

The activity of Selol in multidrug-resistant and sensitive human leukemia cells

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Abstract. Selol is a mixture of selenitetriglycerides synthesized from sunflower oil. As it contains the element selenium in its structure, it is suspected to exhibit chemopreventive and anticancer activity. In this study, the ability of Selol to inhibit cell proliferation and to induce apoptosis was investigated. Three cell lines were used: leukemia HL-60 cell line and multidrug-resistant HL-60/Dox (resistant to doxorubicin) and HL-60/Vinc (resistant to vincristine). Selol was shown to reduce the cell number as a result of treatment with increasing concentrations. For selected concentrations the evidence of apoptosis (changes in mitochondrial potential and caspase activity) was investigated, as well as changes in lysosome distribution. The study has shown that Selol overcame the cell resistance, as doxorubicin-resistant cells were more sensitive towards Selol than sensitive cells.

Introduction

Despite an evident progress in cancer diagnosis and treatment, there is a constant need of a drug which could arrest the premalignant process, thus reducing the risk of cancer. The role of selenium (Se) as an antioxidant and anticancer agent is very well documented in the literature (1-4). In addition, it has been proven that an adequate concentration of selenium can restore the sensitivity to chemotherapeutic agents of cytostatic-resistant malignant cells (5,6). The highest activity as a free radical scavenger and anticancer agent is assigned to selenium compounds containing selenium at the +4 oxidation level.

Selenitetriglycerides are a new semi-synthetic group of compounds containing selenium (+4). Selol is a mixture of selenitetriglycerides from sunflower oil that was obtained as previously described (7). The putative structure of Selol that was devised on the basis of ¹H and ¹³C NMR study is presented in Fig. 1.

The distribution profile of Selol in rats and mice was investigated after oral, subcutaneous and intraperitoneal administration (8,9). Selol was distributed in the whole body. Most of the data were obtained following oral administration as this is the proposed route of administration in the human. The highest concentrations were found in the suprarenal gland, testes, epididymis, brain cortex and white matter of the brain. Lower concentrations were found in the cerebellum, liver, kidneys, lungs and spleen. The heart contained the lowest Selol concentration (8,9). The presence of Selol in the brain indicates that it crosses the blood-brain barrier, which is an important indicator in overcoming plasma transporter activity. These results are probably connected with a high lipophilicity of Selol.

Furthermore, the metabolism of Selol was investigated and experiments revealed complete elimination within 24 h with urine. The results indicate that the pharmacological action of Selol is more complex than 'classical' selenium compounds (Na₂SeO₃, Na₂SeO₄, SeO₂, selenocysteine and selenomethionine). Further studies are required to classify its biological function (10). The studies are being conducted and the results are forthcoming.

In single-dose toxicity studies performed in rats, LD₅₀ was 100 mg Se/kg after oral administration of Selol. The subcutaneous and intraperitoneal administration of Selol showed extremely low toxicity, therefore precise determination was impossible. Selol did not show a cumulative toxicity (11). Selol did not show mutagenicity which was proven with Salmonella strains. Selol's low toxicity, contrary to other selenium compounds, make administration of higher doses of selenium possible, which may lead to a more potent anti-cancer activity.

As Selol or its metabolites cross the brain-blood barrier and selenium compounds are recognized to restore sensitivity of multidrug-resistant neoplastic cells, in the present study, the effect of Selol on human leukemia cell lines, both sensitive and multidrug-resistant, was evaluated. The aim of the present

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Abbreviations: IC₅₀, 50% inhibitory concentration; Dox, doxorubicin; Vinc, Vincristine; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide

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study was to determine the *in vitro* anti-leukemic activity of new selenium compounds against the HL-60 cell line and its two sublines with different MDR phenotypes. HL-60/Vinc has an overexpression of P-glycoprotein (Pgp) and HL-60 has an over expression of MRP1 protein. Both proteins are the main cause of MDR status of cancer cells (12,13).

Changes in cell growth, mitochondrial membrane potential, lysosome distribution, multidrug resistance status and also the cell cycle were evaluated in three cell lines after 48 and 72 h of incubation with Selol. In comparison, the cytotoxicity of Na₂SeO₃ was also evaluated for these cell lines.

Materials and methods

Materials. Selol was synthesized in the Department of Drug Analysis at Warsaw Medical University as described previously (7). In the present study Selol 5% was used, which designates the declared content of Selenium (IV) as 5%.

JC-1, Vybrant™ Multidrug Resistance assay kit and LysoTracker were purchased from Molecular Probes (Eugene, OR). D₂R (Asp₂-rhodamine 110 conjugate) was purchased from Calbiochem. MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide], sodium selenite and all media constituents were purchased from Sigma.

Cells. Human leukemia cells HL-60, vincristine-resistant human leukemia subline HL-60/Vinc and doxorubicin-resistant HL-60/Dox cells were obtained from Kansas State University, Manhattan, KS, USA. These cells were grown in RPMI-1640 medium supplemented with 20% heat-inactivated fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin (250 ng/ml), L-glutamine (2 mM), 1% non-essential amino acids, 1%, 0.5% glucose.

For all experiments, HL-60, HL-60/Vinc and HL-60/Dox were seeded at a density of 2.5x10⁵ cells/ml.

Cell growth measurement. HL-60, HL-60/Vinc and HL-60/Dox cells were plated in 96-well plates. All cells were incubated with increasing concentrations of Selol for 24, 48 and 72 h.

At the end of incubation, before the MTT test was conducted, cells were examined under the microscope and images of cultures were taken. Next, the culture medium was removed, cells were washed with PBS, and the solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromide] in PBS was added. After 3.5 h of incubation, cells were centrifuged and the untransformed MTT solution was removed and acidic isopropanol, containing 0.04 N HCl was added to each well (14). When the MTT reduction product, formazan, was solubilized completely in propanol, the absorption of each well was measured with a Power XS Biotek Spectrophotometer plate reader at a 570-nm test and a 690-nm reference wavelength respectively. The final results are shown as the mean of three independent experiments.

IC₅₀ calculation. The IC₅₀ concentration which causes inhibition of 50% cell viability compared to control cells was calculated using Sigma-Plot 2000. The following equation was fitted to experimental data obtained for cells incubated with increasing Selol concentrations (15):

$$E = E_0 \frac{(E_{\max} \times C_g)}{IC_{50}^g + C_g}$$

Mitochondrial membrane potential ($\Delta\psi_{mt}$) measurements.

$\Delta\psi_{mt}$ was measured using JC-1, which is a mitochondrial dye that stains mitochondria in living cells. The dye accumulates in mitochondria of healthy cells, forming aggregates that fluoresce red. In apoptotic cells it remains in the cytoplasm and fluoresces green. All cell lines were plated in an 8-well coverglass chambered slide (Nunc), and treated with a series of Selol concentrations. Slides were rinsed with PBS and stained with 7.5 mg/ml JC-1 in PBS at 37°C for 10 min (16). Slides were cytospin centrifuged before each step. The fluorescence was measured with a confocal microscope Olympus FV500. An Argon (488-nm) laser was employed to excite both monomers and aggregates of the dye. The fluorescence was collected by 505- to 525-nm and 560- to 610-nm filters for the monomers and aggregates respectively.

Caspase activity. The caspase activity was measured by means of confocal microscopy. Cells, after 48 h of incubation were rinsed with PBS and incubated with 5 μg/ml D₂R for 10 min at 37°C. D₂R is a non-fluorescent substrate for caspases and is intracellularly cleaved to rhodamine 110 (17). The fluorescence was measured with a confocal microscope Olympus FV500. The fluorescence was excited with 488 nm and collected using a 510- to 525-bandpass filter.

Lysosome distribution changes. Lysosomes were stained with fluorescent dye LysoTracker® according to the vendor's protocol. The dye accumulates in the organelles that have acid pH. By examination of the dye disposition, the changes in lysosome location were evaluated. The fluorescence was measured with confocal microscope Olympus FV500. The fluorescence was excited with 488 nm and collected using a 510- to 525-bandpass filter.

Cell cycle phase distribution changes. Following a 24- and 48-h incubation, cells were rinsed twice with PBS (phosphate-buffered saline, pH 7.4), fixed with 70% ice-cold ethanol and stored at -20°C until examined. For analysis, cells were stained with DAPI dye. Cell cycle distribution was measured using FACS Vantage Cytometer, equipped with an emitting 351-nm laser. Cell cycle was analyzed with a ModFit LT program. Experiments were performed in duplicate.

MDR. The activity of Pgp in MDR cells was measured using Vybrant Multidrug Resistance assay kit. The assay quantifies the activity and the degree of inhibition of Pgp activity by the measurement of the increase in intracellular calcein fluorescence. Calcein AM is a nonfluorogenic substrate for esterases, which forms a fluorescent product, calcein, after enzymatic modification. The substrate can penetrate into living cells, but the product is charged and becomes caged inside the cell. Cells expressing a high level of Pgp or other related protein rapidly extrude nonfluorescent Calcein AM from the plasma membrane, in this way reducing the fluorescence from inside the cells.

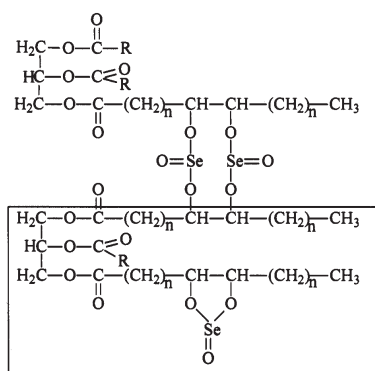


Figure 1. A putative structure of Selol. The structure of Selol majority to 5% Se (IV) is present in the frame (Octadeca-9,11-dienoic acid 1-[7-(5-non-3-enyl-2-oxo-2?4-[1,3,2]dioxaselenolan-4-yl)-heptanoxyloymethyl]-2-octadeca-9,13-dienoyloxy-ethyl ester).

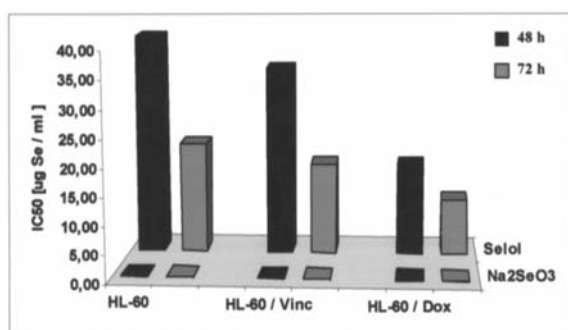


Figure 2. Comparison of IC₅₀ values after 48 and 72 h of Na₂SeO₃ and Selol treatment for HL-60, HL-60/Vinc and HL-60/Dox cell lines.

To validate the assay, cells were incubated with 1, 5 and 10 μ M of verapamil and cyclosporine for 15 min at 37°C. Verapamil is a calcium channel blocker that inhibits Pgp activity noncompetitively and cyclosporin A is a competitive inhibitor of Pgp-drug binding. Subsequently, cells were stained with Calcein AM according to the protocol.

Cells incubated with Selol were also stained with Calcein AM. Cells were examined with a confocal microscope and the fluorescence was measured with an Olympus FV500. The fluorescence was excited with 488 nm and collected using a 510- to 525-bandpass filter.

Results

Cell viability, cell cycle and apoptosis. As shown in Fig. 2, IC₅₀ for sodium selenite was significantly lower than for Selol in each cell line, regardless of the MDR status of the cell line and incubation time. IC₅₀ after Selol treatment was time dependent and it decreased with prolonged incubation. It is worth emphasizing that after a 24-h incubation, the evaluation of IC₅₀ was impossible due to the low cytotoxicity of this compound. Selol displayed the strongest activity toward the HL-60/Dox line, which was also confirmed by

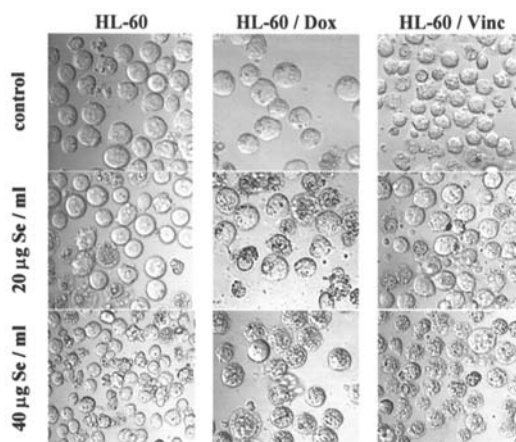


Figure 3. Morphology of HL-60, HL-60/Dox and HL-60/Vinc cells after 72 h of selenium treatment.

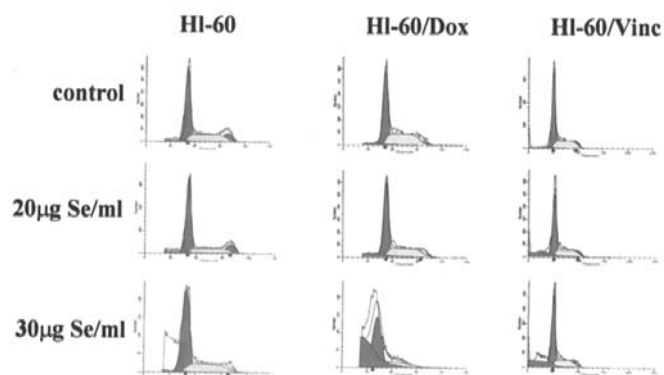


Figure 4. The effect of Selol on cell cycle distribution after 72 h of Selol treatment.

morphology evaluation. In Fig. 3 the changes in cell morphology after 72 h are presented. The changes indicated cell death, thus the apoptosis markers (mitochondria membrane potential changes, caspase activity and DNA content) were studied together with lysosome distribution changes. As shown in Fig. 4, after Selol treatment more green fluorescence was visible which indicated depolarization of mitochondrial membranes. To confirm the visual results, the average ratios green/red fluorescence were calculated using FluoView 4.3 program. This confirmation was especially important in the resistant cells, since after Selol treatment a permeability of plasma membrane changes and more JC-1 dye entered the cells. Both results showed that the most extensive depolarization of mitochondria was in the HL-60/Vinc cells; less but still significant changes were observed successively in the HL-60/Dox and HL-60 cell lines.

The induction of apoptosis was evaluated also by D₂R staining. D₂R is the dye that is used to detect the activity of enzymes from the caspase family. No significant changes between caspase activity in the control and Selol-treated cells were observed.

Cell cycle phase distribution change analysis demonstrated no significant influence of Selol to this parameter, however it

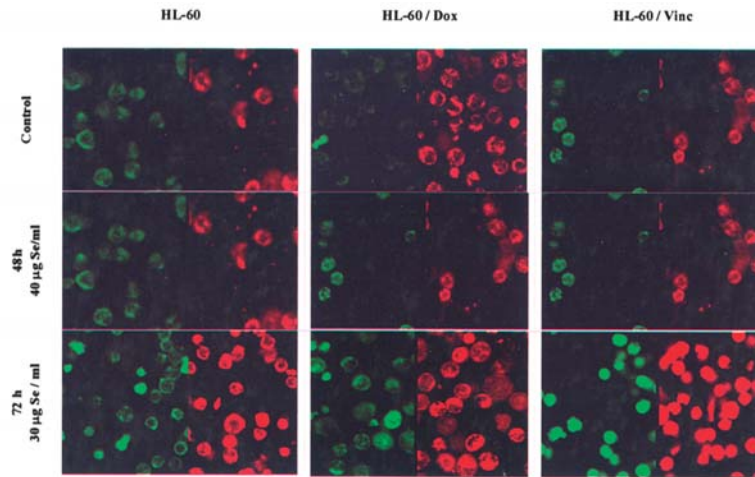


Figure 5. Microscopic images of changes in mitochondrial membrane potential ($\Delta\Psi_m$). Cells were incubated with Selol for 48 and 72 h and examined by confocal microscopy to detect changes in the mitochondrial membrane potential $\Delta\Psi_{mt}$ aggregates (red fluorescence-polarized mitochondria) and monomers (green fluorescence-depolarized mitochondria) of potential sensitive dye JC-1.

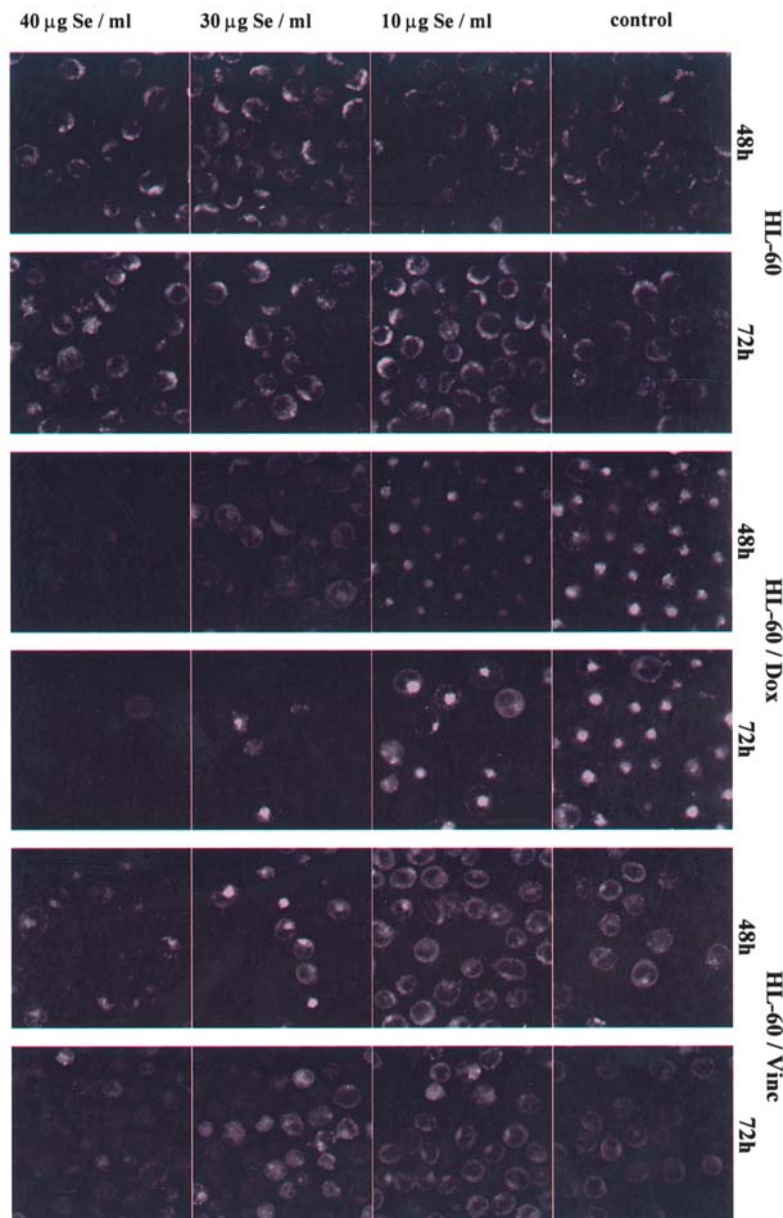


Figure 6. The effect of Selol on lysosome distribution. Cells were incubated for 48 and 72 h with Selol, stained with LysoTracker dye and examined with confocal microscopy.

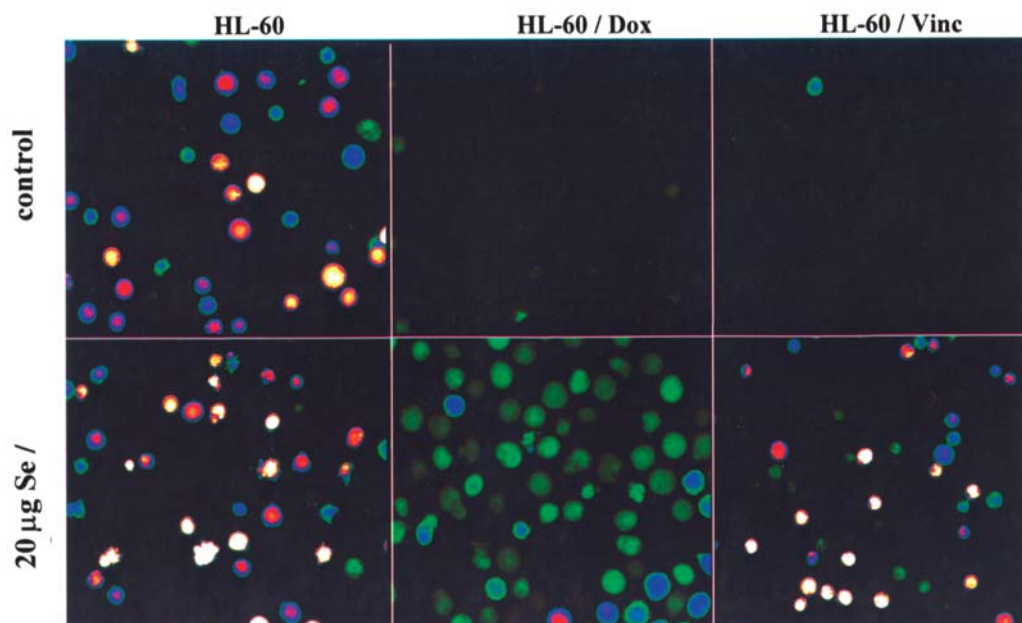


Figure 7. Microscopic images of Calcein AM staining. Cells were treated with Selol for 72 h and stained with Pgp substrate-Calcein AM.

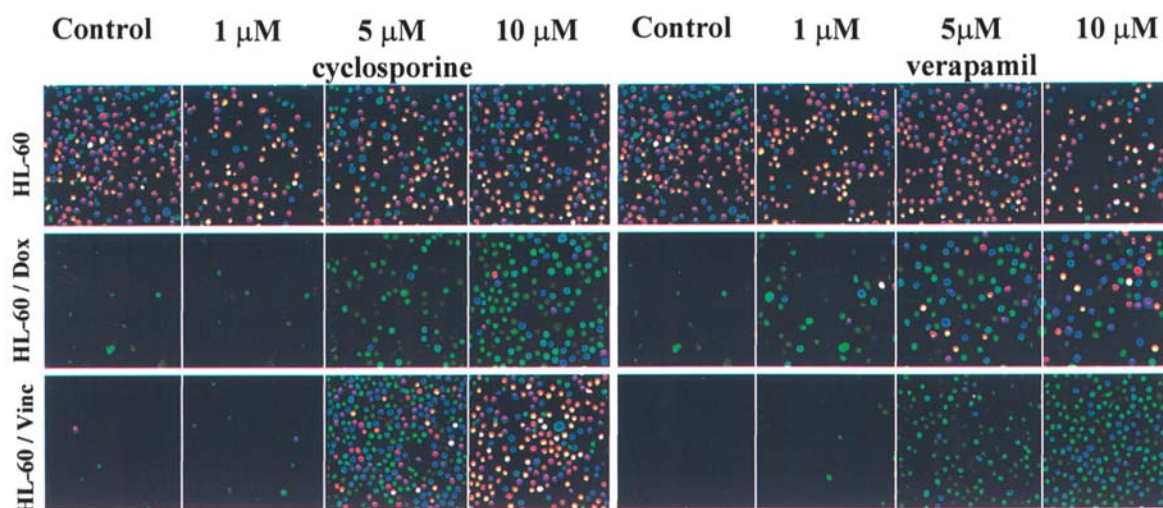


Figure 8. Microscopic images of Calcein AM staining. Cells were treated with increased concentrations of cyclosporin A and verapamil and subsequently stained with Pgp substrate-Calcein AM.

demonstrated the DNA fragmentation. As shown in Fig. 5, the most extensive DNA fragmentation was observed in HL-60/Dox cells, whereas in HL-60 almost no SubG1 peak was observed.

Lysosomes are organelles that are involved in cell death through a degradation of cell compartments. Changes in their location and intensity of fluorescence were evaluated to assess their implication in the cytotoxicity of Selol. As shown in Fig. 6 the changes were dose and time dependent. The largest effect was visible for HL-60/Vinc cells, where lower doses increased and higher doses caused almost the complete decline of fluorescence. A similar effect was observed in the HL-60/Dox cell line. In HL-60 the fluorescence only increased slightly, but no significant decrease was observed.

Evaluation of MDR transporter activity. Firstly, to confirm the MDR status of the cells and to validate the Vybrant MDR assay kit, cells were treated with two reference inhibitors of Pgp activity. The first one was verapamil and the other was cyclosporin A. Results are presented in Fig. 7. In HL-60 cells no significant changes in fluorescence were observed after treatment with any of the Pgp inhibitor concentrations. The HL-60/Dox cells responded to both compounds, however more to verapamil, whereas HL-60/Vinc was more sensitive to cyclosporin A. In both resistant cell lines the effect was dose dependent.

Similarly, the effect of Selol was evaluated. As shown in Fig. 8, the most significant effect of Selol on the activity of proteins involved in MDR was observed in HL-60/Vinc cells, however, it was also effective in HL-60/Dox.

Discussion

Selol is a new semi-synthetic compound containing selenium in its structure. Selenium is an element which was shown to possess chemopreventive activity in humans. It acts via many pathways but the exact mechanism is still not well understood (2).

The aim of this study was to evaluate for the first time the cytotoxicity of Selol and to compare the cytotoxic activity toward neoplastic cell lines that are sensitive and multidrug resistant. Three cell lines were studied: sensitive human leukemia HL-60 and multidrug-resistant HL-60/Dox and HL-60/Vinc. To compare the cytotoxicity of the two compounds containing selenium at the same level of oxidation, we treated cells with sodium selenite and with Selol. Cells responded differently to these compounds and the cytotoxicity of selenite was significantly higher. This is consistent with the results of toxicity *in vivo*, where Selol had a very low toxicity compared to other selenium compounds (1). In the next phase of our study only the activity of Selol was investigated. There was a significant difference between the response of the cell lines to Selol. It exhibited a stronger ability to decrease the cell viability of MDR cells. IC_{50} s calculated for both resistant lines were distinctly lower than those calculated for the sensitive line. Bjorkhem-Bergman *et al* (6) in their study on selenite cytotoxicity found the same relationship.

In addition, when comparing the results of mitochondrial membrane depolarization, a similar dependence was found. Most depolarized mitochondria were observed in the HL-60/Vinc and HL-60/Dox lines. Staining these cell lines after Selol treatment indicated an interesting result. Firstly, for the control, untreated cells, the staining of mitochondria was difficult as the dye, even at high concentrations, did not cause a proper staining. However, when cells were treated with Selol, the staining improved which indicated that Selol eased the dye intake to the interior of the cell. This is consistent with our further result concerning the overcoming of MDR by Selol in resistant cell lines.

The defeat of MDR was demonstrated in two ways: by comparison of IC_{50} values, but also directly by the assay which utilizes Calcein AM. Since both resistant cell lines have overexpression of protein from the ABC transporter family (18-20), we used reference substances to confirm our results. HL-60/Vinc cells have an overexpression of Pgp protein and were more vulnerable to cyclosporin A, a competitive inhibitor of Pgp (21). HL-60/Dox cells were more vulnerable to verapamil that is a calcium channel blocker and inhibits Pgp activity noncompetitively. Selol seemed to strongly overcome the resistance of HL-60/Vinc cells, which suggests that it can influence Pgp protein directly and competitively, however further studies are required to state the mechanism of Selol influence to MDR cells.

Also, the mechanism of cell death induced by Selol needs to be analyzed in more depth. Our results indicated at first that Selol can induce apoptosis. This was confirmed by mitochondria depolarization, however further experiments did not confirm our hypothesis. No caspase activation was observed and also no changes in phosphatidylserine location in cells (data not shown), which indicated another type of cell death (22). Cells also exhibited morphological changes

characteristic to autophagy (displayed a Swiss cheese-like appearance), especially the resistant cell lines (23). The LysoTracker dye dispersed throughout the whole cell, which indicated lysosomal changes that were probably involved in the digestion of the cell. The DNA degradation was mostly present in HL-60/Dox cells that were the most extensively affected by Selol. This can be caused by nucleases present in lysosomes, since in these cells the complete dispersion of lysosomal content was observed. The degradation of DNA was probably not the effect of caspase activity, since we have not detected caspase activation.

Summarizing, our study revealed cell growth inhibition in all cell lines, which suggests that Selol can be a promising anticancer agent. Moreover, the activity was stronger in the resistant cells, which suggests that it can overcome cell resistance to anticancer drugs and could be used as a chemosensitizing agent. It is clear that much additional research will be required before a complete understanding of the anticancer mechanism of Selol will be reached.

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