INTRODUCTION

Selenium (Se) is a trace element which is necessary for the correct functioning of the human body and is found in its every cell. Various analyses showed a link between an insufficient level of selenium in a diet and the occurrence of various illnesses, such as Keshan and Kashin-Beck disease as well as disorders of the circulatory system [1-4]. It is suggested that supplementing the diet of patients in critical condition (e.g. patients suffering from widespread burns or in cases of systemic inflammatory response syndrome - SIRS) with high levels of selenium results in a significant drop in mortality and reduces time to recovery [5-7]. As an important antioxidant, selenium also displays anti-inflammatory properties. It has been reported that in cases of chronic inflammation, such as in patients with mucoviscidose and cystic fibrosis of inflammatory bowel disease, a lowered serum level of selenium is observed [8,9].

A lot of attention has been paid to selenium with regards to anti-tumour prophylaxis. A link has been observed between levels of selenium in the body and the incidence of certain neoplastic diseases (e.g. prostate cancer) along with a positive effect of selenium supplementation, especially in patients with low plasma levels at the outset [10,11].

Selenium occurs naturally in various foods, which results in a relatively low incidence of acute selenium deficiency. The overall levels of selenium within a population, however, are dependent on geographical location. Individual levels of selenium depend on, amongst others, the form in which it is ingested, daily dose, individual metabolism and genotype, i.e. the occurrence of single nucleotide polymorphisms (SNPs) in genes responsible for selenoprotein synthesis [1,12]. In general terms, organic selenium compounds are easier to absorb when compared to inorganic compounds, whilst inorganic selenium compounds display increased toxicity when compared to organic compounds [13]. Compounds containing selenium at the +4 oxidation state display higher tissue affinity. They are capable of forming complexes with proteins as well as being more effectively incorporated into active sites of selenoprotein synthesis [1,12]. In general terms, organic selenium compounds are easier to absorb when compared to inorganic compounds, whilst inorganic selenium compounds display increased toxicity when compared to organic compounds [13].
Our own data, combined with reports in the literature concerning the pro-oxidative activity of selenium, at a specific dose, suggest that Selol could be used as an adjuvant in cancer therapy in the future [16-18]. Additionally, the antioxidising properties of selenium, along with the part it plays in various biological processes through selenodependent enzymes, suggest a possible prophylactic application of selenium in various diseases where a link between incidence or progression and selenium deficiency has been found, as well as those where the aetiology lies in an increased intracellular level of reactive oxygen species (ROS).

Mindful of the need to establish the safety aspects of dietary supplementation with Selol-based selenium, bearing in mind its oxidative properties, the work presented here intended to ascertain the cytotoxicity of Selol for the normal human BJ fibroblast and PNT1A prostate cells, as well as the transformed LNCaP prostate cell line. The aim was to show the differential cytotoxicity of Selol in normal and transformed cell lines.

In vitro studies included measuring cell viability using the MTT assay, measuring total protein levels using the Bradford assay as well as measuring pro-apoptotic properties using flow cytometric methods with annexin V and propidium iodide staining.

The toxicity of Selol was compared with the toxicity of sodium selenite, an inorganic selenium compound approved for use in dietary supplementation and often used in work towards ascertaining the anti-neoplastic mechanisms and preventative properties of selenium.

**MATERIALS AND METHODS**

**Compounds and reagents**

Selol was synthesized at the Department of Bioanalysis and Drug Analysis at the Medical University of Warsaw, as described in the patent [15]. A micellar solution of Selol was used (based on lecithin, water and Selol) with a declared selenium concentration of 5% (w/v).

Commercially available sodium selenite (Sigma-Aldrich) was used in the assays.

**Cell lines**

BJ (ATCC) - normal human fibroblasts.

PNT1A (ATCC) - normal human prostate cells.

LNCaP (ATCC) - human prostate adenocarcinoma cells.

**Reagents**

MTT (Sigma-Aldrich), isopropanol (Labscan), Bradford reagent: Bio-Rad Protein Assay (Bio-Rad), human albumin (Biomed). FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen), PBS, PBS without Mg²⁺ and Ca²⁺ ions (Institute of Immunology and Experimental Therapy, Polish Academy of Sciences), trypsin, EDTA (Lonza), EMEM (Lonza), RPMI (Lonza), Fetal Bovine Serum (Lonza), antibiotics: penicillin, streptomycin, amphotericin B (Lonza), water for injection (Polfa Lublin).

**Cell culture**

Cells were cultured at 37°C, 5% CO₂ in either EMEM (BJ cells) or RPMI (PNT1A and LNCaP cells), with appropriate additions of PBS and antibiotics. In the case of LNCaP cells, RPMI was further supplemented with HEPES, sodium pyruvate and glucose. Due to the differences in doubling times between the cell lines used, the initial density of the cell suspension was optimised to confer 70% coverage of the well (for either 96- or 6-well plates) prior to experiments being carried out, regardless of cell line or time of incubation with the compounds. PNT1A cells were cultured in the conditions described above for 24 hours prior to exposure, whilst BJ and LNCaP cells were cultured for 48 hours.

**Incubation with the compounds**

After initial incubation the cell media was replaced with media containing the compounds being investigated, at a range of concentration set based on pilot studies: Selol from 1.25 to 625 µM and sodium selenite from 1 to 20 µM, with regards to selenium concentration. The impact of Selol and was assessed after 24 and 48 hours of incubation for all cell lines, whilst the impact of sodium selenite was assessed after 24 and 48 hours of incubation for the normal cell lines, but due to the high cytotoxic activity, the impact of sodium selenite on the transformed cell line could only be assessed following 24 hours of incubation. The controls consisted of cells cultured in appropriate media (for sodium selenite) and in media with "empty" micelles (without Selol) - a placebo at a volume equivalent to the highest concentration of Selol employed in a given experiment.

**MTT assay**

Following incubation on a 96 well plate with either one of the compounds or a placebo, medium was aspirated from the cells, which were then washed twice with PBS without Ca²⁺ and Mg²⁺ ions, after which 1 ml of of water was added and left for approximately 10 minutes. Following this, the contents of each well was thoroughly mixed by pipetting until a uniform solution was achieved. In order to quantify the protein content, cell suspensions were lysed using 1 M NaOH. After thorough mixing, lysates were placed in wells of a 96 well plate, with 6 repeats prepared for each sample. A standard curve based on a solution of human albumin for a range of concentrations from 1 to 10 µg/ml was prepared in parallel. 180 µl of Bradford reagent was added to each well, following which the absorbance was measured using a 600 nm filter on a Multiscan Plus Type 314 spectrophotometer (Labystem).

**Bradford protein quantification assay**

Following incubation on a 6 well plate with either one of the compounds or a placebo, medium was aspirated from the cells, which were then washed twice with PBS without Ca²⁺ and Mg²⁺ ions, after which 1 ml of water was added and left for approximately 10 minutes. Following this, the contents of each well was thoroughly mixed by pipetting until a uniform solution was achieved. In order to quantify the protein content, cell suspensions were lysed using 1 M NaOH. After thorough mixing, lysates were placed in wells of a 96 well plate, with 6 repeats prepared for each sample. A standard curve based on a solution of human albumin for a range of concentrations from 1 to 10 µg/ml was prepared in parallel. 180 µl of Bradford reagent was added to each well, following which the absorbance was measured using a 600 nm filter on a Multiscan Plus Type 314 spectrophotometer (Labystem).

**Cytometric assessment of pro-apoptotic activity**

Following incubation with either one of the compounds or a placebo, the cells along with the medium were placed in centrifuge tubes, following which they were centrifuged. The resulting pellet was washed with PBS and centrifuged again. After the supernatant was removed, the remaining cells were suspended in a binding buffer (supplied with the kit), at a volume which would result in a concentration of 1×10⁶ cells/ml. Following this, set volumes of each suspension were transferred to cytometric tubes in accordance to the procedure outlined by the manufacturer, after which annexin V and propidium iodide were added to each tube. The tubes were then mixed and incubated in darkness for 15 minutes. Following incubation, 400 µl of binding buffer was added and the samples were then analysed using a FACS Calibur flow cytometer (Becton Dickinson) with CellQuest software.

**Statistical analysis**

For all the methods used to assess the cytotoxic activity of the examined compounds: Selol and sodium selenite, a number of independent repeats were carried out (from n=4 to n=6) for each experimental setup. The values quoted in the Results section are average values for each of the experimental points. No cytotoxic activity was noted for the placebo, which makes it a suitable control for the experiments in question with a value set at 100%. Statistical analysis was performed using SYSTAT software ver. 13.00.05 for Windows (Systat Software Inc., IL, USA). The data were expressed as mean ± standard deviation (SD). Statistical differences between groups were assessed using an ANOVA with appropriate post-hoc test selection: the Dunnett test for multiple comparisons in relation to the control group and the Bonferroni test for comparison of the effect of incubation time. Simple Pearson correlation analysis was used to indicate the strength of the relationship between the effect observed and the selenium concentration applied. p-values lower than 0.05 were considered statistically significant.
RESULTS

The impact of Selol and sodium selenite on the viability different cell lines (MTT assay).

The MTT assay was used to assess the viability of BJ, PNT1A and LNCaP cell lines following 24 and 48 hours of incubation with Selol and sodium selenite. The graphs depicting the percentage of viable cells depending on the concentration of selenium in the compounds used were used to assign an IC₅₀ value, which is a concentration at which cell growth is limited to 50%. This value is used to characterise the cytotoxic activity of analysed compounds.

Following a 24 hour incubation of BJ and PNT1A cells with Selol (Fig. 2), within the described range of concentrations, a limitation of cell growth to 50% was not observed, which made it impossible to assign an IC₅₀ value.

Extending the incubation to 48 hours had a visible impact on the viability of BJ cells, and resulted in an IC₅₀ value of 625 µM Se (SD = 6.1). In the case of PNT1A cells, extending the incubation time to 48 hours did not yield an IC₅₀ value, which is a concentration at which cell growth is limited to 50%. This value is used to characterise the cytotoxic activity of analysed compounds.

Following a 24 hour incubation of LNCaP cells with Selol for the highest investigated concentration of selenium (625 µM), the viability of LNCaP cells was 61.7% (SD = 3.1). Extending the incubation to 48 hours yielded an IC₅₀ value of 365 µM Se (SD = 3.7), which is almost half of the value calculated for the BJ cell line.

Following a 24 hour incubation of cell lines with sodium selenite, in a range of concentrations from 1 to 20 µM Se, a statistically significant drop in the number of viable cells when compared to the control was seen at concentrations as low as 10 µM Se and 6 µM Se, for BJ and PNT1A cells respectively, and 3 µM Se for LNCaP cells (Fig. 3).

The drop in viability correlated with the concentration of selenium added to the media (BJ: R² = 0.827; p<0.001, PNT1A: R² = 0.946; p<0.001 and LNCaP: R² = 0.866; p<0.001). Experimentally assigned IC₅₀ values for this compound were approximately 19 µM Se and approximately 11 µM Se, for BJ and PNT1A cell respectively, and 6 µM Se for LNCaP cells. As was the case with Selol, the transformed LNCaP cells displayed the greatest sensitivity towards the compound. Due to the high toxicity of sodium selenite observed after 24 hours of incubation, it was decided not to carry out an MTT assay following a 48 hour incubation.

The impact of the investigated compounds on total protein content

The results of the quantification of the total protein content, measured using the Bradford assay, for each of the cell lines following incubation with the investigated compounds have been presented in Figures 4 and 5.

The total protein content of the control was defined as 100%.

In the case of BJ cells, no statistically significant change in total protein content was observed following 24 hours of incubation for the entire explored range of Selol concentrations (125 - 625 µM Se), with the protein content at the highest concentration amounting to 97.8% of the control (SD = 1.7; p = 0.070).

In the investigated range of concentrations of selenium from Selol, a small yet statistically significant lowering of total protein content was observed in PNT1A cells following a 24 hour incubation (Fig. 4).

For the highest investigated concentration of selenium (625 µM) the protein content amounted to 88.2% of the control (SD = 2.6; p<0.001). Extending the incubation with Selol to 48 hours resulted a statistically significant drop in total protein content was observed, at concentrations ranging from 500 to 625 µM for BJ cells and from 250 to 625 µM for PNT1A cells. Following incubation at the highest concentration of selenium (625 µM Se), the total protein content of PNT1A cells dropped to 67.4% of the control (SD = 3.3,
The total protein content of the transformed LNCaP cells dropped significantly following a 24 hour incubation with Selol at a concentration of 125 µM Se (SD = 1.25; p<0.001). For the highest of the applied concentration of Selol, the total protein content was 63.1% of the control (SD = 3.05; p<0.001) and was, as was the case with cell viability measured using the MTT assay, approximately 20% lower than that of normal PNT1A cells. Extending the incubation time of LNCaP cells to 48 hours resulted in a further drop in the total protein content by approximately 10% for a selenium concentration of 125 µM and 20% for subsequent concentrations. The total protein content following incubation with Selol at a concentration of 625 µM Se was 41.0% of the control (SD = 2.54; p<0.001).

The results obtained for sodium selenite (Fig. 5) showed a concentration of 625 µM Se was 41.0% of the control (SD = 2.54; p<0.001) and was, as was the case with cell viability measured using the MTT assay, approximately 20% lower than that of normal PNT1A cells. Extending the incubation time of LNCaP cells to 48 hours resulted in a further drop in the total protein content by approximately 10% for a selenium concentration of 125 µM and 20% for subsequent concentrations. The total protein content following incubation with Selol at a concentration of 625 µM Se was 41.0% of the control (SD = 2.54; p<0.001).

The data presented in Figure 6 shows that no significant pro-apoptotic activity was recorded for both BJ and PNT1A cell lines, despite increasing the Selol concentration, for both incubation times.

The percentage of apoptotic cells remained at an average level of approximately 10.5% (SD = 1.2) after 24 hours and 14% (SD = 2.4) after 48 hours for BJ cells, and 7.5% (SD = 1.7%) after 24 hours and 9.6% (SD = 1.9) after 48 hours for PNT1A cells. There was also no observable statistically significant impact of Selol on the number of necrotic cells present (p=0.16). The percentage of necrotic cells did not exceed 1.5% throughout all the tested concentrations of selenium coming from Selol (Fig.7).

The pro-apoptotic activity of Selol and sodium selenite was assessed following incubation with the examined compounds, using annexin V and propidium iodide. This experimental setup allowed the identification of living, apoptotic and necrotic cells.

In the case of BJ cells, following 24 hours of incubation at a concentration of 10 µM Se, the total protein content was 66.1% of the control (SD = 3.0; p<0.001). A similar drop in protein content was seen with PNT1A cells incubated at a concentration of 6µM Se. The same concentration of selenium (i.e. 6µM) used with LNCaP cells resulted in a total protein content of 29.6% of the control (SD = 5.3; p<0.001), over twice the drop seen in normal cells for the same incubation time. The observed drop in total protein content was dependent on the concentration of selenium added to the experimental setup (BJ): R² = -0.906; p<0.001, PNT1A: R² = -0.959; p<0.001 and LNCaP: R² = -0.979; p<0.001. It was also observed that the length of incubation with selenium selenite had a statistically significant (p<0.001) effect on the lowering of total protein content in the cell cultures. The total protein content of BJ and PNT1A cell cultures, following the addition of sodium selenite at a concentration of 20 µM Se, was approx. 50% after 24 hours and approx. 25% after 48 hours of incubation. Due to the trace amounts of protein remaining in the LNCaP cultures following 24 hours of incubation at the highest sodium selenite concentration, a 48 hour incubation was not carried out.

The pro-apoptotic activity of Selol and sodium selenite was assessed following incubation with the examined compounds, using annexin V and propidium iodide. This experimental setup allowed the identification of living, apoptotic and necrotic cells.
A statistically significant effect of incubation time on inducing apoptosis was recorded for all cell lines \((p<0.001)\). The level of apoptotic BJ cells at the highest concentration of sodium selenite \((20 \, \mu M \text{ Se})\) was 8.3\% \((SD = 4.6)\) and 36.5\% \((SD = 5.0)\) following 24 and 48 hours of incubation respectively. The same experiment with PNT1A cells resulted in respectively 27.7\% \((SD = 4.0)\) and 42.1\% \((SD = 2.3)\) of apoptotic cells within the population. The highest pro-apoptotic effect of sodium selenite was seen with the LNCaP cells, where the level of apoptotic cells following 24 hours of incubation was 82.7\% \((SD = 9.5)\).

As was the case with apoptotic cells, there was an observable effect of the concentration of selenium (from either of the compounds tested) on the percentage of necrotic cells following incubation (Fig.9).

DISCUSSION
Selol is a half-synthetic organic compound containing selenium at the +4 oxidation state. The particular impact of selenium is important when considering its applications to the biochemical processes taking place in living cells, including uptake into the active sites of seleno-enzymes. The process of metabolic interconversion of selenium compounds is extremely complex and not fully understood. Evidence suggests that the absorbability and bioavailability of dietary selenium is dependent on whether it comes from an organic or inorganic compound \([1]\).

The results of the MTT and total protein content assays (Fig. 2 and 4) show that Selol did not display any strong toxicity towards cultures of two normal cell line: BJ and PNT1A, for the tested concentrations of up to 625 \(\mu M\). The recorded drop in cell survival brought on by exposure to Selol was not very significant, with even 48 hours of incubation not leading to a 50\% drop in PNT1A cell proliferation. In the case of BJ cells the 50\% drop was only achieved following 48 hours of incubation at the highest concentration of Selol \((equivalent \, to \, 625 \, \mu M \text{ Se})\).

Under the same experimental parameters, LNCaP cells displayed a significantly higher sensitivity towards the activity of Selol, which is clear from the IC\(_{50}\) value of approx. 36.5 \(\mu M\) Se, almost half of the value measured for BJ cells. It is worth noting that in the case of normal PNT1A cells it was not possible to establish an IC\(_{50}\) value.

Flow cytometry identification of live, apoptotic and necrotic cells in BJ and PNT1A cultures following the addition of Selol showed that, in the investigated range of concentrations of up to 500 \(\mu M\) Se, there was no induction of apoptosis or necrotic effect of the compound on these normal cell lines. In the case of LNCaP cultures, the percentage of apoptotic cells significantly increased with Selol concentration.

In comparison, sodium selenite displayed a clear pro-apoptotic activity as well as had a necrotic effect on both normal and transformed cells at a significantly lower concentration, i.e. 10 \(\mu M\) Se.

The results described above show a clear difference between the response to Selol of normal BJ fibroblasts and PNT1A prostate cell line and that of the transformed LNCaP cell line. In all three assays, for the range of concentration investigated, Selol was more toxic towards the transformed LNCaP cells, with the toxicity being dependent on both the concentration of selenium and the incubation time. The results recorded for LNCaP cells support earlier findings concerning transformed cells being more sensitive to the activity of Selol than normal cells \([16]\).

Sodium selenite displayed strong toxicity towards both normal and transformed cells after 24 hours of incubation in a much narrower range of concentrations, i.e. 1-20 \(\mu M\).

The significantly lower toxicity of Selol, compared to sodium selenite, it most likely caused by the relatively large size of the molecule (Fig.1) having an impact \textit{in vitro}, and possibly also \textit{in vivo}, on the ability and rate of transport through the cell membrane, as well as the different metabolism of this organic selenium compound.

This hypothesis may be supported by previous research into the absorption of both compounds by HeLa cells in \textit{in vitro} culture \([16]\). The investigation used ICP-MS to show a significant difference in the dynamics of membrane crossing between selenium derived from Selol and selenium derived from sodium selenite. It was shown that the small inorganic selenium molecule penetrated the cell much faster than the large organic Selol molecule. Increasing the incubation time with Selol allows the compound to penetrate the cell membrane to a point where a similar level of selenium is present inside the cell as in the case of the highest used concentration of sodium selenite. Analysis of the data concerning the cytotoxicity of Selol and sodium selenite towards HeLa cells \([16]\) shows that the presence of a clear cellular response is dependent on the rate at
which selenium penetrates into the cells as well as on the time needed to liberate selenium from the its complex compound form.

CONCLUSION

Taking into account the literature concerning the increasing absorption of organic selenium compounds by the human organism, as well as the results of the investigation of the cytotoxic effect of Selol on normal BJ and PNT1A cells and transformed LNCAp and HeLa cells, it can be concluded that, taking into account safety and effectiveness in being used in intracellular processes, Selol has superior properties compared to sodium selenite [12, 16]. Additionally, the increased toxicity towards transformed cells encourages further investigation into understanding the activity mechanism of Selol. The potential to use Selol as a prophylactic and therapeutic source of selenium requires further work, including carrying out in vivo experiments. These investigations are currently underway, and will be the subject of subsequent publications.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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