Study of the effect of Selol and sodium selenite on HeLa cells *in vitro*

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1. Introduction

Selenium is of fundamental importance to human health, it participates in many physiological processes and is therefore regarded as one of the microelements essential for the proper functioning of higher organisms [1]. The results of epidemiologic studies suggest that increased risk of tumour formation is accompanied by insufficient selenium supply, whereas clinical and experimental studies indicate clearly that selenium protects against tumour growth [2, 3].

The biological role of selenium was unravelled when it was discovered that this element was a constituent of active sites of many enzymes. The most known example of selenium-dependent enzymes is the family of glutathione peroxidases (GSH-px), the main function of which is protection of the organism against oxidative stress. Selenium deficiency is manifested by strongly reduced level of GSH-px and by increased production of active oxygen compounds. It was also found that it were the selenites in erythrocytes, rather than selenomethionine or selenocysteine that reacted with glutathione, leading to an active form of selenium: selenodiglutathione. Selenodiglutathione, formed in the reaction between glutathione and selenium, the oxidation state of the latter being +4, has strong antioxidative and antitumour properties. It induces apoptosis in human tumour cells [4÷7]. In this form it is also specifically built into active sites of enzymes.

One of the potential antitumour compounds, over which research is currently conducted to elucidate the mechanism of its action, is Selol 5% – a selenium(IV)-containing organic compound, which is a mixture of selenitetriglycerides synthesized from sunflower oil [8].

The basic screening test of a potential medicament is the evaluation of its cytotoxic activity. The methods used in such tests enable the measurement of changes associated with the disturbances of physiological processes of cells induced by the substance under study. The aim of such tests is the determination of the effect of the substance on the survival rate of cells as related to a control sample, which usually is a cell culture in a plain medium or a culture exposed to other medication of known cytotoxicity.

The aim of the study described herein was the comparison of the cytotoxic action on HeLa tumour cells of a seleno-organic compound, Selol 5%, with that of sodium selenite, a known inorganic selenium compound. The following methods were used in this study: MTT, Bradford protein assay and flow cytometry using Annexin V and propidium iodide as the dye. ICP-MS was applied to determine penetration of selenium into cells.

2. Experimental

2.1 Compounds studied and reagents used

Selol 5% (in the form of liposomes, obtained from the Drug Analysis Department, Warsaw University of Medicine), sodium selenite (Sigma), MTT (Sigma), isopropanol (Labscan), Bradford reagent (Bio-Rad Protein Assay), human albumin (Biomed), FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen), PBS (buffered salt solution), complete and exclusive of Mg^{2+} and Ca^{2+} ions (Institute of Immunology and Experimental Therapy), trypsin, EDTA (Lonza), 1 mg/ml Se standard solution for ICP-MS (Inorganic Ventures), internal standard solution: 10 mg/l Co, nitric acid of purity adequate for ICP (Merck), redistilled water additionally purified in Nanopure Deionization System (Barnstead), argon 99.995% vol. purity (BOC GAZY, Warsaw).

2.2 Cell culture

The cultures of HeLa cells (*Human Cervix Carcinoma Cells*), cells of human cervical neoplasm (*German Collection*) was grown in a 5% CO_2 atmosphere at 37°C on an EMEM medium (*Eagle's Minimum Essential Medium - Lonza*) with an addition of 10% FBS (*Fetal bovine serum - Lonza*) and 1% of antibiotics (Penicillin, Streptomycin, Amphotericin B - Lonza).

The cells were inoculated, depending on the time of incubation with the compounds studied, in an amount of 1 or 1.5 x 10^5 per well in a 6-well plate and 3 or 4.5 x 10^5 per well in a 96-well plate (Nunc, Greiner).

2.3 Incubation with the drug

After 24 h incubation of the cells under conditions described above, the medium was replaced with a fresh one containing the compounds studied, at a range of Selol concentrations selected on the basis of pilot tests and a range of sodium selenite concentrations selected on the basis of literature data. The following concentration ranges were used, expressed as selenium concentration: sodium selenite – 1 to 12 μM Se, and Selol – 6.25 to 500 μM Se. The effect of the compounds studied on HeLa cells was observed after 24 and 48 h of incubation. The control sample was a cell culture grown on a plain medium (in the case sodium selenite) and on a medium with "empty" liposomes added (in the case of Selol), in an amount corresponding to the highest concentration of the given compound used in the given test.

2.4 Cell viability assessment - MTT test

After 24 or 48 h of incubation with the placebo or the substance studied, the medium was removed from over the cells and each well was washed with 75 μl Ca^{2+}/Mg^{2+}-free PBS. Then to each well 50 μl of MTT reagent, 0.25 mg/ml (tetrazolium salt solution in Ca^{2+}/Mg^{2+}-free PBS prepared beforehand) were added. After 4 hours the formed formazan crystals were dissolved by adding to each well 200 μl of 99.7% isopropanol. The absorbance of solutions was measured spectrophotometrically at the wavelength of 540 nm, using a plate reader (Labsystems iEMS Reader MF).

Assuming the cell viability in the control culture as 100%, the viability of cells in samples with the compounds studied were computed on the basis of the absorbance measured.

2.5 Total protein determination – Bradford method

After 24 or 48 h of incubation with the placebo or the substance studied, the cells in the wells were washed with Ca^{2+}/Mg^{2+}-free PBS and then removed from the surface of the wells by scraping, then centrifuged for 7 min. at 1500 rpm. The sediment obtained was washed with PBS and centrifuged again. The cells were then suspended in deionized water.

In order to determine the total proteins, 0.5 ml of cell suspension was subjected to lysis with 0.5 ml NaOH (1 M). After intimate mixing

of the cells, 20 μl of the lysate were transferred into each well of a 96-well plate in 6 turns. At the same time a standard curve for albumin was plotted within the range of 1 to 10 μg/ml. 180 μl of Bradford reagent were added to each well and absorbance was measured using a 600 nm filter on a Multiscan Plus 314 instrument (Labsystem). Assuming the total protein content in control cells as 100%, the viability rate of cells in tested samples was calculated.

2.6 Cytometric determination of cell apoptosis induction – Annexin V and propidium iodide test

After 24 or 48 h of incubation with the placebo or the substance studied, the medium from over the cells and the cells, after trypsinizing thereof, were collected into centrifuge test tubes. All this was centrifuged for 7 min. at 1500 rpm. The sediment was washed with PBS and centrifuged again. Upon removing the supernatant, the cells were suspended in a binding buffer (supplied with the kit), in an amount corresponding to the concentration of 1 x 10^6 cells/ml. Afterwards 100 μl portions of the suspension were transferred into cytometric test tubes. 5 μl Annexin V and 5 μl of propidium iodide were added to each test tube. The contents of the test tubes were stirred in darkness for 15 minutes. After that time 400 μl of binding buffer were added and analysis was carried out on a FACS Calibur flow cytometer (Becton Dickinson), using CellQuest software.

2.7 Determination of selenium penetration rate – ICP-MS method

0.5 ml of cell suspension, prepared as described in item 2.5 above, were digested in nitric acid, using microwave energy, in a closed 4-stage system. The digested matter was transferred quantitatively to 20-ml measuring flasks. 20 μl of internal standard solution (Co standard, 10 μg/l) were added and made up to the mark with water. Selenium determination was performed on a Thermo Electron X Series II inductively coupled plasma mass spectrometer from Thermo Electron Corporation. The optimum measuring range in the ICP-MS method for this element is 0.1-2.5 μg/l.

3. Discussion of results

In order to quantitatively assess the cytotoxic activity of potential chemotherapeutics, various cell tests are applied in vitro cultures, wherein various parameters associated with cell growth (proliferation) are determined. In this study we have used indirect measurement of activity of mitochondrial dehydrogenase (MTT), determination of total protein, based on the ability of cell proteins to bind with Coommasie Brilliant Blue dye and on the ability of the compounds studied to induce apoptosis in HeLa cells.

3.1 Effect of sodium selenite and Selol on viability of HeLa cells (MTT test)

The toxic effect of Selol and sodium selenite on HeLa cells was assessed by means of the MTT test, on the basis of changes in the activity of succinate dehydrogenase, a mitochiondrial enzyme present in living cells. This enzyme transforms a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into its reduced form, insolube formazan, which precipitates as crystals, the latter being soluble in isopropanol. Absorbance of isopraponol solution measured at the wavelength of ca. 540 nm is proportional to the number of living cells in the culture. The results obtained were used to assess the viability of cells in samples with the compounds studied after 24 or 48 h incubation.

The concentration of selenium inhibiting cell growth by 50% (IC_{50} – a measure of cytotoxic activity of substances) was determined from the cell survival rate vs. selenium concentration curves (Fig. 1).

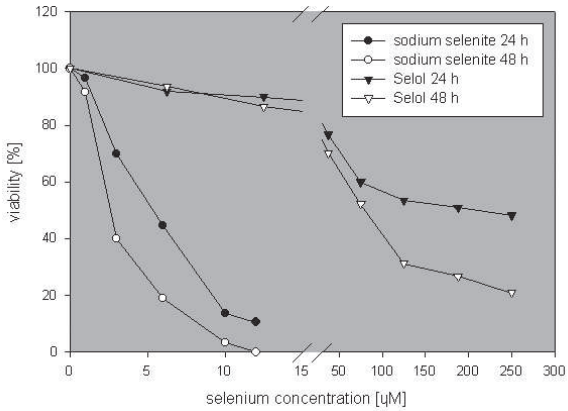

Fig. 1. Effect of selenium in sodium selenite and Selol on the survival rate of HeLa cells (MTT test)

It was found that after only 24 h of incubation in the presence of sodium selenite a significant decrease in the number of living HeLa cells was observed, that decrease depending on the concentration of selenium added to the medium. Depending on the time of incubation (24 or 48 h), IC_{50} for this compound was 5.9 μM and 2.6 μM Se, respectively. In the case of 24 h incubation with Selol, the decrease of cell survival rate to 50% (IC_{50}) was only observed at selenium concentration of ca. 216 μM. The diagram presented shows that the survival rate curve is much flatter in this case and that for the highest concentration used (250 μM Se) the percentage of living cells after 24 h incubation is still ca. 45%. Extending incubation time to 48 h caused the shifting of IC_{50} value towards lower selenium concentrations in the medium, $IC_{50} \sim 80$ μM Se. No cytotoxic effect of "empty" liposomes, used as the carrier for Selol, has been observed in this study. This means that the liposomes alone have no toxic effect and that they may be assumed to be an appropriate control reference in this experiment and that 100% survival rate may be adopted in this case. In view of the high toxicity of sodium selenite, 48 h incubation was abandoned in further study.

3.2 Effect of the compounds studied on total protein content (Bradford method)

The decrease in the number of living cells in the cultures, observed in the MTT test, which depended on the concentration of selenium in the medium and on incubation time, was also reflected in the determined total protein content. The total protein content in cell cultures incubated for 24 h with sodium selenite and for 24 and 48 h with Selol, with regard to the control sample, decreased with increasing selenium concentration (Fig. 2).

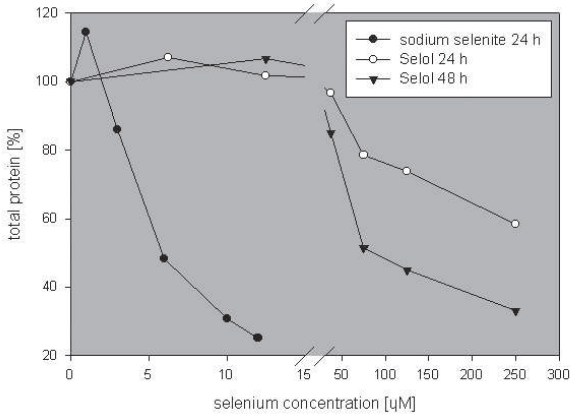

Fig. 2. Effect of selenium in sodium selenite and Selol on total protein content (Bradford method)

In the case of both compounds, and both incubation times, only at the lowest selenium concentration used (1 μM for sodium selenite and 12.5 μM for Selol) did the protein content increase as compared to the control sample, by ca. 14 and 7%, respectively.

Comparison between the rates of total protein content decrease caused by increased concentration of the compounds studied in the medium demonstrates much higher activity of sodium selenite in this regard. In the case of this compound, a significant difference in total protein content, that is ca. 15% in relation to the control sample, is already observed at selenium concentration of 3 μM. In the case of Salol, comparable decrease in protein content occurs after 24 h incubation at the concentration of more than 37.5 μM Se. In the experiment with sodium selenite, at the concentration of ca. 6 μM Se, 50% total protein decrease was observed after 24 h. In the case of Selol, at the highest concentration used, protein content after 24 h incubation was about 60%. Total protein content was decreased to 50% in the case of selenium concentration of ca. 75 μM after extending exposition time to 48 h.

3.3 Proapoptotic activity of sodium selenite and Selol (cytometric study)

The ability of the compounds studied to induce apoptosis was investigated, after 24 h incubation with sodium selenite and after 24 and 48 h incubation with Selol, in a test with Annexin V and propidium iodide. The results indicate a distinct difference in the proapoptotic activity of the compounds (Fig. 3), which correlates with the data obtained in the MTT test and total protein content determination. The experimental setup used enabled differentiation between living, apoptotic and necrotic cells.

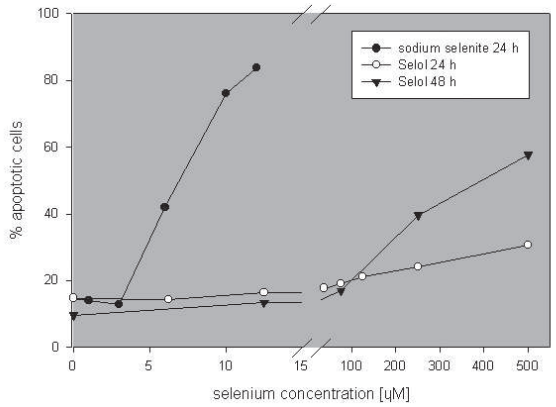

Fig. 3. Percentage of apoptotic HeLa cells vs. concentration of selenium supplied in the form of sodium selenite and Selol (Annexin V and propidium iodide test)

The data obtained and presented in Table 1 indicate that in the case of sodium selenite, with increasing concentration thereof, a distinct increase of the number of apoptotic cells is observed, which attains the level of 83.6% at the highest concentration used. In the case of Selol, for comparable selenium concentration (12,5 μM), the percentage of apoptotic cells is only 15%. Extending cell exposition time to Selol to 48 h has significantly intensified apoptosis at higher concentrations, i.e. above 100 μM Se. For the highest concentration used (500 μM Se), the number of apoptotic cells has nearly doubled in the population after 48 h. From the viewpoint of cytotoxic mechanism of the action of the compounds studied, only a small percentage of necrotic cells were observed after both incubation times. The percentage of necrotic cells in the case of sodium selenite and Selol varied between 0.4÷2.5% and 0.3÷5.5%, respectively.

Table 1

Percentage of HeLa cells (live (L), apoptic (A) and necrotic (N)) in culture subjected to the action of sodium selenite and Selol in the selected range of selenium concentrations

Percentage										
Se conc., μM	Sodium selenite			Se conc., μM	Selol					
	L	A	N		L	A	N	L	A	N
	24 h				24 h			48 h		
0	85.1	14.5	0.4	0	83.9	14.8	1.3	90.0	9.7	0.3
1	85.3	14.1	0.6	6.25	84.0	14.4	1.6	-	-	-
3	85.8	13.0	1.2	12.5	84.1	15.0	0.9	86.1	13.4	0.5
6	56.6	42.0	1.4	37.5	81.3	17.8	0.9	-	-	-
10	21.5	76.0	2.5	75.0	82.7	16.5	0.8	82.6	17.0	0.4
12	14.5	83.6	1.9	125.0	78.0	21.3	0.7	-	-	-
				250.0	72.1	24.2	3.7	59.7	39.6	0.7
				500.0	63.8	30.7	5.5	41.0	57.7	1.3

3.4 Penetration of selenium into HeLa cells (ICP-MS method)

The ability of selenium of the compounds studied to penetrate into HeLa cells was assessed using ICP-MS. After 24 h incubation it was found that there was a distinct differentiation of the amount of selenium detected in cells depending on the compound used. The data obtained indicate that selenium of the sodium selenite solution has a higher ability to permeate through the cell membrane than selenium delivered in the form of Selol in liposomes. The mean values (n=3) of the determined content of selenium derived from both compounds and normalized with regard to the results of total protein determination in cells (item 3.2.), in relation to initial selenium concentrations are shown in Figure 4.

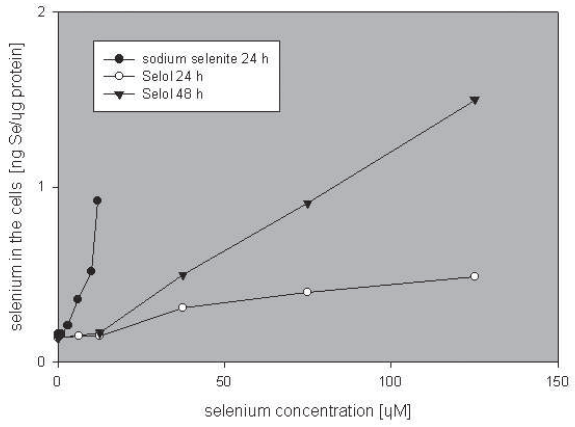

Fig. 4. Penetration of selenium from sodium selenite and Selol into HeLa cells (ICP-MS)

After 24 h incubation of HeLa cells with the compounds studied at the lowest concentrations used (1 μM for sodium selenite and 12.5 μM for Selol), the amount of selenium determined in cells was comparable to that in the cells of the control sample. Consistent increase of selenium detected in cells was observed with the growing concentration of selenium in the solution, starting from 3 μM Se for sodium selenite and 37.5 μM Se for Selol. In the case of sodium selenite the penetration curve after 24 h incubation (Fig. 4) is close to exponential, whereas in the case of Selol it is close to logarithmic. This means that the difference in penetration rate within the studied range of concentrations increases with increasing selenium concentration. Similar selenium content, i.e. ca. 0.3 ng Se/μg protein, was detected for initial concentrations of 6 μM Se and 37.5 μM Se, for sodium selenite and Selol, respectively. The difference found in penetration rate showed that selenium absorption from sodium selenite solution was 6 times faster.

Much higher selenium concentrations in cells after administering Selol were observed when incubation time was extended to 48 h. In this case, with initial selenium concentration of 75 μM, penetration was comparable with the highest value obtained for sodium selenite, i.e. ca. 0.9 ng Se/μg protein. After 48 h the amount of selenium detected in cells for the highest Selol concentration used (125 μM Se) rises significantly: a threefold increase in relation to the amount detected after 24 h.

Table 2 summarizes the results of the MTT test, cytometric test and total protein determination for similar amounts of selenium

Table 2

Comparison of HeLa cells survival rate determined by three methods for comparable penetration rates of selenium into cells: 0.3 ng Se/μg protein, determined after 24 h incubation of cells with sodium selenite at a concentration of 6.0 μM Se, and with Selol at a concentration of 37.5 μM Se

Method	Percentage of live cells, %	
	Sodium selenite	Selol
MTT	44.7	78.0
Bradford	48.4	96.7
Annexin V and propidium iodide test	56.6	81.3

penetrating into the cells from both compounds, i.e. ca. 0,3 ng Se/μg protein, corresponding to initial concentrations of 6 μM Se and 37.5 μM Se for sodium selenite and Selol, respectively. These data indicate that after 24 h incubation, sodium selenite inhibits cell growth ca. 1.7 times stronger than Selol.

Table 3 lists the results of the above tests for comparable selenium content determined in cells, i.e. ca. 0.9 ng/μg protein. The values listed were obtained for the highest seleniumconcentration used with sodium selenite (12 μM), after 24 h incubation, and with Selol (75 μM Se) after 48 h incubation. In the case of sodium selenite, the amount of selenium penetrating into the cells caused significant reduction of cell survival rate and strongly induced apoptosis, which was reflected in the amount of total protein determined. In the case of identical quantity of selenium determined after 48 h incubation with Selol, the cell survival rate obtained in MTT tests and resulting from total protein determination was ca. 50%, whereas the number of living cells obtained in the Annexin and propidium iodide test was ca. 80%.

Table 3

Comparison of HeLa cells survival rate determined by three methods for comparable penetration rates of selenium into cells: 0.9 ng Se/μg protein, determined after 24 h incubation of cells with sodium selenite, at a concentration of 12.0 μM Se, and after 48 h incubation with Selol, at a concentration of 37.5 μM Se

Method	Percentage of live cells, %	
	Sodium selenite	Selol
MTT	10.6	52.2
Bradford	25.2	51.6
Annexin V and propidium iodide test	14.4	82.6

4. Summary and Conclusions

The studies made so far on HeLa tumour cells and the two selenium compounds, sodium selenite and Selol, confirm the differences reported in the literature on the utilization by the cell of selenium(IV) from an inorganic and an organic compound [9÷11].

When comparing the results obtained for the two compounds after 24 and 48 h incubation, the MTT test demonstrated much higher toxicity of sodium selenite, evidenced by the determined IC_{50} values. For both periods of incubation duration, 50% inhibition of cell viability occurred in the case sodium selenite at selenium concentration ca. 30 times lower than in the case of Selol. Similar difference in the activities of both compounds was shown by total protein determination using the Bradford method, whereas it was also observed that the antiproliferation effect produced by sodium selenite on HeLa cells was several dozen times stronger than that of Selol. As regards the results of tests on penetration of the two compounds into cells in an in vitro culture, attention should be drawn to the distinct difference in the penetration rates of selenium from sodium selenite solution and from suspension of Selol-containing liposomes. ICP-MS investigations have shown that the small inorganic molecule of sodium selenite penetrates into the cell much faster, and that it is probably transferred in a passive manner due to concentration differences. The transfer mechanism of liposomes with Selol is presumably different. After 24 h incubation with Selol, only a slight increase of selenium concentration was observed. This indicates that the cell cannot absorb, within a defined period of time, more of the compound of a more complex structure and higher molecular weight. Extending incubation time to 48 h strongly changed the shape of the curve, making it similar to that of sodium selenite. This indicates that extending incubation time of cells with Selol improves penetration to such a degree that the level of selenium determined in cells becomes similar to that obtained with the highest sodium selenite concentration used. Analysis of the data in Tables 2 and 3 leads to the conclusion that in the case of Selol, which is a mixture of selenitetriglycerides, supplied in the form of liposomes, the antiproliferation effect of this compound does not depend solely on selenium penetration into the cell. The final effect in the cell depends also on the time necessary to release selenium from the complex organic compound. This is confirmed by the results obtained in the MTT test and total protein content determination performed after 48 h incubation with Selol.

Taking into consideration the concentration ranges of both compounds, as used in the studies, i.e. 1÷12 μM Se for sodium selenite and 6.25÷500 μM Se for Selol, and the results obtained, it may be concluded that the therapeutic index of Salol is probably much higher than that of sodium selenite. In the case of Selol a distinct decrease of survival rate observed in the MTT test and total protein determination occurred within the concentration range of 37.5÷75 μM Se and 12.5÷37.5 μM Se, after 24 and 48 h incubation, respectively. In the case of sodium selenite the decline in cell survival rate appeared already at Se concentration of 3 μM (Figs. 1 and 2).

The results obtained for sodium selenite in the study of proapoptotic activity of both compounds are similar to those of the MTT test and total protein determination. Substantial decrease in the number of living cells, accompanied by increase of the number of cells in the apoptosis phase, occurred after 24 h incubation with this compound in doses corresponding to selenium concentration 3÷6 μM and was intensified with growing concentration. Such distinct tendency was not observed for Selol. In the case of 48 h incubation with Selol, within the concentration range (in terms of selenium) of 6.25÷125 μM, increase of proapoptotic activity was not observed. This activity increased strongly only at concentrations above 125 μM Se, significantly reducing cell survival rate. The results obtained confirm literature reports on sodium selenite activity and allow to formulate a conclusion that the cytotoxic mechanism of the action of both compounds on HeLa cells is associated, *inter alia*, with apoptosis induction, which presumably is the cause of survival rate decrease [12, 13].

Total protein determinations carried out in the experiments confirm the literature reports on the stimulating effect of low selenium doses on the growth of tumour cells [14]. In the case of both compounds studied, for the first concentration used, i.e. 1 μM Se and 6.25 μM Se, for sodium selenite and Selol, respectively, a slight increase of protein content was observed in comparison to the control sample.

To summarize, diverse toxic effect on HeLa tumour cells has been identified in the case of both compounds, and the effect of their interaction with the tumour cell depends on the chemical structure, concentration and time of action of the compound. The studies conducted also indicate that the significant difference between the cytotoxic activities of sodium selenite and Selol are the result of different degree of penetration of selenium from these compounds into the cell. This translates into acute cytotoxicity of sodium selenite within a narrow range of concentrations, and mild cytotoxicity over a longer period of time of Selol, the efficacy of the latter being associated with prolonged release of selenium from the oily form of the compound after penetrating into the cell.

In view of the above it may be stated that therapeutic application of sodium selenite is limited due to the toxicity of the compound. Selol, on the other hand, shows moderate toxic action towards the cells, acts

longer and within a broader range of concentrations, which is probably due to its more complex mechanism of action on cells, and raises hopes for its successful therapeutic application.

The differences found in cytotoxic activity of sodium selenite and SeIol require confirmation by tests on a larger number of tumour and healthy cells. Applicability of SeIol in tumour therapy depends, to much extent, on the proof of its selective and toxic action limited to pathologically altered cells. The research in this area is pending, and the results obtained will be the subject of a subsequent paper.

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