carboplatin and sodium butyrate on the cell cycle and apoptosis was assessed. Additionally, magnetic stem cell sorting was performed, positive melanoma CD133 cells were isolated and the effects of carboplatin and sodium butyrate on cell viability with heterogeneous population of melanoma cells (CD133+/CD133-) was compared. For carboplatin LC_{50} and LC_{90} were 1.2 μmol/L and 4.58 μmol/L, respectively. For sodium butyrate LC_{50} and LC_{90} were 65.73 mmol/L and 275.06 mmol/L. The value for LC_{10} could not be determined. Sodium butyrate at the highest concentration (100.0 mmol/L) resulted in only 57.36% mortality of cells. A synergistic effect of both compounds was observed in low concentrations of sodium butyrate and carboplatin. That synergism disappeared at concentrations corresponding to LC_{50}. At the concentration corresponding to LC_{50}C and high concentration of sodium butyrate, a decrease of cell numbers in phase G2/M was observed (r = -0.97). Cells were arrested in phase G1/G0 and S.
The presented results exclude the possibility of the combined application of sodium butyrate and carboplatin in cancer therapy.

Keywords: carboplatin. sodium butyrate, melanoma, cancer stem cells

The standard treatment for a primary lesion of malignant melanoma is surgery. In the case of disseminated disease, treatment is based on the use of chemotherapy and immunotherapy, but the results are still disappointing. Monochemotherapy shows the effectiveness in only 15-20% of patients with advanced and metastatic cancer (1, 2). In multi-drug chemotherapy, different mechanisms of action are used. This can prevent the development of resistances to applied cytotoxic drugs. The most often used chemotherapeutics in the management of melanoma include; dacarbazine, temozolomide (analog of dacarbazine), derivatives of nitrosourea and platinum compounds. Amongst all synthesized platinum compounds, cisplatin (C) is distinguished by having the greatest activity. In spite of the broad spectrum of activity against cancers of epithelial origin, testicular cancer, ovarian and bladder cancer, cisplatin demonstrates high toxicity (3). Cisplatin and carboplatin show a similar spectrum of action. However, carboplatin is less nephrotoxic than cisplatin and has improved water solubility, which allows for its convenient dosage. Carboplatin belongs to the platiunum(II) complex group and has two amine ligands in *cis*-position and dicarboxylic groups. Carboplatin has a similar mechanism of

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action to cisplatin, and forms the same adducts. Adducts are formed by carboplatin as a result of bonds created in the N-7 position of guanine or adenine in DNA (4). Carboplatin is used in much higher concentrations and requires a duration several times longer than cisplatin. Nevertheless, the side effect profile is better than for cisplatin (5).

With the object of improving the cancer treatment effectiveness and a patients' quality of life, there is a serious need to identify new anticancer compounds. A number of experimental trials have been undertaken with naturally derived compounds such as sodium butyrate.

Naturally, sodium butyrate is formed in the human colon as a byproduct of bacterial fermentation (6). The therapeutic effect of sodium butyrate results principally from its ability to produce histone dephosphorylation and the accumulation of histone H1 (7). HDAC (histone deacetylase) inhibitors block the enzymatic activity of deacetylase and result in hyperacetylation of histones, cell growth inhibition and differentiation, apoptosis evoking, changing expression of the genes and finally inhibiting tumor growth. Therefore, HDAC inhibitors are new and promising anticancer compound generations (8-12).

Cancer stem cells (CSCs) represent only a small proportion of a tumor's mass, with high proliferative potential enabling the growth of the tumor, its recurrence and the ability to spread. CSCs have properties which confer a resistant phenotype to chemotherapeutics agents (11, 13-15).

There is a high probability that a malignant melanoma develops as a result of normal melanocyte stem cell transformation (16). Melanoma stem cells (MSC) represent a small population of tumor cells, which show high proliferative potential. The increased expression of characteristic antigens for the MSC, such as: CD20+, CD133+ or the transport proteins from the ABC group has been demonstrated for these cells (17, 18). Although the molecular and biological characteristic of MSC is poorly understood, their impact on the development of cancer seems to be tremendous. It seems that the emergence of tumor recurrence responds to cancer stem cells. Therefore, the main purpose of anticancer therapy should be directed at CSCs, whose destruction would probably be effective for preventing cancer relapse.

Due to the fact that butyrate does not exhibit such strong anticancer properties as platinum compounds, attempts of usage of sodium butyrate in combination with other chemical compounds are made (19). The aim of this study was to assess the combined impact of carboplatin and sodium butyrate on the B16 melanoma viability *in vitro*.

EXPERIMENTAL

Cytotoxic influence of carboplatin and sodium butyrate on the unsorted B16 cell line

B16 cell line (CD133+/CD133-) was established from the study of B16 tumors excised from a C57BL/6J mouse. B16 cells were cultured in DMEM/HAM'S F-12 medium supplemented with 10% fetal bovine serum (FBS) and 5 µg/mL amphotericin B, 100 µg/mL streptomycin and 100 U/mL penicillin at 37°C in 5% CO_2 and 98% humidity.

Stock solution of carboplatin (C) was 10 mg/mL. Concentrations were obtained by diluting stock solution in complete medium. Sodium butyrate (SB) (Sigma) in powder was dissolved in culture medium to obtain the desired concentrations.

B16 cell line was exposed to various concentrations of carboplatin (0.001–10 µmol/L) and sodium butyrate (1-100 mmol/L) in short time intervals. Cells were seeded on 24-well plates at a density of 5×10^4 cells per well. Twenty four hours after seeding, the cells were exposed to different concentrations of carboplatin and counted after 24 and 48 h. Cell viability was assessed by the trypan blue exclusion test. After incubation, medium with carboplatin was removed from the wells, each well was flushed with 0.5 mL PBS and then the cells were detached from the wells using 0.5 mL 0.05% trypsin. After centrifugation at 300 × g for 5 min, the cells were suspended in 1 mL of medium. Then, 50 µL of the cell suspension was taken and combined with the same volume of trypan blue. Cells were counted by two independent researchers in Neubauer chamber under the inverted microscope at 100× magnification. Application of the dye allowed to distinguish the living cells from the dead cells. The number of living cells was calculated using formula:

$$X = \frac{L_c}{L_k} \times 100\%$$

X – cytotoxicity of drug. L_c – the average number of cells in the test sample L_k – the average number of cells in control.

Because of the high toxicity of carboplatin and the need to use very high concentrations of sodium butyrate in order to obtain a lethal effect, studies on the cytotoxicity of both compounds were limited to 24 h. LC values were calculated.

Based on the regression curves, survival rates of B16 cells after a 24 h incubation period with carboplatin and sodium butyrate were estimated. LC_{10},

LC_{50} and LC_{90} values were calculated based on equations derived from regression curves.

Carboplatin and sodium butyrate influence on cell cycle

Cells were seeded on 6-well plates at a density of 1.5×10^5 cells/well, and exposed to carboplatin, sodium butyrate and their mixture at concentrations corresponding to LC values. After 24 h of incubation, the cells were washed twice with PBS solution, detached from the wells with trypsin and centrifuged for 5 min at 300 × g in 4°C. In the next stage, 500 µL of hypotonic, coloring the DNA solution of PI/Triton X-100 was added. The solution consisted of: 50 µg/mL PI, 0.1 mg/mL RNAase and 0.05% Triton X-100. The cells were incubated for 30 min in the dark and at room temperature. Then, to stop the reaction, tubes with reactive mixture were placed on the ice and then were transferred to a flow cytometer equipped with System IITM Software, Version 1.0. (Coulter Electronics, Krefeld, Germany). Each measurement was done three times. Comparative analysis was performed.

Cell cycle arrest was specified on the basis of results obtained form cell cycle analysis. Cell accumulation in the individual cell cycle phase was identified as a cell cycle arrest in this phase.

Magnetic stem cell sorting

CD133+ cells were isolated using CD133 Cell Isolation Kit (Milenyi Biotec SRL, Italy). Briefly, to avoid nonspecific binding of antigens, cell population counting 1×10^8 cells was labeled by adding 100 µL of FCR reagent (blocking reagent). Then, the cells were incubated for 30 min. at 4°C in the presence of 100 µL "magnetic beads" with ligands that bind CD133 antigen (microbeads). After incubation, the cells were rinsed with buffer and centrifuged for 10 min at 300 × g. Then, 500 µL of cell suspension was put on the column (MS Column) and placed in a magnetic field. B16 CD133- cells were flushed with 500 µL of buffer. B16 CD133+ cells were immobilized on the column. To evoke B16 CD133+ cells, the column was removed from magnetic field and rinsed with 1000 µL of buffer.

Apoptosis detection

To assess the apoptosis, cells were seeded on 6-well plates at a density of 1.5×10^5 cells/well. Carboplatin, sodium butyrate as well as mixture of these compounds in LC were studied. Cells were incubated for 24 h and then detached from the wells using trypsin, suspended in PBS solution and centrifuged twice for 4 min at 300 × g at 4°C. Then, 490 µL of binding buffer (Immunotech, Canada) diluted tenfold with distilled water was added. Cells were then placed on ice and incubated for 10 min. After incubation, 5 µL of annexin V-FITC solution and 5 µL of propidium iodide solution (25 µg of propidium iodide in 1 mL of diluted binding buffer) were added. Cells were shaken for 5 s and then placed on ice in dark conditions for 10 min. Cytometric measurements were made with a flow cytometer equipped with System IITM Software, Version 1.0. (Coulter Electronics, Krefeld, Germany). Each experiment was repeated fivefold and comparative analysis was done. The correlation between the number of apoptotic and necrotic cells and cells in different cell cycle phases was calculated.

Influence of sodium butyrate on CD133+ expression

Cells were seeded on 24-well plates at a density of 5×10^4 cells/well. After 24 h, the seeded cells were exposed to different concentrations of sodium butyrate 0 (control), LC_{10} and LC_{50}. After 24 h, the cells were detached from the wells and magnetic stem cell sorting was performed. The percentage of isolated CD133+ cells from the heterogenous population of CD133+/CS133- B16 cells in different concentration of sodium butyrate was established.

Statistical analysis

Percentage of cells in cell cycle phases, and CD133+ cells after sodium butyrate influence was calculated from three independent measurements and after apoptosis analysis from five independent measurements as an average value, as described in previous studies.

RESULTS

Survival rate of B16 melanoma cells after 24-hours incubation with carboplatin

B16 melanoma cells in control had a regular shape and size. Cells were elongated and spindle-shaped, with numerous cytoplasmatic lamellipodia. Cells were dividing intensively (Fig. 1A). After incubation with carboplatin, there was observed a decrease in cell number, compared to the control. The cells began to detach from the well surface. Cells lost their regular shape and size, cell to cell contact and attachment to the surface of the culture's surface. Many of the cells were rounded in appearance and grouped into larger clusters (Fig. 1D). In higher concentrations of carboplatin, the percentage of living cells decreased (Fig. 1E). In the highest concentration, corresponding to LC_{90}, we didn't

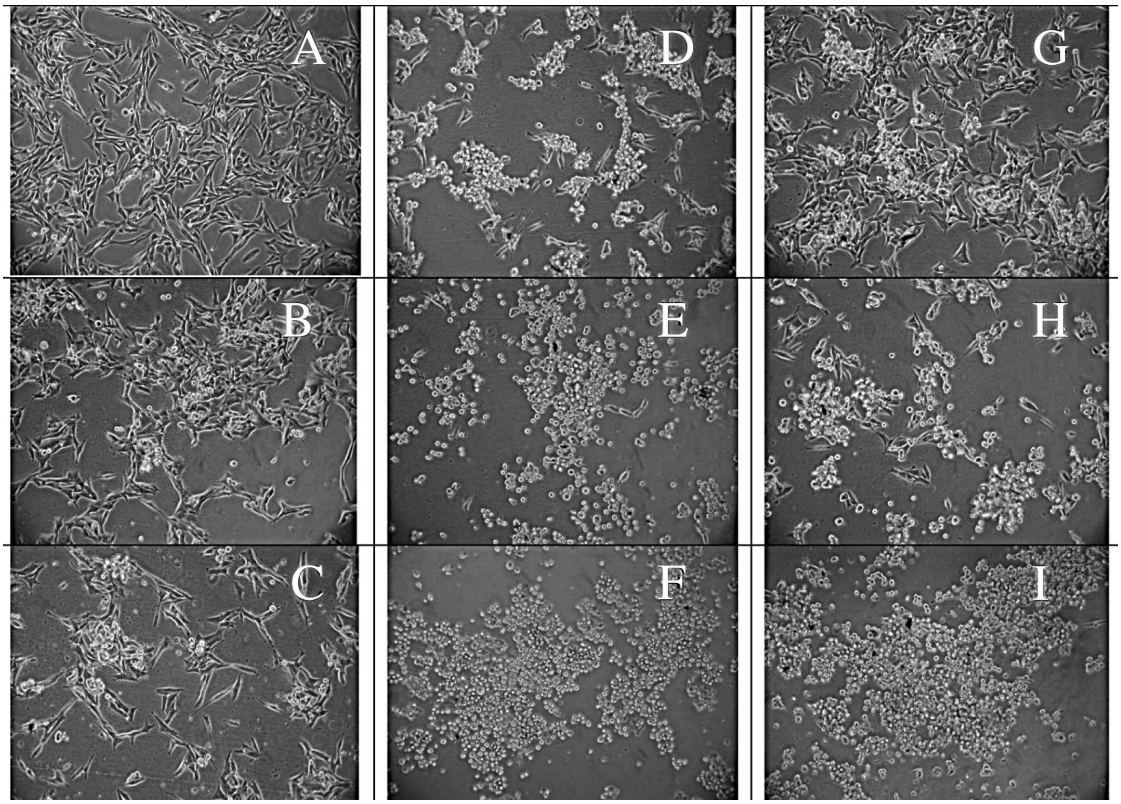

Figure 1. Morphology of B16 cells. **A** - control, **B** - $LC_{10}SB$, **C** - $LC_{50}SB$, **D** - $LC_{10}C$, **E** - $LC_{50}C$, **F** - $LC_{90}C$, **G** - $LC_{10}C$ $LC_{10}SB$, **H** - $LC_{50}C$ $LC_{50}SB$, **I** - $LC_{90}C$ $LC_{50}SB$

observe cells with the typical morphology of the B16 cell line (Fig. 1F).

Survival curve of B16 melanoma cells after 24 h incubation with carboplatin was prepared (Fig. 2A). LC_{50} and LC_{90} were 1.2 µmol/L and 4.58 µmol/L, respectively. LC_{10} value could not be determined. The lowest tested carboplatin concentration was selected as a theoretical value of LC_{10} and amounted 0.001 µmol/L.

Survival rate of B16 melanoma cells after 24 h incubation with sodium butyrate

Sodium butyrate was tested in the concentration ranged from 1.0 to 100.0 mmol/L. LC_{50} and LC_{90} were 65.73 mmol/L and 275.06 mmol/L, respectively (Fig. 2B). LC_{10} value could not be determined. One mmol/L, the lowest tested carboplatin concentration was selected as a theoretical value of LC_{10}. Sodium butyrate at a highest concentration (100.0 mmol/L) resulted in only 57.36% mortality of cells. B16 melanoma cells have proved to be resistant to sodium butyrate and that is why further research was limited only to the LC_{10} and LC_{50} concentrations.

B16 melanoma cells after 24 h incubation with sodium butyrate at the concentration corresponding to the LC_{10} value showed a small reduction in cell number compared to the control (Fig. 1B). In comparison to the LC_{10} carboplatin value (Fig. 1D), many cells with normal morphology of B16 melanoma cells were observed. At concentrations corresponding to the LC_{50} value (Fig. 1C) the number of cells was lower. Moreover, cells were rounded in appearance compared to the LC_{10} value (Fig. 1B).

B16 cells viability after exposure to carboplatin and sodium butyrate

The next step was to examine cytotoxic carboplatin concentrations ($LC_{10}C$, $LC_{50}C$ and $LC_{90}C$) with increasing concentrations of sodium butyrate ($LC_{10}SB$ and $LC_{50}SB$). Results gained after cells incubation with $LC_{10}C$ and $LC_{10}SB$ (Fig. 1G) were similar to those obtained after incubation with $LC_{10}SB$ (Fig. 1B). Furthermore, more spherical structure, which could indicate additional cytotoxic effects of carboplatin were observed. After exposure to $LC_{50}C$ and $LC_{50}SB$ values (Fig. 1H), a decrease in

the number of cell with normal morphology was present. A lot of cells were rounded in appearance. After the incubation at the concentration of $LC_{90}C$ and $LC_{50}SB$ just single cells with normal morphology of B16 line (Fig. 1I) were detected.

Influence of carboplatin on the cell cycle of B16 melanoma cells

At the concentration corresponding to $LC_{50}C$, the percentage of cells arrested in G1/G0 phase was 70%. At $LC_{90}C$ concentration, a decrease of cell number in G1/G0 phase (60%) and an increase number of cells in S phase was noticed. Raising carboplatin concentration resulted in a significant decrease in the number of cells in phase G2/M. There were no cells in phase G2/M after incubation with LC_{50} and LC_{90} concentrations of carboplatin; in these concentrations cells were stopped in S phase. The correlation was negative, $r = -0.98$, $p < 0.01$ (Tab. 1).

Influence of sodium butyrate on cell cycle of B16 melanoma cells

Increasing of sodium butyrate concentration resulted in the decrease of cell population in phase G1/G0. At the same time, an increase in the percentage of cells in phase S was observed ($r = 0.99$, $p < 0.002$). After applying a high concentration of butyrate corresponding to the LC_{50}, percentage of the cells in phase G2/M was reduced to zero (Tab. 1).

Carboplatin and sodium butyrate influence on cell cycle of B16 cells

At the concentration corresponding to $LC_{50}C$ and the high concentration of sodium butyrate, a decrease of cell number in phase G2/M was observed ($r = -0.97$). Cells were arrested in phase

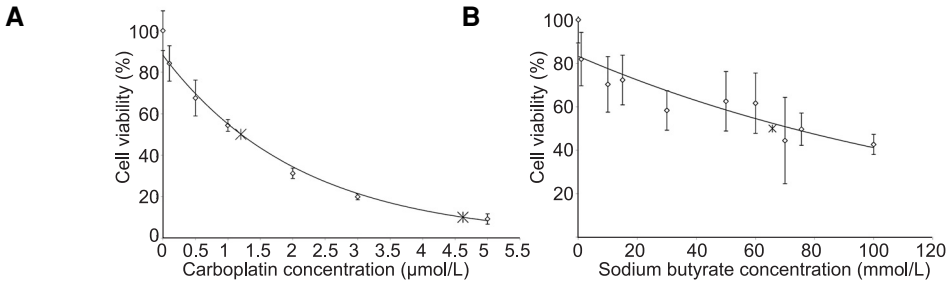

Figure 2. **A**. Survival curve of melanoma B16 cells after 24 h incubation with carboplatin. **B**. Survival curve of melanoma B16 cells after 24 h incubation with sodium butyrate

Table 1. Cell cycle phases of B16 melanoma cells after 24 h incubation with carboplatin and sodium butyrate (LC - lethal concentration, C - carboplatin, SB - sodium butyrate).

LC	Concentration [µmol/L]	Percentage of cells in phase		
		G1/G0	S	G2/M
Control	0.000	51.2 ± 2.4	41.4 ± 1.2	7.4 ± 0.0
$LC_{10}C$	0.001	34.4 ± 3.1	61.1 ± 2.3	4.5 ± 0.2
$LC_{50}C$	1.200	70.7 ± 5.2	29.3 ± 5.1	0.0 ± 0.1
$LC_{90}C$	4.580	58.1 ± 2.8	41.9 ± 3.1	0.0 ± 0.1
$LC_{10}SB$	1.00	41.4 ± 1.3	51.6 ± 0.2	7.3 ± 1.7
$LC_{50}SB$	65.73	46.0 ± 1.5	54.0 ± 2.1	0.0 ± 0.1
$LC_{10}C + LC_{10}SB$		21.1 ± 2.2	69.9 ± 3.1	8.9 ± 0.1
$LC_{10}C + LC_{50}SB$		55.5 ± 2.0	1.0 ± 4.1	43.5 ± 2.3
$LC_{50}C + LC_{10}SB$		70.2 ± 1.7	29.8 ± 0.1	0.0 ± 1.8
$LC_{50}C + LC_{50}SB$		62.2 ± 2.8	33.8 ± 3.1	4.0 ± 0.1
$LC_{90}C + LC_{10}SB$		48.0 ± 6.2	51.1 ± 4.3	0.9 ± 3.1
$LC_{90}C + LC_{50}SB$		53.6 ± 2.3	41.4 ± 1.1	4.9 ± 1.1

G1/G0 and S (r = 0.9 and r = 0.89). After incubation with $LC_{10}C$ and $LC_{10}SB$, an increase of cell numbers in phase G2/M was noticed (Tab. 1).

Increasing concentration of sodium butyrate caused a decrease in the number of G1/G0 cells treated with $LC_{50}C$ and at the same time caused an increase in cell numbers in phase S.

After incubation with $LC_{50}SB$ (together with $LC_{50}C$), the number of the cells in phase G2/M increased. After incubation with $LC_{90}C$ and $LC_{10/50}SB$, a rise of cell numbers in phase G2/M was also observed.

Carboplatin and sodium butyrate influence on apoptosis

In this part only the results of incubation of B16 cells and B16 CD133+ cell population in high carboplatin concentration were presented. Thus, we tried to emphasize the trend, which occurred when a combination of high concentrations of both tested compounds were used. The results of the experiment are shown in Table 2. There were no differences in morphology between cells potentially having phenotype of cancer stem cells and B16 cells. CD133+ cells show a higher resistance to a combination of carboplatin and sodium butyrate than the heterogeneous cell population (Tab. 2). The percentage of living B16 CD133+ cells highly correlated with the concentration of sodium butyrate ($r = 0.89$. $p < 0.001$). In B16 cells, the correlation coefficient was lower ($r = 0.7$, $p < 0.5$). Over 25% more CD133+ cells were detected after carboplatin and sodium butyrate treatment ($LC_{90}C + LC_{50}SB$), compared to carboplatin ($LC_{90}C$) treatment alone. Over 5% more B16 CD133+/Cd133- cells were detected after carboplatin and sodium butyrate treatment ($LC_{90}C + LC_{50}SB$), compared to carboplatin ($LC_{90}C$) treatment alone (Tab. 2). Apoptosis detection showed similar results ($r = 0.66$, $p < 0.02$ for B16 CD133+/CD133-; $r = 0.95$, $p < 0.001$ for B16 CD133+). The correlation coefficient was 0.66 ($p < 0.82$) for B16 cells.

Influence of sodium butyrate on CD 133 expression

More CD133+ cells were isolated after incubation with sodium butyrate in LC_{50} concentration than in LC_{10} and the control.

DISCUSSION

Traditional methods of cancer treatment are random, associated with a high risk of recurrence and what is more, their efficacy is unsatisfactory in advanced stages of the disease. Discovery of cancer stem cells changed the look on cancer and methods of its treatment. There are many theories about cancer stem cells. One of them says that CSCs are mutated normal stem cells having the ability to initiate tumor growth and post-treatment relapse (20). CD133 is a pentaspam membrane glycoprotein and marker of cancer initiating cells, first discovered in human glioblastoma (21, 22). This marker is also present on the cell surface of other types of cancer, including melanoma (11, 13, 23). In this study we isolate melanoma CD133 positive cells and compared the effects of carboplatin and sodium butyrate on cell viability with heterogeneous population of melanoma cells (CD133+/CD133-).

Carboplatin decreases viability of B16 cells. Since carboplatin is still an imperfect drug, there is a need for searching new compounds with better therapeutic index or compounds enhancing its effect. Sodium butyrate, an HDAC inhibitor (iHDAC), seemed to be a good candidate. iHDAC increases histone acetylation in melanoma cells. Only melanoma cell lines are sensitive to apoptosis induced by histone deacetylase inhibitors, while melanocytes are resistant to apoptosis induced by these compounds (9).

Sodium butyrate showed a cytotoxic effect on B16 cells. LC_{50} allowed to stop cell proliferation in G1/G0 and S phases (no cells in G2/M phase), with a predominance of cells in S phase. Higher concentration of sodium butyrate can not be used due to

Table 2. The influence of SD on apoptosis and necrosis in the B16 cells and B16 CD133+ melanoma cells after 24 h exposure to $LC_{90}C$ depending on the concentration of sodium butyrate ($LC_{10}/LC_{50}SB$).

	Living cells (%)		Apoptotic cells (%)	
LC	B16	B16(CD133+)	B16	B16(CD133+)
Control	99.8 ± 0.8	92.2 ± 0.5	0.4 ± 0.2	7.9 ± 0.6
$LC_{90}C$	27.4 ± 2.4	27.1 ± 1.2	72.6 ± 1.7	72.9 ± 1.8
$LC_{90}C + LC_{10}SB$	27.9 ± 4.1	29.7 ± 2.1	72.1 ± 3.7	70.3 ± 3.9
$LC_{90}C + LC_{50}SB$	33.3 ± 1.2	53.4 ± 1.4	66.7 ± 4.9	46.7 ± 2.0

high hypertonicity of solution (24). *In vitro* studies showed that sodium butyrate at low concentrations could stimulate, and at higher could inhibit cell proliferation (25).

We expected a synergistic effect of sodium butyrate and carboplatin similar to that observed with sodium butyrate and actinomycin D on melanoma B16 (26). Cisplatin and sodium butyrate had synergistic effect on HSC-3 cells (Human Oral Squamous Cell Carcinoma line) (27). These observations have led to undertake research on the expected synergistic effect of carboplatin and sodium butyrate. Based on the results presented in our work it was found that in low concentrations of sodium butyrate and carboplatin corresponding to the LC_{10} it was a synergistic effect of both compounds as in the other studies. That synergism disappeared at concentrations corresponding to the LC_{50}, but the cytotoxic effect was still observed. This effect is not greater than the total cytotoxic effect caused by both compounds separately. At higher concentrations of carboplatin and increasing concentrations of sodium butyrate, the expected synergism has not appeared. Moreover, we observed increases of cell viability under these conditions.

Carboplatin interferes with the cell cycle process of B16 cells through the effective cell blocking in G1 and S phases. There were no cells in G2/M phase at LC_{50} and LC_{90} of carboplatin. Seventy percent of cells were stopped in G1/G0 and 30% were in phase S. When cells were treated with LC_{90} we found a slight displacement from G1 (58 ± 2.8%) to phase S (41.9 ± 3.1%).

According to many studies, sodium butyrate antiproliferative potential and its ability to induce apoptosis in cancer cells were proved. Furthermore, it was found that sodium butyrate caused an increased sensitivity to ionizing radiation in melanoma cells, which is particularly important from the clinical perspective (7, 25, 28-30). Sodium butyrate is able to inhibit the cell cycle both in the G1/G0 and G2/M phase, which probably depends on individual sensitivity of the cells to this compound. Molecular mechanisms of the action of butyrate also showed differences depending on the cells type (10). The cytotoxic effect of sodium butyrate raises with the concentration and incubation time (9, 31).

iHDAC activate the expression of inhibitor P21WAF/Cip1 due to the strong acetylation of chromatin at the Sp1 gene promoter. This is a mechanism independent of p53 protein. P21WAF/Cip1 inhibits the activity of CDK2 at G1/S checkpoint and activates the RB protein. This action leads to cell cycle arrest in G1 phase ultimately (10). P21WAF/Cip1 knockout cells are more susceptible to iHDAC-induced apoptosis, while the wild strain cells die by necrosis. This fact suggests that the P21 protein is responsible for the arrest of cells in G1/G0 phase, but not for apoptosis induction. P21WAF/Cip1 protein also affects apoptosis, and this mechanism may be both p53-dependent and p53-independent (9, 32-34). Sodium butyrate stops cells at G2/M phase in the presence of p53, while cycle arrest at G1/G0 phase occurs in the absence of p53 protein activation. In our study, sodium butyrate confirmed previously described resistances of melanoma cells to this type of iHDAC. After 24 h incubation of the cells with sodium butyrate, LC_{50} value amounted more than 65 mmol. That concentration allowed for cells arrest at G1/G0 and S phase with accumulation in S phase. There was no clear percentage advantage for the cells number at G1/G0 phase (24). Therefore, the expected biological effect would be false.

Research on the fibroblast cell lines, Jurkat, L929 and HeLa, showed that sodium butyrate at low concentrations (0.15-0.31 mmol) can stimulate cell proliferation, and at higher concentartion (2.5 mmol) cause inhibition of cell proliferation (25). Incubation of the cells with sodium butyrate at low concentrations entailed cell proliferation at S phase. This is probably due to the stimulation of expressions of proteins involved in the cell cycle. In most cases, the greatest induction of apoptosis occurs in high concentrations of butyrate and over a longer time incubation (over 20 h). A smaller number of cells in G0/G1 and S phase is observed after incubation with high concentrations of sodium butyrate (9, 35, 36). In our study similar dependences were observed.

The high concentrations of sodium butyrate reduce the level of G1 phase regulators (cyclin A, cyclin E, CDK2, and CDK6 cdk4) and activate P21 protein. A larger number of cells at the G2/M phase are caused by sodium butyrate action on the G2/M cycle regulators (cyclin B and p27KIP1) (25).

The influence of sodium butyrate at concentration 5 mmol on the cell cycle process of B16 melanoma cells was studied by Rousseu (36). He observed the growth in cell population at G1/G0 phase. The percentage of cells at G1/G0 phase (81.4%) was greater with a longer incubation time (more than 20 h). Similar results were obtained in different studies in which B16 melanoma cells were incubated with sodium butyrate at a concentration of 10 mmol (35). In both studies, a temporary increase in cell numbers at G2/M phase was noticed (35, 36). In our study, we observed adverse effect.

In different studies, HSC-3 cells were incubated with sodium butyrate (0.5 mmol) and cisplatin (3.48 μg/mL - LC_{50}) simultaneously (27). A synergistic cytotoxic effect was observed. It is believed that a stronger effect on the cell cycle is exerted by cisplatin, which stops the cell cycle in G1/G0 phase. Cell cycle arrest in G1/G0 phase is most likely the result of rapid proteolysis of cyclin D1 (p53-independent) or slow growth and stabilization of p53 protein. Sodium butyrate does not significantly influence the percentage of cells at G1/G0 phase compared to cisplatin, although there is a slight increase of cells at phase G2/M. Our results show similar dependence. A slight increase in cell numbers at G2/M phase was observed after B16 melanoma incubation with high carboplatin $LC_{50/90}$ and sodium butyrate concentrations.

Influence of sodium butyrate LC_{50} and carboplatin at LC_{90} on melanoma cancer stem cells showed increased survival of these cells under *in vitro* conditions and lack of synergistic effect of both compounds. The obtained results show that sodium butyrate at a higher concentration (LC_{50}) reduced carboplatin cytotoxic effect. Cytometric analysis of the CD133+ cells survival rate after exposure to LC_{90} of carboplatin and LC_{50} of sodium butyrate showed an increase in cell number compared to incubation with carboplatin alone. The growth of the living cell population in this case was more than 25%. This phenomenon can be explained by the fact that the cancer stem cells are more resistant to carboplatin in comparison to the heterogeneous population (CD133+/CD133-) which contains progenitor cells and other cells building the tumor mass. Similar results were obtained with A549 cell line (37). In this study A549 cells with increased expression of CD133 were more resistant to cisplatin treatment. Moreover, A549 CD133+ cells were characterized by increased expression of multidrug resistance genes. Our results also indicate that increasing concentrations of sodium butyrate causes an increase in the number of apoptotic cells and a decrease in number of necrotic CD133+ cells. Cancer stem cells are characterized by intense activity of membrane transport and ion exchange pumps, and other factors beneficial to the survival of stem cells (CD133+) after the exposure to high concentrations of butyrate and carboplatin. Results obtained thanks to research on CD133+ cells seem to rule out physical and chemical factors, which could explain the increased survival of cells after incubation with carboplatin and sodium butyrate. Cancer stem cells (CD133+) and heterogeneous population (CD133+ and CD133-) survived similarly after incubation with high concentrations of carboplatin and low concentration of butyrate ($LC_{90}C$ and $LC_{90}C + LC_{10}SB$). Results obtained for CD133+ cells incubation with $LC_{90}C + LC_{50}SB$ were more pronounced (more living cells), which could be predicted from the cancer stem cells properties.

The number of cancer stem cells in the melanoma population under *in vitro* conditions is small, but their presence can be critical to the success of anticancer therapy (17, 38). CD133 is not the only marker of the CSC. In the literature there is also information about other markers, such as CD24, CD166, CD44 and nestin (13, 23). Our results indicate that HDAC inhibitor sodium butyrate does not show synergistic action with carboplatin. It is not known whether this effect occurs only with carboplatin, that is why it is possible in the future to study the effects of sodium butyrate on the other platinum compounds.

HDAC inhibitors can stimulate differentiation of cancer cells or cause apoptosis (39). In the study performed by Cinatl et al., HDAC inhibitor decreased tumor growth and induced a more differentiated phenotype of neuroblastoma cells (40). Cytotoxic drugs, like cisplatin and carboplatin, act on non differentiated cells, that is why higher concentrations of sodium butyrate and carboplatin act inversely on B16 viability. To evaluate sodium butyrate influence on melanoma stem cells differentiation, incubation of B16 cells with sodium butyrate and subsequent isolation of CD133+ cells was performed. According to literature, CD133+ cells are cancer stem/progenitor cells (21). Addition of sodium butyrate can activate stem cell niche as we shown for LC_{50} (Tab. 3). This activation can influence an unwanted effect of carboplatin and sodium butyrate on B16 melanoma cells. The cytostatic effect of sodium butyrate alone can be related to increased cells differentiation (40) but it can be weaker when chemotherapeutic drug is added.

Table 3. Percentage of isolated CD133+ cells from heterogenous population of CD133+/- B16 cells in different concentrations of sodium butyrate.

Sodium butyrate concentration	0 (Control)	LC_{10}	LC_{50}
CD133+ (%)	1.4 ± 0.85	1.5 ± 1.2	3.5 ± 0.42

CONCLUSIONS

The presented results exclude the possibility of combined application of sodium butyrate and carboplatin in cancer therapy and tend to doubt that the combination of other HDAC inhibitors with chemotherapeutic drug would have any clinical meaning.

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