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Antiproliferative Potential of Officinal Forms and Nanoparticles of Lithium Salts

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We studied the effect of officinal forms and nanoparticles of lithium carbonate and lithium citrate on proliferative activity of hepatoma-29 cells. Lithium carbonate nanoparticles suppressed proliferation of hepatoma-29 cells in lower concentrations than officinal form of this salt. The antiproliferative effect of lithium salts i activation of apoptosis and arrest of hepatoma-29 cells in the G_2/M phase of the cell cycle.

Key Words: *lithium salts; hepatoma-29; antiproliferative effect; nanoparticles*

Tumor cells mediate the growth and production of growth factors through signaling pathways, including Notch1-HES-ASCL1, Ras-Raf-MEK-MAPK, PI3K-Akt-MAPK, and glycogen synthase kinase-3β (GSK-3β) [2,3]. Lithium salts suppress activity of GSK-3β [6]. Lithium chloride (LiCl) used in the therapy of pheochromocytoma and medullary thyroid cancer significantly inhibit tumor growth [3]. Moreover, lithium salts can arrest tumor cells in the G_2/M phase of the cell cycle [7]. However, the cytotoxic potential of lithium salts nanoparticles remains unknown.

This work was designed to compare the *in vitro* effects of officinal forms and nanoparticles of lithium salts on hepatoma-29 (H29) cells.

MATERIALS AND METHODS

H29 cells were isolated and verified in the Institute of Cytology and Genetics (Siberian Division of the Russian Academy of Sciences). Nanoparticles of lithium carbonate (Li_2CO_3) and lithium citrate ($Li_3C_6H_5O_7$) were obtained by mechanical activation in an AGO-2 planetary mill [1]. Activation of apoptosis in H29 cells by lithium salts was studied on a FACSCanto II cytofluorometer (BD) using Annexin V-FITC Apoptosis Detection Kit (BD). The cell cycle of H29 cells was estimated on a FACSCanto II cytofluorometer. The antiproliferative potential of officinal forms and nanoparticles of lithium salts in relation to H29 cells was evaluated from MTT (Sigma) incorporation on a Stat Fax 2100 spectrophotometer at 492 nm.

The results were analyzed with Statistica 6.0 software. The measures of central tendency and dispersion were described by the median (Me) and lower (LQ) and upper quartiles (UQ). The significance of differences was determined by Mann–Whitney *U* test. The differences were significant at $p<0.05$.

RESULTS

The proliferative potential of tumor cells was reduced by 30% after 72-h exposure to LiCl in doses of 10-25 mM [4]. Preliminary evaluation of the antiproliferative effect of LiCl showed that this agent in a concentration of 20 mM inhibits proliferation of H29 cells by 26% (18-27%), which is consistent with published data.

Officinal forms and nanoparticles of lithium salts had opposite effects on the proliferative potential of H29 cells (Table 1). Antiproliferative activity of officinal Li_2CO_3 form increased in a dose range of 0.1-25 mM. Further increasing the dose of this agent was accompanied by a decrease in its antiproliferative effect. Antiproliferative activity of Li_2CO_3 was most pronounced in a concentration of 1 mM. Increasing the

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concentration of Li_2CO_3 to 50 mM was also accompanied by a decrease in its antiproliferative potential. However, Li_2CO_3 nanoparticles in a concentration of 100 mM produced a potent antiproliferative effect on H29 cells. Officinal form of $Li_3C_6H_5O_7$ salt in a concentration of 1 mM produced maximum inhibitory effect on H29 cell proliferation. The antiproliferative effect of this salt decreased with increasing its concentration. However, inhibition of H29 cell proliferation became more pronounced after treatment with this agent in concentrations of 25 and 100 mM. $Li_3C_6H_5O_7$ nanoparticles in low concentrations exhibited lower antiproliferative potential. The inhibition of H29 cell division was most significant after treatment with $Li_3C_6H_5O_7$ nanoparticles in a concentration of 100 mM.

Comparison of the effects of officinal form and nanoparticles of Li_2CO_3 produced the following results. Li_2CO_3 nanoparticles (100 mM) had a greater inhibitory effect on H29 cell proliferation than officinal form. However, the antiproliferative potential of Li_2CO_3 nanoparticles in lower concentrations (50 and 25 mM) was less pronounced than that of officinal form. No statistically significant differences were revealed in the effect of officinal form and nanoparticles of Li_2CO_3 nanoparticles on H29 cells. Officinal form and nanoparticles of $Li_3C_6H_5O_7$ (50 mM) had various inhibitory effects on H29 cell proliferation. The antiproliferative potential was less pronounced for $Li_3C_6H_5O_7$ nanoparticles in concentrations of 25, 10, and 1 mM. No statistically significant differences were observed in the effect of officinal form and nanoparticles of $Li_3C_6H_5O_7$ in concentrations of 100, 5, and 0.1 mM on H29 cell proliferation. Therefore, the antiproliferative effect on H29 cells was revealed after treatment with Li_2CO_3 nanoparticles in lower concentrations as in comparison with those of officinal form of Li_2CO_3. These differences were not observed for $Li_3C_6H_5O_7$.

The count of necrotic H29 cells, degree of apoptosis, and cell death were shown to increase significantly in the presence of lithium salts (Table 2).

A statistically significant decrease in the number of early apoptotic cells was typical only for $Li_3C_6H_5O_7$ nanoparticles (as in comparison with officinal form). Studying the dynamics of activation of apoptosis in H29 cells under control conditions (30 min – 48 h) showed an increase in the count of cells in various phases of necrosis (to 22%). Necrosis of 69 and 85% H29 cells was induced by the 48th hour of exposure to 1 mM Li_2CO_3 salts (officinal form and nanoparticles, respectively). No differences were found in the induction of H29 cell death by $Li_3C_6H_5O_7$ salts (87.5 and 97.7% necrotic cells for officinal form and nanoparticles of lithium citrate, respectively). Hence, lithium salts activate apoptosis in H29 cells.

Taking into account that LiCl can arrest tumor cells in the G_2/M phase of the cell cycle, it was important to study the effect of Li_2CO_3 and $Li_3C_6H_5O_7$ on the cell cycle of H29 cells. Lithium salts in a concentration of 1 mM significantly increased the count of apoptotic H29 cells (<2n) in comparison with the control (Table 3). Moreover, we revealed a significant decrease in the number of H29 cells in the G_0/G_1 (2n) and S phases (4n)

TABLE 1. Antiproliferative Potential of Lithium Salts

Concentration, mM	Li_2CO_3	Li_2CO_3n	$Li_3C_6H_5O_7$	$Li_3C_6H_5O_7$n
100(1)	26.23* (25.11-63.84)	28.24 (28.24-65.10)	30.25 (26.67-67.11)	33.23 (28.46-68.31)
50(2)	14.95[1]* (12.38-58.47)	5.48[1] (-6.20-55.38)	10.71[1]* (-12.68-45.87)	-2.14[1] (-5.26-50.07)
25(3)	57.24[1,2]* (53.98-80.45)	-4.97[1] (-11.10-49.64)	38.56[2]* (32.43-71.83)	6.45[1,2] (3.05-54.85)
10(4)	3.50[1,2,3]* (-0.74-54.07)	16.02[1,3,4] (12.07-59.07)	11.92[1,2,3]* (8.42-58.10)	1.34[1,2] (-0.67-52.06)
5(5)	24.81[3,4,5] (16.90-39.00)	23.10[2,3] (11.57-24.38)	-2.83* (-7.91-46.44)	19.42 (1.18-21.61)
1(6)	19.14[3] (-1.50-34.28)	33.80[1,2,3,5,6] (23.21-46.28)	52.60[2,5+] (38.56-58.74)	15.80 (-0.75-27.70)
0.1	0.67[1,2,3] (-1.04-51.54)	-0.22[5] (-1.12-50.42)	3.80[1,2,3,4,6] (-0.74-53.48)	2.60[1] (-2.38-54.07)

Note. [1,2,3,4,5,] and [6] shows significant difference (p<0.05) from the corresponding concentration of the salt. *p<0.05 in comparison with nanoparticles of lithium salts. Here and in Table 2 and 3: Li_2CO_3, officinal form of lithium carbonate; Li_2CO_3n, lithium carbonate nanoparticles; $Li_3C_6H_5O_7$, officinal form of lithium citrate; $Li_3C_6H_5O_7$n, lithium citrate nanoparticles .

TABLE 2. Parameters of Apoptosis in H29 Cells

Lithium salts	Necrosis	Apoptosis	Early stage of apoptosis
Control	0.15 (0.10-0.20)	2.60 (0.70-3.60)	0.35 (0.20-0.70)
Li_2CO_3, 1 mM	2.00* (2.00-2.00)	4.50* (3.80-7.50)	4.95* (1.00-7.50)
Li_2CO_3n, 1 mM	1.00* (1.00-2.00)	5.85* (1.00-19.60)	2.50* (1.00-4.90)
$Li_3C_6H_5O_7$, 1 mM	3.65* (1.00-3.80)	6.80* (6.80-7.50)	0.60 (0.50-0.60)
$Li_3C_6H_5O_7$n, 1 mM	2.80* (1.00-4.60)	5.25 (2.30-8.20)	3.40*+ (1.00-3.40)

Note. $p<0.05$: *in comparison with the control; +in comparison with officinal form of salt.

of the cell cycle. The count of cells in the G_2/M phase (>2n) was greater than in the control. The exception was officinal form of Li_2CO_3. Li_2CO_3 nanoparticles in a concentration of 1 mM were more potent in arresting H29 cells in the G_2/M phase. By contrast, $Li_3C_6H_5O_7$ nanoparticles decreased the number of G_2/M phase H29 cells (in comparison with officinal form).

Studying the cell cycle of H29 cells after exposure to 1 mM lithium salts (30 min – 48 h) produced the following results. Officinal form and nanoparticles of Li_2CO_3 increased the number of cells in the G_2/M phase (80 and 50%, respectively). However, $Li_3C_6H_5O_7$ in the same concentration mainly induced apoptosis in 70-80 cells. Only 15-20% cells were arrested in the G_2/M phase.

Our results show that lithium carbonate and citrate have an inhibitory effect on the proliferative potential of H29 cells, which is consistent with published data. Previous observations revealed that lithium salts inhibit tumor cell growth, cause an arrest of tumor cells in the G_2+M phase, and activate the pro-apoptotic signaling pathways [2-5,7,8]. Lithium initiates HL60 apoptosis, which is mediated by activation of expression of p53, Rb, and Bax, inhibition of Bcl-2 expression, increase in the production of proinflammatory cytokines (IL-6 and TNF-α), and decrease in the production of anti-inflammatory cytokines (IL-2 and IL-10) [8]. LicCl induces apoptosis in tumor cells and causes an increase in the content of ROS in patients with colorectal cancer [5]. DU145 prostate cancer cells are resistant to cytostatics. Combined treatment with cytostatics in low doses and LiCl causes tumor cell death, which is manifested in an increase in the count of apoptotic cells. A combination of LiCl and doxorubicin arrests tumor cells in the S phase of the cell cycle. These cells are arrested in the G_2/M phase of the cell cycle after combined treatment with LiCl and doxorubicin [4].

TABLE 3. Phase of the Cell Cycle for H29 Cells

Lithium salts	<2n	2n	>2n	4n
Control	16.40 (7.70-17.40)	70.00 (68.95-77.30)	3.50 (3.50-6.25)	9.05 (8.35-10.05)
Li_2CO_3, 1 mM	80.80* (80.50-89.50)	11.30* (4.70-14.15)	1.90*+ (1.25-2.30)	3.15* (1.70-5.90)
Li_2CO_3n, 1 mM	79.30* (79.20-82.20)	9.75* (4.05-12.00)	8.05 (5.80-10.75)	3.00* (2.90-3.10)
$Li_3C_6H_5O_7$, 1 mM	59.05* (54.05-70.55)	10.25* (1.35-18.10)	25.00*+ (21.20-31.80)	3.50* (3.20-4.20)
$Li_3C_6H_5O_7$n, 1 mM	71.25* (66.20-80.05)	8.85* (0.85-16.80)	13.25* (12.60-16.25)	3.75* (2.25-4.40)

Note. $p<0.05$: *in comparison with the control; +in comparison with nanoparticles of this salt.

We conclude that lithium salts produce an antiproliferative effect mediated via induction of apoptosis and arrest of H29 cells in the G_2/M phase of the cell cycle. Nanoparticles of lithium carbonate exhibit antiproliferative potential in lower concentrations than officinal form. These data are of importance for combined antitumor therapy.

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